Isolation of an intact DNA polymerase-primase from embryos of Drosophila melanogaster

(DNA replication/proteolysis/multisubunit enzyme)

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ABSTRACT A procedure has been devised for the purification of intact DNA polymerase α from early embryos of Drosophila melanogaster. The purified enzyme consists of at least three polypeptides with Mr's of 182,000, 60,000, and 50,000. These are related antigenically to the α (Mr, 146,000), β (Mr, 58,000), and γ (Mr, 46,000) subunits, respectively, of the DNA polymerase described previously [Banks, G. R., Boezio, J. A., & Lehman, I. R. (1979) J. Biol. Chem. 254, 9866–9872]. The α subunit (Mr, 182,000) has a molecular weight indistinguishable from that observed in extracts of freshly harvested embryos and presumably present in vivo. As in the previous preparation, the α subunit is required for DNA polymerase activity and is very likely the catalytic subunit of the enzyme. The ratio of primase to polymerase remains constant throughout the purification. Thus, the primase is very likely an integral component of the Drosophila DNA polymerase α. The purified DNA polymerase-primase contains no detectable endo- or exodeoxyribonuclease and has pH, MgCl₂, (NH₄)₂SO₄, and NaCl optima identical to those reported previously. In contrast, the Km for dTTP is 3.7 μM as compared with 17.5 μM for the previous enzyme. Sensitivities to aphidicolin and N-ethylmaleimide and resistance to deoxy TTP are unchanged.

DNA polymerase α as purified from a variety of eukaryotes consists of two or more polypeptides. Several polymerases (1–7) have been isolated as multisubunit proteins consisting of a catalytic core with an approximate Mr of 130,000 in association with three or four smaller subunits whose Mr's range from 40,000 to 60,000. However, other polymerase preparations are lacking a high molecular weight subunit (8, 9). Our recent studies (10) and those of others (11–14) have shown that a DNA primase activity is associated with the α polymerase and may be part of the multisubunit molecule. Uncontrolled proteolysis during purification has prevented the unambiguous determination of the subunit structure of DNA polymerase α and the structure-function relationships of its individual polypeptides. That proteolysis may yield an active but highly degraded form of the enzyme has been clearly demonstrated both in Drosophila melanogaster embryos and in calf thymus (4, 15–17). Several years ago purification to near-homogeneity of the DNA polymerase α from early embryos of D. melanogaster was described. This enzyme consisted of subunits with Mr's of 148,000 (α), 58,000 (β), 46,000 (γ), and 43,000 (δ). However, more recently, the α subunit was found to be derived from polypeptides of Mr's 185,000 and 166,000 as a result of in vitro proteolysis (18). We describe here a purification procedure, starting with freshly harvested embryos, that yields a DNA polymerase α with an α subunit of Mr, 182,000. DNA primase activity remains quantitatively associated with the polymerase through each of the steps of the purification procedure, indicating that it is an integral component of the Drosophila DNA polymerase α.

MATERIALS AND METHODS

Materials. Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. [3H]dTTP and [3H]dATP were purchased from New England Nuclear. [32P]dTTP, [32P]dATP, [32P]dCTP, and [32P]dGTP were purchased from Amersham. Phenylmethylsulfonyl fluoride (Phenylmethansulfonyl fluoride (PhMeSO₂F), Sigma), sodium metabisulfite (Baker), urea (ultrapure, Schwarz/Mann), and Amberlite MB-3 (Mallincrodt) were prepared for use as described (19). Leupeptin was purchased from the Peptide Institute (Minoh-shi, Japan). Aphidicolin was prepared as a stock solution at 1 mg/ml in 40% ethanol/10% dimethyl sulfoxide. N-Ethylmaleimide (Schwarz/Mann) was dissolved in water immediately before use.

Antiserum. Antiserum directed against Drosophila DNA polymerase α and its isolated α and β subunits were prepared as described (18). Purified IgG was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (20).

