Subunit Structure of Mitochondrial DNA Polymerase from Drosophila Embryos

PHYSICAL AND IMMUNOLOGICAL STUDIES*

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The subunit structure of mitochondrial DNA polymerase from Drosophila embryos has been examined by a combination of physical and immunological methods. A highly specific rabbit antiserum directed against the native enzyme was developed and found to recognize specifically its two subunits in immunoblot and immunoprecipitation analyses. That and the potent inhibition by the rabbit antiserum of the DNA polymerase and 3′ → 5′ exonuclease activities of the nearly homogeneous mitochondrial DNA polymerase provide strong evidence for the physical association of the 3′ → 5′ exonuclease with the two subunit enzyme. An immunoprecipitation analysis of crude enzyme fractions showed that the two subunits of Drosophila mitochondrial DNA polymerase are intact, and an in situ gel proteolysis analysis showed that they are structurally distinct. Template-primer DNA binding studies demonstrated formation of a stable and discrete enzyme-DNA complex in the absence of accessory proteins. Photochemical cross-linking of the complexes by UV light indicated that the α but not the β subunit of mitochondrial DNA polymerase makes close contact with DNA, and limited digestion of the native enzyme with trypsin showed that an ~65-kDa proteolytic fragment of the α subunit retains the DNA binding function.

Of the five eucaryotic DNA polymerases (α, β, γ, δ, ε), the mitochondrial DNA polymerase (pol γ) is the least abundant and perhaps the least well studied. With the recent discovery of mtDNA diseases (1), and the realization that drugs used to combat cancer and viruses affect mtDNA function (2), there is renewed interest in it. Studies describing the identification and relative abundance of pol γ have demonstrated that it is the only DNA polymerase found in animal mitochondria (3), and that it accounts for only about 1% of the total cellular DNA polymerase activity (4). Notwithstanding the enzyme’s low relative abundance, in surveying Drosophila at six developmental stages, we showed that the level of pol γ activity varies 180-fold during development and is greatest in early embryos (5). This allowed its purification to near-homogeneity (5), and characterization of its subunit structure and catalytic mechanism (6–12). It is now apparent that other animal mitochondrial DNA polymerases including mammalian enzymes have similar catalytic and structural features (13–15).

We proposed that Drosophila pol γ is a heterodimer comprising a 125-kDa polymerase catalytic subunit and a 35-kDa polypeptide of unknown function (5). Likewise, pol γ from frog (13), pig (14), and human cells (15) has been shown to contain a large catalytic subunit and several smaller polypeptides, some of which appear to result from in vitro proteolysis. A genomic clone of the polymerase catalytic subunit of yeast mtDNA polymerase encodes a 140-kDa polypeptide (16); whether or not the yeast enzyme contains a small subunit is unknown.

We and others have shown that pol γ contains a potent and highly mispair-specific 3′ → 5′ exonuclease, which proofreads errors during in vitro DNA synthesis (6, 14, 17–19). Ito and Braithwaite (20) have shown that the deduced amino acid sequence of the yeast catalytic subunit (MIP1) can be aligned with the family A DNA polymerases, of which Escherichia coli DNA polymerase I (Eco pol I) and bacteriophage T7 DNA polymerase are members, and that some amino acid residues that are critical in the DNA polymerase and 3′ → 5′ exonuclease domains of Eco pol I are invariant in MIP1. In fact, Foury has shown by site-directed mutagenesis, that substitutions in conserved exo-domain asparagines result in a mutator phenotype upon production of the recombinant protein in yeast (21). Thus, although a subunit assignment for the 3′ → 5′ exonuclease has not been made in any of the animal mitochondrial DNA polymerases, it is most likely that the 3′ → 5′ exonuclease function resides in the polymerase catalytic subunit. Interestingly, however, while the 3′ → 5′ exonuclease resides in the polymerase catalytic subunit in Bacillus subtilis DNA polymerase III, it exists as a separate subunit in E. coli DNA polymerase III (22).

