Catalytic Subunit of Mitochondrial DNA Polymerase from Drosophila Embryos

CLONING, BACTERIAL OVEREXPRESSION, AND BIOCHEMICAL CHARACTERIZATION*

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A full-length cDNA of the catalytic subunit of mitochondrial DNA polymerase from Drosophila embryos has been obtained, and its nucleotide sequence was determined. The cDNA clone encodes a polypeptide with a deduced amino acid sequence of 1145 residues and a predicted molecular mass of 129.9 kDa. Amino-terminal sequence analysis of the mature catalytic subunit of the heterodimeric mitochondrial enzyme from Drosophila embryos identified the amino-terminal amino acid at position +10 in the deduced amino acid sequence, indicating a mitochondrial presequence peptide of only nine amino acids. Alignment of the catalytic subunit sequence with that of Escherichia coli DNA polymerase I Klenow fragment indicated a high degree of amino acid sequence conservation in each of the three DNA polymerase and three 3'→5' exonuclease domains identified by biochemical studies in the latter enzyme. Bacterial overexpression, purification, and biochemical analysis demonstrated both 5'→3' DNA polymerase and 3'→5' exonuclease in the recombinant polypeptide. This represents the first demonstration of 3'→5' exonuclease activity in the polymerase catalytic subunit of animal mitochondrial DNA polymerase.

Mitochondria are essential in animal cells and are the only organelles outside the nucleus that contain a DNA genome. Animal mtDNA is a double-stranded circular molecule with a highly conserved gene content and organization (1). Notably, although the animal mtDNA genome encodes 13 polypeptides that are required for the oxidative phosphorylation function of the mitochondrion, it does not encode a single protein required for its replication or expression. All of the proteins involved in the replication, transcription, and translation of animal mtDNA are encoded in nuclear genes.

We have studied the key replicative enzyme in mitochondria, mitochondrial DNA polymerase (pol γ). Drosophila pol γ is a heterodimer comprising two enzyme activities, 5'→3' DNA polymerase and 3'→5' exonuclease, in subunits of 125 and 35 kDa (2–4). Based on our work on Drosophila pol γ (2–4) and that of others on the Xenopus (5), pig (6), and human (7) enzymes, we proposed 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 (4). In biochemical studies to date, the role of the small subunit has not been determined nor has a subunit assignment for the 3'→5' exonuclease been made.

To address these issues and to elucidate structure-function relationships in animal mitochondrial DNA polymerase, we have pursued the molecular cloning of the two subunits of Drosophila pol γ. We report here the cloning, bacterial overexpression, and biochemical characterization of the polymerase catalytic subunit.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. [3H]dUTP was purchased from ICN Biochemicals; [32P]dATP was purchased from DuPont NEN. M13, pUC19, pET-11a, and pgt11 DNAs were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides as indicated in the text were synthesized in an Applied Biosystems model 377 oligonucleotide synthesizer.

Enzymes and Proteins—Drosophila pol γ Fraction VI was prepared as described by Wernette and Kaguni (2). T4 polynucleotide kinase and Escherichia coli DNA polymerase I and its Klenow fragment were purchased from New England Biolabs. Tag DNA polymerase and exonuclease III were purchased from Life Technologies, Inc. Nuclease S1 and T4 DNA ligase were purchased from Boehringer Mannheim and Pharmacia Biotech Inc., respectively. Protein A-alkaline phosphatase conjugate, bovine serum albumin, and pre-stained and unstained SDS molecular weight protein markers were purchased from Sigma. Human serum albumin and bovine carbonic anhydrase were purchased from Worthington.

Bacterial Strains and Bacteriophage—E. coli LE392 hsdR514, hsdM, supE44, supF58, lac Y1 or Δ(lacIZY)6, galK2, galT22, metB1, trpR55 was used for screening a λgt11 ovarian cDNA library from Drosophila melanogaster (the generous gift of Dr. Chuen-Sheue Chiang, Stanford University). E. coli XL-1 Blue recA1, endA1, gyr969, thi, hsdR17, supE44, relA1, lac (F' proB lacI51 ΔM15, Th10 (tetR)) was used to subclone the catalytic subunit cDNA for DNA sequence analysis. E. coli BL21 ΔDE3 ompT, rha ΔM9 was used for expression of pET-11a constructs.

Chemicals—Isopropylthio-β-D-galactoside, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma. Sodium metabisulfite and leupeptin were purchased from the J. T. Baker Chemical Co. and the Peptide Institute, Minoh-Shi, Japan, respectively.