Enzymes. Escherichia coli DNA polymerase I and E. coli RNA polymerase were the generous gifts of J. Kelly and J. Kaguni, respectively, of this department. Pancreatic DNase I was purchased from Worthington.

Nucleic acids. Calf thymus DNA (grade A, Calbiochem) was treated with pancreatic DNase until it was rendered 20% acid soluble (21). Poly(dT), N,1,000 residues long, was purchased from P-L Biochemicals. T₇ and φX174 [3H]DNAs were gifts of P. Riddles and D. Soltis, respectively, of this department.

Chromatography and buffers. Phosphocellulose P11, hydroxylapatite HTP, and blue A agarose were purchased from Whatman, Bio-Rad, and Amicon, respectively. Single-stranded DNA cellulose was prepared as described (22). All potassium phosphate buffers were at pH 7.6 and contained 1 mM 2-mercaptoethanol, 0.2 mM EDTA, 1 mM PhMeSO₂F, 10 mM sodium metabisulfite, leupeptin at 2 μg/ml, and 10% glycerol.

Methods. Processing of Drosophila embryos. D. melanogaster (Oregon R) embryos (average age, 9 hr) were collected immediately before use and washed and dechorionated as described (15).

DNA polymerase assay. Reaction mixtures (0.1 ml) contained 50 mM Tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, 20 mM (NH₄)₂SO₄, 10 mM MgCl₂, 200 μg of bovine serum albumin, 25 μg of activated calf thymus DNA, 40–100 μM (each) dATP, dGTP, dCTP, and [3H]dTTP (200–6,000 cpm/pmol), and enzyme, unless otherwise indicated. Incubation was for 30 min at 37°C. One unit of activity is that amount that catalyzes the incorporation of 1 nmol of dNTP into acid-insoluble material in 60 min at 37°C.

Abbreviation. PhMeSO₂F, phenylmethylsulfonyl fluoride.

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DNA primase assay. Reaction mixtures (25 μl) contained 50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 4 mM dithiothreitol, 5 μg of bovine serum albumin, 1.0 μg of poly(dT), 2 mM ATP, 100 μM [³H]dATP (300 cpm/pmol), 0.6 unit (23) of E. coli DNA polymerase I, and enzyme. Incubation was for 30 min at 30°C. One unit of activity is that amount that catalyzes the incorporation of 1 nmol of dATP into acid-insoluble material in 60 min at 30°C.

Nuclease assays. Exonuclease assays were performed as described (1) except that they contained either T7 [³H]DNA (1.0 μg; 9.4 × 10⁶ cpm/μg) or linear φX174 [³H]DNA (0.5 μg; 1 × 10⁶ cpm/μg) as the substrates. Endonuclease assays were performed as described for polymerase assays except that dNTPs were omitted, φX174 form I DNA (0.3 μg) was the substrate, incubation was for 15 min at 37°C, and products were analyzed by agarose gel electrophoresis after addition of NaDodSO₄ to 1.5% and heating for 3 min at 65°C.

Protein determinations. Protein was determined by the method of Bradford (24); bovine serum albumin was the protein standard.

Gel electrophoresis and protein transfers. Polyacrylamide gel electrophoresis in the presence of NaDodSO₄ was performed according to Laemmli (25). Proteins were transferred electrophoretically from polyacrylamide gels to activated amionophenylthioether paper with a Hoeffer Transphor apparatus (26). Transfers were probed with purified IgG at a concentration of 20 μg/ml as described (26).

RESULTS

Purification of D. melanogaster DNA Polymerase-Primase. All operations were performed at 0–4°C.

Preparation of the high-speed supernatant fraction. Freshly harvested dechorionated embryos (76 g) were suspended (4 ml/g of embryos) in 15 mM Hepes, pH 8.0/5 mM KCl/0.5 mM Mg acetate/0.05 mM EDTA/0.35 M sucrose/0.5 mM dithiothreitol/1 mM PhMeSO₄/F/10 mM sodium bisulfite/leupeptin at 2 μg/ml, homogenized in 40-ml portions by five strokes of a 40-ml stainless steel Teflon homogenizer, and filtered through a 75-μm Nitex screen. The retenate was rehomogenized in the same buffer (1 ml/g of embryos) and filtered. The combined filtrates were centrifuged at 10,000 × g for 15 min; the supernatant fluid was recentrifuged at 127,000 × g for 60 min, and the resulting supernatant fluid was filtered through eight layers of cheesecloth to remove residual lipid-like material (fraction I).