Here we report further studies of the subunit structure and enzymatic activities of Drosophila pol γ using a combination of physical and immunological approaches. We have also explored the role of the two subunits in template-primer DNA binding.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. [3H]dATP was purchased from ICN Biochemicals; [32P]dATP was purchased from Du Pont NEN. A recombinant M13 viral DNA (10,650 nt) was prepared by standard laboratory methods for use in DNA polymerase and 3′ → 5′ exonuclease assays. Synthetic oligodeoxynucleotides (15 nt) complementary to M13 viral DNA and containing a 3′-terminal base pair (dAMP/template dTMP primer) or a 3′-terminal mispair (dAMP-dAMP), and the 36-mer 5′-TCTCCATAATTTAGGGCGTCAGCTACCAGCCGTC-3′ were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer.

Enzymes and Proteins—Drosophila pol γ was prepared as described.
by Wernette and Kaguni (5). T4 polynucleotide kinase and E. coli DNA polymerase I Klenow fragment were purchased from New England Biolabs. Bovine serum albumin and pre stanched and unstained SDS molecular weight marker proteins were purchased from Sigma. Protein A-alkaline phosphatase conjugate and protein A-agarose were purchased from Sigma and Boehringer Mannheim, respectively. 125I-Protein A was purchased from ICN.

Preparation of Antiserum—Antiserum directed against Drosophila pol γ was prepared with the nearly homogenous Fraction VI enzyme (5). A virgin female New Zealand White rabbit was immunized with 2.5 μg of pol γ in Freund’s complete adjuvant by injection at or near the popliteal lymph node. Seven booster immunizations, each containing 2 μg of Fraction VI enzyme were administered in Freund’s incomplete adjuvant at 2–4-week intervals. Bleedings were performed 8–10 days after each boost.

Preparation of Drosophila Extracts—All operations were performed at 0–4 °C. Drosophila melanogaster (Oregon R) embryos of average age (9 h) were collected immediately before use and suspended at a ratio of 4 ml/g in 25 mM Hepes (pH 8.0), 10% glycerol, 0.3 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM sodium metabisulfite, 2 μg/ml leupeptin, and 2% sodium cholate and homogenized by 8 strokes in a 7-ml glass homogenizer. The homogenate (embryo extract) was frozen immediately in liquid nitrogen and stored at −80 °C. Mitochondrial extracts were prepared from freshly harvested embryos as described by Wernette and Kaguni (5).

Polyacrylamide Gel Electrophoresis, Transfer, and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (23). Proteins were transferred to nitrocellulose membranes (0.45 μg, Schleicher & Schuell) using a Hoefer Transphor electrophoresis unit model TE22 for 6 h at 70 V in 190 mM glycine, 25 mM Tris base, and 20% (v/v) methanol. The membranes were washed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20 (TBST) for 1 min and blocked for 2 h in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5% (w/v) nonfat milk. The membranes were probed with anti-DNA polymerase γ serum (1:1000 in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% nonfat milk) for 6–10 h and then washed three times for 10 min each in TBST and once for 5 min in TBS. After the washes were complete, incubation (2.5 h) was performed with protein A-alkaline phosphatase conjugate (1:2000 (v/v) or 125I-protein A (2 μCi/ml) in TBS (pH 8.0). The protein A-alkaline phosphatase-treated membranes were washed three times for 10 min each in TBST, once for 5 min in TBS and once for 5 min in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl2 (alkaline phosphatase buffer). The membranes were developed by incubation in alkaline phosphatase buffer containing nitro blue tetrazolium (330 μg/ml) and 5-bromo-chloro-3-indolyl phosphate (165 μmol/l). The 125I-protein A-treated membranes were washed three times for 10 min each in TBST and exposed to Kodak X-Omat AR x-ray film using a DuPont NEN Quanta III intensifying screen.

Immunoprecipitation of Drosophila DNA Polymerase γ—Polymerase γ (Fraction I, III, or embryo extract, as indicated in the legends to Figs. 1 and 2) was incubated with 10 μM NaCl, 10 μg bovine serum albumin (PBS) and incubated with preimmune serum or polyclonal antiserum overnight on ice. Immune complexes were precipitated by incubation with preswollen protein A-agarose (60 μl of a 50% slurry) for 2 h with gentle rotation. The precipitates were collected by centrifugation and washed once with PBS containing 0.05% (v/v) Tween and twice with PBS, suspended in Laemmli sample buffer (23), heated for 10 min at 85 °C, and re-centrifuged. The supernatant fractions were then subjected to immunoblot analysis as described above.