Methods

Sequence Analysis of D. melanogaster pol γ—D. melanogaster pol γ was prepared as described by Wernette and Kaguni (2). The enzyme (255 pmol), purified from 1120-g embryos of average age 9 h, was denatured for 3 min at 65 °C in 1× Laemmli sample buffer and was
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electrophoresed in a 5–15% linear gradient SDS-polyacrylamide gel (13). The gel was stained with silver and destained for 30 min in a buffer containing 2% SDS, 10% (v/v) methanol, and 0.05% (v/v) acetic acid, and the protein-containing membrane (80 mm²) was excised and destained for 1.5 min in 1% (v/v) acetic acid and rinsed with water. The membrane was submitted for protein sequence analysis to Harvard MicroChem (Harvard University), where the membrane-bound protein was digested with trypsin, and the resulting polypeptide fragments were fractionated by microbore HPLC and sequenced by automated sequential Edman degradation using an Applied Biosystems model 477A pulse liquid peptide sequenator with an on-line model 120A PTH-AA Analyzer. The sequences obtained for four tryptic peptides are indicated in Fig. 1. The amino-terminal sequence of the intact 125 kDa polypeptide was obtained directly after transfer to polyvinylidene fluoride membrane.

Cloning of the Catalytic Subunit of D. melanogaster pol γ—We used the peptide sequence DDAFMDFLPLQQEQLH to generate two degenerate oligonucleotide probes, 5′-GA/T/C-A/GTGGCAAT/GTC/CTC/T/ T/GA/T/CATG-3′ (forward) and 5′-A/G/A/G/A/GTG/C/TT/C/TG/C/AT/GAC/T/CATG-3′ (reverse), for use in PCR synthesis on a T/A (48°C for 90 s, and 72°C for 1 min in a reaction volume of 0.1 ml containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 4 mM MgCl₂, 1 mM dithiothreitol, 200 μM each dATP, dGTP, dCTP, and dTTP, 0.5 μg of a 1711 cDNA, 25 pmol 5′-32P-labeled forward oligonucleotide primer (56,000 cpm/μmol), 100 pmol of reverse oligonucleotide primer, and 1.25 units Taq DNA polymerase. The expected product of 44 bp was obtained, purified by electrophoresis in a 12% denaturing polyacrylamide gel, and sequenced by the Maxam and Gilbert method (9). Based on the nucleotide sequence obtained, a 23-nucleotide deoxyoligomer, 5′-TC-GACCTGCAACGGCGAGCATATC-3′, was synthesized and used to screen the 1711 ovarian cDNA library by the Benton and Davis method (10) using E. coli LBB-2 as the bacterial host.

Fifty-three primary-screen positive colonies were obtained at a frequency of ~5 × 10⁻⁶. 26/28 of these remained positive in secondary/tertiary screenings. One plaque pure isolate with a 3.7-kilobase pair insert was subcloned and sequenced in its entirety. To do so, the 3.7-kilobase pair insert was amplified by PCR, and DNA fragments of 2.3 and 1.4 kilobase pairs were generated by cleavage with EcoRI restriction endonuclease sites at its ends, and the resulting DNA fragment was purified by gel electrophoresis, cleaved with NdeI, and cloned into the bacteriophage T7 promoter-based expression vector pET-11a (Novagen) at its unique NdeI site. The E. coli strain BL21 (DE3) (Novagen) was used for transformation, and ampicillin-resistant plasmid-containing cells were screened for insert size and orientation of recombinant DNA by restriction analyses.

For overexpression of the catalytic subunit, pET-11a recombinant plasmid-containing BL21 (DE3) cells were grown at 37°C with 0.2% tryptone, 2% yeast extract, 1 mM MgCl₂, and 10% (v/v) glycerol, with shaking at 250 rpm for 30–45 min. The cells were harvested by centrifugation at 12,000 × g for 10 min, and the supernatant fluid was recovered.

Phosphocellulose chromatography of the soluble extract was performed essentially as described by Wernette and Kaguni (2), except that the linear gradient was from 50–600 mM potassium phosphate. The catalytic subunit eluted at ~400 mM potassium phosphate; fractions were pooled and adjusted to 80% (v/v) sucrose with 10% (v/v) final concentration of hen egg white lysozyme (Boehringer Mannheim) and 0.25 M NaCl. The suspension was then sonicated with a Ultrasonic Processor model W-225 (Heat Systems, Ultrasonics, Inc.) for 10 pulses using the microtip at maximum output and 50% usage, followed by cooling in an ice water-salt bath. The sonication was repeated twice, and the sample was then centrifuged at 20,000 × g for 15 min. The supernatant fluid was recovered for use as the soluble protein fraction for phosphocellulose chromatography (see above). The pellet was resuspended in 1/80 volume of original cell culture in buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium metabisulfite, 2 μg/ml leupeptin) containing 10 mM NaCl and sonicated once. The sample was then centrifuged at 20,000 × g for 15 min, and the resulting supernatant fluid was removed and discarded. The pellet was suspended in the same volume of buffer A containing 1 M NaCl and recentrifuged. The previous step was repeated, and then the resulting pellet was washed by the same procedure in buffer A containing 10 mM NaCl. The final washed, insoluble pellet was extracted by incubation for 2 h in 1/300 volume of original cell culture in buffer A lacking glycerol and containing 2 mM urea (deionized by stirring at 25°C for 20 min with 0.2 g Amberlite MB-3 (Mallinckrodt)/ml of solution and then filtered). The extract was then centrifuged at 12,000 × g for 10 min, and the supernatant fluid was recovered.