Phosphocellulose chromatography and ammonium sulfate precipitation. Fraction I was adjusted to an ionic equivalent of 80 mM potassium phosphate and loaded onto a phosphocellulose column (5 ml of packed phosphocellulose per g of embryos) equilibrated with 80 mM potassium phosphate buffer at a flow rate of 200 ml/hr. The column was washed with 100 mM potassium phosphate at a flow rate of 300 ml/hr until no protein could be detected in the effluent. A 600-ml linear gradient from 100 to 300 mM potassium phosphate was applied. The column was then washed with 600 ml of 500 mM potassium phosphate. In this preparation, enzyme activity eluted in the 500 mM potassium phosphate wash and the active fractions were pooled (fraction II). However, depending on the biological state of the embryos used, enzyme activity frequently elutes at ~200 mM potassium phosphate. Solid (NH₄)₂SO₄ was added (0.313 g/ml of fraction II) over 30 min, and the suspension was stirred for an additional 45 min; the precipitate was collected by centrifugation at 32,000 × g for 40 min and was stored for 4–5 hr before resuspension in 10 mM potassium phosphate (fraction III).

Hydroxylapatite chromatography and ammonium sulfate precipitation. Fraction III was dialyzed against 10 mM potassium phosphate in several collection bags (M, cutoff, 25,000) until an ionic equivalent of 100 mM potassium phosphate was reached. The dialyzed fraction was centrifuged at 13,000 × g for 5 min and the supernatant fluid was loaded onto a column (1.7 × 5.5 cm) of hydroxylapatite equilibrated with 100 mM potassium phosphate at 23 ml/hr. The column was washed with 15 ml of this buffer and a 100-ml linear gradient from 100 to 200 mM potassium phosphate was applied at 36 ml/hr. Enzyme activity was eluted at 145 mM potassium phosphate and active fractions were pooled (fraction IV). Ammonium sulfate precipitation was performed as before and the precipitate was stored overnight before resuspension in 20 mM potassium phosphate (fraction IVb).

DNA-cellulose chromatography and ammonium sulfate precipitation. Fraction IVb was dialyzed as before until an ionic equivalent of 20 mM potassium phosphate containing 60 mM NaCl was reached. The dialyzed fraction was centrifuged to remove insoluble material and the supernatant fluid was loaded at 4 ml/hr onto a column (1.4 × 3.0 cm) of single-stranded DNA-cellulose equilibrated with 20 mM potassium phosphate containing 60 mM NaCl. The column was washed with 4 ml of this buffer at 9 ml/hr, followed by 12 ml each of buffers containing 100 and 350 mM NaCl. Active fractions, eluted with the latter buffer, were pooled (fraction V) and mixed with saturated and neutralized (NH₄)₂SO₄ (1.2 ml/ml of fraction V). The precipitate was collected after 60 min by centrifugation at 35,000 rpm at 3°C for 45 min in a Beckman SW 41 rotor and was resuspended in 0.5–1.0 ml of 20 mM potassium phosphate (fraction Vb).

Glycerol gradient sedimentation. Fraction Vb was layered onto one or two preformed 10–30% glycerol gradients containing 50 mM potassium phosphate, 200 mM (NH₄)₂SO₄, 1 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PhMeSO₄/F, 10 mM sodium bisulfite, and leupeptin at 2 μg/ml, prepared in polyallomer tubes suitable for use in a Beckman SW 41 rotor. Centrifugation was at 35,000 rpm at 3°C for 38.5 hr, after which 15-drop fractions were collected. Peak activity fractions were pooled (fraction VI) and stored at −80°C.