Chemical Cleavage of Drosophila DNA Polymerase γ—Polymerase γ Fraction VI (3 μg) was subjected to SDS-polyacrylamide gel electrophoresis, and gel slices containing the 125- and 35-kDa polypeptides were excised and subjected to proteolysis with N-chlorosuccinimide as described by Lischwe and Ochs (24). The proteolytic products were electrophoresed in a 5–15% linear gradient SDS-polyacrylamide gel and stained with silver as described by Wray et al. (25).

RESULTS

Specificity of Antiserum Developed against Native Drosophila DNA Polymerase γ—We have described Drosophila mitochondrial DNA polymerase as a heterodimer of 125- and 35-kDa subunits, containing both 5′ → 3′ DNA polymerase and 3′ → 5′ exonuclease activities (5, 8). To begin to elucidate structure-function relationships in pol γ, we developed a highly specific rabbit antiserum against the nearly homogenous enzyme (see "Methods"). In an immunoblot analysis, the rabbit antiserum detects only the 125- and 35-kDa subunits of pol γ in the Fraction IV enzyme, which is only 8% pure (Fig. 1A, and Ref. 5). Immunoprecipitation of pol γ Fraction III yields the same two polypeptides upon subsequent immunoblotting (Fig. 1B). The data indicate that neither the α nor the β subunits of Drosophila pol γ have been proteolyzed during the course of purification from the Fraction III to Fraction IV stage, and that the antiserum is highly specific and recognizes the native enzyme. Further, notwithstanding the non-quantitative nature of the immunological procedures employed, both the immunoblot and immunoprecipitation analyses of the crude enzyme fractions are consistent with the 1:1 subunit stoichiometry that we determined for the nearly homogeneous Drosophila pol γ by SDS-polyacrylamide gel electrophoretic analysis, and in hydrodynamic studies that yield a calculated native molecular mass of 160 kDa (5).

Inhibition of DNA Polymerase and 3′ → 5′ Exonuclease in...
Drosophila pol γ by Rabbit Antiserum—The DNA polymerase and 3′ → 5′ exonuclease in Drosophila pol γ copurify quantitatively (8). To provide further evidence that the two activities reside in the two subunit enzyme, we performed enzyme inhibition studies of nearly homogeneous pol γ using the rabbit antiserum. The rabbit antiserum is a potent inhibitor of both DNA polymerase and 3′ → 5′ exonuclease activity (Fig. 2). In contrast, no inhibition of the DNA polymerase or 3′ → 5′ exonuclease activities of E. coli DNA polymerase I is observed (data not shown). Because the antiserum is highly specific for the two subunit enzyme (Fig. 1) and inhibits its DNA polymerase and exonuclease activities to similar extents at equal antiserum concentrations, we conclude that both activities reside in native Drosophila pol γ.

In order to make a subunit assignment for the 3′ → 5′ exonuclease function, we carried out extensive studies by gel filtration and velocity sedimentation in the presence of denaturants, to achieve dissociation and separation of the two subunits of Drosophila pol γ with retention of catalytic activity. However, we found that subunit dissociation in the presence of guanidineHCl, urea, or ethylene glycol occurs only upon partial denaturation and substantial loss of enzyme activity; enzyme assay and immunoblot analyses indicate that ≥95% of both DNA polymerase and 3′ → 5′ exonuclease activity is lost before subunit dissociation occurs (data not shown).

The Two Subunits of Drosophila pol γ Are Intact and Distinct—To confirm the proposed subunit structure of native Drosophila pol γ, immunoprecipitation and in situ gel proteolysis studies were pursued. Immunoprecipitation of native pol γ from crude enzyme fractions shows that its α and β subunits have most likely not been proteolyzed during the course of purification (Fig. 3). In this analysis, freshly harvested embryos were processed rapidly into whole embryo (lanes 2–4) and mitochondrial (lanes 5–7) extracts which were precipitated with either rabbit antiserum (lanes 2, 3, 5, and 6) or preimmune (lanes 4 and 7). It is evident that only the α and β polypeptides in the nearly homogeneous enzyme are identified in the crude protein fractions upon subsequent immunoblotting. Notably, no polypeptides larger than the 125-kDa α subunit are observed, and the α/β polypeptide ratio is similar in the crude versus nearly homogeneous fractions.