Table 1. Purification of DNA polymerase-primase from freshly harvested embryos of D. melanogaster

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume, ml</th>
<th>Protein, mg</th>
<th>Activity, units × 10⁻²</th>
<th>Specific activity, units/mg</th>
<th>Activity, units × 10⁻²</th>
<th>Specific activity, units/mg</th>
<th>Polymerase/primase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-100 (I)</td>
<td>360</td>
<td>2,581</td>
<td>1,792</td>
<td>69.4</td>
<td>792</td>
<td>30.7</td>
<td>2.3</td>
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<td>Phosphocellulose (II)</td>
<td>493</td>
<td>261</td>
<td>704</td>
<td>269</td>
<td>266</td>
<td>86.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Ammonium sulfate (III)</td>
<td>50</td>
<td>65</td>
<td>772</td>
<td>1,188</td>
<td>189</td>
<td>291</td>
<td>4.1</td>
</tr>
<tr>
<td>Hydroxylapatite (IV)</td>
<td>41</td>
<td>8.3</td>
<td>284</td>
<td>3,425</td>
<td>66.4</td>
<td>800</td>
<td>4.3</td>
</tr>
<tr>
<td>DNA-cellulose (V)</td>
<td>5</td>
<td>0.78</td>
<td>142</td>
<td>18,256</td>
<td>34.6</td>
<td>4,431</td>
<td>4.1</td>
</tr>
<tr>
<td>Glycerol gradient (VI)</td>
<td>1.5</td>
<td>0.19</td>
<td>98.3</td>
<td>51,737</td>
<td>24.8</td>
<td>12,947</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Blue A agarose chromatography. DNA polymerase-primase (1,550 units; fraction VI) was dialyzed into 10 mM potassium phosphate containing 20% glycerol and was loaded onto a column (0.5 × 2.2 cm) of blue A agarose equilibrated with 10 mM potassium phosphate at 0.8 ml/hr. The column was washed at 1.2 ml/hr with 1.6 ml each of 20 mM potassium phosphate containing 0.5 and 1 M NaCl. Enzyme activity was eluted with 1.6 ml of 20 mM potassium phosphate containing 2 M NaCl.

As shown in Table 1, the DNA polymerase activity of fraction VI was purified ∼750-fold with a yield of 6%. The procedure yielded 0.19 mg of purified enzyme from 76 g of embryos as contrasted with 0.23 mg from 540 g of embryos by our previously reported procedure (1). The polymerase/primase ratio remained constant throughout the purification.

Physical Properties. Electrophoresis of the fraction VI enzyme on a 5–10% NaDodSO4/polyacrylamide gel (Fig. 1 Left, lane 2) yielded predominantly five polypeptides, with Mr of 215,000, 182,000, 73,000, 60,000, and 50,000. Chromatography on blue A agarose prior to electrophoretic analysis (Fig. 1 Left, lane 1) resulted in removal of most of the minor contaminants and in a 40% decrease in the relative amount of the Mr 215,000 polypeptide; the relative abundance of the remaining four major polypeptides was unchanged. Remarkably, although the Mr 60,000 and Mr 50,000 polypeptides had the molecular weights of the β and γ subunits of the enzyme described previously (1), the Mr 148,000 α subunit was absent. Instead, a Mr 182,000 polypeptide was found whose molecular weight is consistent with that predicted for an undegraded α subunit based on an immunological analysis of embryo extracts (18). Densitometric scanning of the stained gel revealed that the relative abundance of the Mr 182,000, 73,000, 60,000, and 50,000 polypeptides in the two preparations shown (Fig. 1 Right) was 1.0/1.0/1.6/1.2, respectively; these four polypeptides together account for about 85% of the protein applied (Fig. 1 Left, lane 1). The amount of Mr 215,000 polypeptide was variable in these and other preparations.

To determine which of the polypeptides revealed by NaDodSO4/polyacrylamide gel electrophoresis were related antigenically to the previous DNA polymerase, the polypeptides were transferred electrophoretically from a gel to diazotized paper. The paper was probed with antiserum directed against the

Fig. 1. NaDodSO4/polyacrylamide gel electrophoresis of D. melanogaster DNA polymerase-primase. (Left) Fraction VI, prior to (5.0 μg, lane 2) and after (2.0 μg, lane 1) chromatography on blue A agarose, was denatured and electrophoresed in a 5–10% linear gradient NaDodSO4/polyacrylamide slab gel. Marker proteins electrophoresed in adjacent lanes and indicated by their molecular weights (×10^-3) were: myosin (a gift from J. Spudich, Stanford University), E. coli RNA polymerase β' and β subunits, E. coli β-galactosidase (Worthington), rabbit muscle glycogen phosphorylase (Worthington), bovine serum albumin (Pentex), rabbit muscle pyruvate kinase (Worthington), E. coli alkaline phosphatase (Worthington), and bovine carbonic anhydrase (Worthington). (Right) Densitometric scans of lane 1 (blue A) and lane 2 (glycerol gradient) shown in Left. The molecular weights (×10^-3) of the four major polypeptides are indicated.

Fig. 2. Antigenicity of polypeptides in the purified DNA polymerase-primase and in an embryo extract. Fraction VI (2.5 μg, lanes 1–3) and extract derived from homogenization of freshly harvested embryos in NaDodSO4 (150 μg, lane 4) were electrophoresed in a 5–10% linear gradient NaDodSO4/polyacrylamide gel and the polypeptides were transferred electrophoretically to diazotized paper and probed with α subunit (lanes 1 and 4), β subunit (lane 2), and DNA polymerase-primase-specific (lane 3) IgGs as described by Reiser (26). The paper was then incubated with 125I-labeled protein A and autoradiographed. The molecular weights of the radioactive polypeptides are as indicated (×10^-3).
α and β subunits as well as the intact DNA polymerase and then was incubated with 125I-labeled Staphylococcus aureus protein A and autoradiographed. When probed with α-subunit-specific IgG (Fig. 2, lane 1) one predominant and one minor species were observed, with Mr's of 182,000 and 160,000, respectively. Incubation with β-subunit specific-IgG (Fig. 2, lane 2) revealed a single band of Mr, 60,000. Anti-DNA polymerase-specific IgG reacted with polypeptides of Mr's 182,000, 60,000, and 50,000 and, to a lesser extent, species of Mr's 160,000 and 148,000 (Fig. 2, lane 3). The latter two species were also found upon longer exposure with the α-subunit-specific IgG and are proteolytic products of the Mr, 182,000 α subunit.

In summary, three polypeptides with Mr's of 182,000, 60,000, and 50,000 related antigenically to the α, β, and γ subunits, respectively, of the earlier DNA polymerase α (1, 19) were identified in the new enzyme preparation. Of these, the β and γ subunits had unaltered molecular weights, whereas that of the α subunit increased from Mr, 148,000 (1) to Mr, 182,000. Neither the Mr, 215,000 nor the Mr, 73,000 polypeptide observed in the NaDodSO4/polyacrylamide gel (Fig. 1) was related antigenically to the previous enzyme nor were the other minor polypeptides. A parallel experiment was performed with freshly harvested embryos homogenized with NaDodSO4 and using α-subunit-specific IgG as a probe (Fig. 2, lane 4). In this case, a single polypeptide of Mr, 182,000 was observed, indicating that this species, present in embryo extracts and presumably in vivo, represents the intact α subunit.

Inasmuch as immunological characterization of the DNA polymerase-primase showed the α subunit to be a polypeptide of Mr, 182,000, we wished to determine whether the α subunit was also the catalytic core of the enzyme. As in the previous analysis (19), glycerol gradient sedimentation of the enzyme was performed in the presence of 2.8 M urea. Sedimentation of the DNA polymerase-primase in the absence of urea yielded a single sharp peak with a sedimentation coefficient of ≈8.3 S (data not shown). When the enzyme was incubated for 2 hr at 0°C in 2.8 M urea and then sedimented, two peaks of activity were observed (Fig. 3).