To demonstrate that the α and β subunits of Drosophila pol γ are structurally distinct, we performed an in situ gel proteolysis analysis with N-chlorosuccinimide (Fig. 4). The α and β...
subunits of Drosophila pol γ were purified by SDS-polyacrylamide gel electrophoresis and then cleaved with N-chlorosuccinimide and re-electrophoresed. They yield completely distinct polypeptide patterns; partial cleavage of the α subunit yields 11 polypeptides ranging from 9 to 33 kDa (lane 3), none of which correspond to the four products derived from the β subunit (lane 5).

Drosophila pol γ Forms a Discrete Complex with Template-Primer DNA That Involves Close Contact with the α Subunit—

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Subunit Structure of Mitochondrial DNA Polymerase

The subunit structure of mitochondrial DNA polymerase is an unresolved issue. Based on recent studies of the Drosophila (5), Xenopus (13), pig (14), and human (15) enzymes, we can propose a consensus subunit structure for animal mitochondrial DNA polymerase, in which a large polypeptide of 125–140 kDa containing the DNA polymerase function, is associated quantitatively with a smaller subunit of 35–50 kDa. However, because the frog (13) and pig (14) preparations contain polypeptides of intermediate size, some of which retain DNA polymerase activity, it is possible that all of the smaller polypeptides result from proteolysis of the polymerase catalytic subunit. To address this issue, we examined the intactness of Drosophila pol γ in a comparison by immunoprecipitation of crude versus nearly homogenous enzyme fractions. We found that both the size and the apparent stoichiometry of the α and β subunits are the same in extracts as compared to that observed in the isolated enzyme. Furthermore, we found by limited in vitro proteolysis of the two subunits that they are structurally distinct. We conclude that the 125- and 35-kDa polypeptides that copurify with the DNA polymerase activity are bona fide subunits of Drosophila pol γ. However, the possibility remains that the two subunits are derived from a transient precursor polypeptide that is not detected in whole embryo extracts. This issue can only be resolved when the nuclear genes encoding the two subunits are isolated.

We explored structural and functional relationships in native Drosophila pol γ, in enzyme inhibition studies, and by limited in vitro proteolysis and photochemical cross-linking. We developed a potent and highly specific rabbit antiserum against native pol γ and found it to inhibit its 5′ → 3′ DNA polymerase and 3′ → 5′ exonuclease activities of bacteriophage T4 DNA polymerase (27), E. coli DNA polymerase III (28), and calf thymus DNA polymerase δ (26) do not this form of the enzyme exhibits either DNA polymerase or 3′ → 5′ exonuclease activity, or if the apparently intact β subunit remains associated, remains to be determined.

FIG. 6. Photochemical cross-linking of trypsin-digested Drosophila pol γ to template-primer DNA. Polymerase γ Fraction VI (7 units, 0.6 pmol) was incubated for 15 min at 20 °C with radiolabeled BrdUMP-substituted template-primer DNA (1.5 pmol), in the absence (lanes 1 and 2) or presence of 200 ng (lane 3) or 400 ng (lanes 4–6) of trypsin, and the digestion was terminated by addition of a large excess of protease inhibitors as described under “Methods.” Following incubation, the samples were irradiated with UV light for 15 min at 0 °C, processed, and electrophoresed in a 7.5% SDS-polyacrylamide gel, and the gel was autoradiographed. Lane 1, a UV-irradiated, no protein control; lane 2, pol γ irradiated without prior trypsin digestion; lanes 3 and 4, pol γ digested with 200 or 400 ng of trypsin, respectively, prior to UV irradiation; lane 5, a UV-irradiated, trypsin only control; lane 6, as in lane 2 except that bovine serum albumin was substituted for pol γ.  