The faster sedimenting activity appeared at a much decreased level in an analysis in which the enzyme was incubated for 4 hr in 3.4 M urea prior to sedimentation (Fig. 3 Inset) and is therefore most likely the result of incomplete dissociation. NaDodSO4/polyacrylamide gel electrophoresis of fractions taken across the urea/glycerol gradient showed a separation of subunits (Fig. 4 Left). DNA polymerase activity co sedimented with the isolated Mr, 182,000 α subunit (Fig. 4 Right).

![Image](https://via.placeholder.com/150)
Biochemical Properties. Reaction requirements. The reaction requirements and NaCl, (NH₄)₂SO₄, pH, and MgCl₂ optima for DNA polymerase activity of the undegraded enzyme were nearly identical to those described for the previous enzyme (1). However, the Kₘ for dTTP was substantially lower (3.7 vs. 17.5 μM). Resistance to deoxy TTP and sensitivities to aphidicolin and N-ethylmaleimide remained the same.

Other activities. The purified enzyme showed no detectable exonuclease activity with native T7 [³H]DNA or with linear φX174 [³H]DNA as substrates in the presence or absence of the four unlabeled deoxynucleotide triphosphates (<10 pmol of acid-soluble nucleotide produced by 12 units of polymerase). In addition, no endonuclease activity could be detected when assayed by the conversion of φX174 form I DNA to form II or III by agarose gel electrophoresis.

DISCUSSION

Purification of a DNA polymerase α from D. melanogaster embryos consisting of a M, 182,000 α subunit and two or three smaller subunits has relied both on the development of a very rapid purification procedure and on the use of freshly harvested embryos. Immunological analysis of enzyme fractions obtained at various stages in the previous purification procedure showed that proteolysis had occurred in all but the final steps (18). Further, such studies with extracts of embryos, frozen both before and after dechorionation, have shown that the M, 182,000 α subunit is only a minor species in frozen embryos (unpublished data; ref. 18), indicating that it is exceedingly sensitive to proteolysis.

The subunit structure of the DNA polymerase-primase purified by the procedure described here is similar to that of the earlier enzyme. However, the immunological studies presented here show that the α subunit has a M, of 182,000 rather than a M, of 148,000, while the molecular weights of the β and γ subunits have remained at M, 60,000 and M, 50,000, respectively. An additional polypeptide of M, 75,000, which is not related antigenically to the previous enzyme, now routinely copurifies with the polymerase and may have dissociated, possibly as a result of diminished interaction with the partially degraded enzyme. On the other hand, the M, 43,000 β subunit (1, 19) appears not to be associated with our current polymerase preparation. This polypeptide is highly abundant in embryo extracts and in early enzyme fractions (18) and may have been a contaminant in purified enzyme fractions prepared by the previous procedure. These issues will be resolved only when functions can be ascribed to the various polypeptides.

That the α subunit is required for DNA polymerase activity was shown by urea/glycerol gradient sedimentation of the enzyme followed by NaDODSO₄/polyacrylamide gel electrophoresis of gradient fractions: DNA polymerase activity co-sedimented with the M, 182,000 α subunit, whereas activity was decreased 60-93% in fractions enriched in the β and γ subunits; it was absent in fractions enriched in the M, 73,000 species. This result is in agreement with that of Villani et al. (19), who showed that polymerase activity was associated with the M, 148,000 degradation product of the M, 182,000 α subunit.

The undegraded polymerase exhibits a substantially lower Kₘ for dTTP. This change may signify more profound differences in the enzymatic capacity of the polymerase and its interactions with other proteins involved in DNA replication.

Finally, DNA primase copurifies quantitatively with the DNA polymerase. Therefore, it is very likely associated with one or more of the subunits of the enzyme. Preliminary experiments suggest that the primase activity does not reside solely in the M, 182,000 α subunit but may require or reside in the β or γ subunits, or both (unpublished data). Further work is clearly required to resolve this issue.

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