We showed previously in an in situ gel assay that the α subunit of Drosophila pol γ contains the DNA polymerase function (5). To begin to dissect functional domains in pol γ, we subjected the native enzyme to limited trypptic digestion in the presence of template-primer DNA, followed by UV cross-linking and SDS-polyacrylamide gel electrophoresis and autoradiography. We found that limited trypptic digestion of native pol γ produces a form of the enzyme that retains DNA binding activity, at a level that produces a cross-linked product comparable in intensity to the intact enzyme (Fig. 6, lanes 3 and 4 versus lane 2). In the proteolyzed form, the polymerase catalytic subunit is trimmed from a 125- to an ~65-kDa DNA-binding polypeptide. Staining of the SDS-polyacrylamide gel with silver indicates two predominant digestion products of the α subunit of ~65 and 55 kDa, and an intact β subunit (data not shown). Notably, the same result is obtained when template-primer DNA is added before or after digestion with trypsin. However, the binding of template-primer DNA appears to protect the enzyme from further degradation, because while nearly quantitative conversion of the α subunit from a 125- to an ~65-kDa DNA-binding polypeptide is observed in the presence of template-primer DNA (Fig. 5, lanes 3 and 4), it is cleaved further to yield smaller polypeptides in the absence of template primer DNA, at a point where ~50% of the α subunit remains intact (data not shown). These data suggest that the 65-kDa polypeptide represents both a structural and a functional domain of the α subunit with respect to DNA binding. Whether or not this form of the enzyme exhibits either DNA polymerase or 3′ → 5′ exonuclease activity, or if the apparently intact β subunit remains associated, remains to be determined.

DISCUSSION

The subunit structure of mitochondrial DNA polymerase is an unresolved issue. Based on recent studies of the Drosophila (5), Xenopus (13), pig (14), and human (15) enzymes, we can propose a consensus subunit structure for animal mitochondrial DNA polymerase, in which a large polypeptide of 125–140 kDa containing the DNA polymerase function, is associated quantitatively with a smaller subunit of 35–50 kDa. However, because the frog (13) and pig (14) preparations contain polypeptides of intermediate size, some of which retain DNA polymerase activity, it is possible that all of the smaller polypeptides result from proteolysis of the polymerase catalytic subunit. To address this issue, we examined the intactness of Drosophila pol γ in a comparison by immunoprecipitation of crude versus nearly homogenous enzyme fractions. We found that both the size and the apparent stoichiometry of the α and β subunits are the same in extracts as compared to that observed in the isolated enzyme. Furthermore, we found by limited in vitro proteolysis of the two subunits that they are structurally distinct. We conclude that the 125- and 35-kDa polypeptides that copurify with the DNA polymerase activity are bona fide subunits of Drosophila pol γ. However, the possibility remains that the two subunits are derived from a transient precursor polypeptide that is not detected in whole embryo extracts. This issue can only be resolved when the nuclear genes encoding the two subunits are isolated.
not associate stably with template-primer DNA in the absence of the auxiliary proteins. That Drosophila pol γ does so, and catalyzes relatively efficient DNA synthesis on a variety of template-primer DNAs in the absence of accessory proteins (5, 6), might suggest that such factors are not required for mitochondrial DNA replication. In that regard, however, we have shown recently that single-stranded DNA-binding protein increases ~20-fold the rate of primer recognition and binding by pol γ (11, 12), raising the possibility that polymerase accessory proteins may enhance its function in vivo, but not be required under the in vitro conditions examined.

Photochemical cross-linking of the pol γ·template-primer DNA complexes revealed that the α but not the β subunit makes close contact with the DNA. Furthermore, limited proteolysis of the complexes with trypsin identified an ~65-kDa proteolytic intermediate of the α subunit, which retains DNA binding activity and is stabilized by the presence of DNA during protease digestion. We are currently evaluating the possibility that this form of pol γ retains enzymatic activity. Notably, the Klenow fragment of Eco pol I (68 kDa) retains both DNA polymerase and 3′ → 5′ exonuclease activity, while a 46-kDa C-terminal fragment of the Klenow enzyme retains only DNA polymerase activity (29). Given the structural similarity identified among DNA polymerases for which the three-dimensional structures have been determined (30), and the fact that both Eco pol I and pol γ belong to the family A DNA polymerase group (31), it seems reasonable to predict conservation of structure-function relationships between the two enzymes. That considered, it will be important to discern the structural features in mitochondrial DNA polymerase that impart its high fidelity and processivity in DNA synthesis, which distinguish it catalytically from Eco pol I.

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