DNA Polymerase and 3′ → 5′ Exonuclease Assay—DNA polymerase and 3′ → 5′ exonuclease activities were assayed on DNAse I-activated calf thymus DNA at 200 mM KCl and on M13 DNA at 120 mM KCl, respectively, as described by Olson et al. (4).

RESULTS

Cloning of the Catalytic Subunit of D. melanogaster DNA Polymerase γ—The heterodimeric mitochondrial DNA polymerase from D. melanogaster was purified to near-homogeneity from embryonic mitochondria as described by Wernette and Kaguni (2). The enzyme derived from 1120-g embryos was subjected to SDS-polyacrylamide gel electrophoresis, and the catalytic subunit polypeptide was transferred to polyvinylidene fluoride membrane. Following tryptic digestion and fractionation of the resulting peptides by microbore HPLC, amino-terminal sequence information was obtained on four tryptic peptides. We then used a 20-amino acid peptide sequence from one of these to generate two degenerate oligonucleotides for use as primers in PCR synthesis on a 1711 cDNA library derived from D. melanogaster ovarian mRNA. The expected product was recovered, and based on its DNA sequence, a 23-nucleotide DNA probe was synthesized and then used to screen the cDNA library. The largest cDNA obtained was 3.7 kilobase pairs in length, and its nucleotide sequence was determined in its entirety on both DNA strands.
co-workers in the crystal structure of Klenow to anchor two divergent metal ions (19). A similar conservation of acidic residues is found in the three polymerase domains. Thus, although we found in dissociation studies that the small subunit is important for exonuclease activity, the sequence analysis indicates that the exonuclease catalytic site also resides in the large subunit of the Drosophila enzyme.

Genomic DNA Structure of the Catalytic Subunit—A data base search revealed an unidentified genomic sequence containing the catalytic subunit of mitochondrial DNA polymerase that was derived from a D. melanogaster P1 genomic DNA library (GenBank accession numbers L39624 and L39625). The genomic sequence encoding the α-subunit is located on the left arm of chromosome 2 (subdivision 34D–34E) in the region containing the alcohol dehydrogenase gene (subdivision 35B). The gene contains three exons and two small introns. The first intron is 55 bp in length and splits the codon specifying G927 in the deduced amino acid sequence. The second intron is 59 bp in length and splits the codon specifying A927. Both introns are located in the region corresponding to the polymerase domain (see above). The sequences of the intron/exon boundaries are GTACGT and GTAAGT (donor site), and CAG (acceptor site), respectively. The nucleotide sequence of the exon in the genomic clone is 99% identical to that of the corresponding region of the cDNA.

A search for RNA polymerase II promoter elements in the genomic DNA sequence in the region upstream of the translation initiation codon failed to reveal a consensus TATA sequence. The ATG codon is located 58 bp downstream from the 5′-end of the cDNA. Within this region, a consensus transcriptional initiator TGAGT, is located 39 bp upstream from the translational start. No other initiator element is located within the 500 bp upstream of the initiation codon, nor were we able to identify cDNAs with longer 5′-leader sequences in PCR analyses of the cDNA library (data not shown). Taken together, these data suggest that the catalytic subunit gene is transcribed from a TATA-less promoter, with transcriptional initiation occurring at several sites in the upstream region immediately proximal to the translational start site.

The 3′-end of the cDNA clone contains a 12-nucleotide poly(A) sequence located 161 bp downstream from the TAG termination codon. No match to the consensus poly(A) signal sequence (AATAAA) is present, suggesting that another sequence, AATAGT, is located 39 bp upstream from the translational start. No other initiator element is located within the 500 bp upstream of the initiation codon, nor were we able to identify cDNAs with longer 5′-leader sequences in PCR analyses of the cDNA library.

Bacterial Overexpression and Purification of the Catalytic Subunit—To elucidate further structure-function relationships in pol γ, we pursued bacterial overexpression and purification of the catalytic subunit. To do so, we engineered by PCR an NdeI site surrounding the ATG at +1 of the α-subunit and at a site distal to the termination codon and inserted the coding region fragment into the NdeI site of the pET11a promoter-based expression vector pET-11a. Overexpression upon isopropylthio-β-D-galactoside induction of plasmid-containing BL21 (DE3) cells yielded —10 μg of α-subunit polypeptide/mL of cell culture. Despite the use of a variety of induction and lysis conditions, only 10–20% of the overproduced protein remained in the soluble fraction upon cell lysis. The soluble fraction derived from optimal induction and lysis conditions was subjected to chromatography on phosphocellulose, and fractions containing the recombinant polypeptide were pooled and concentrated by ammonium sulfate precipitation. At the same time, we pursued the purification of the catalytic subunit from insoluble fractions. Here, we used the salt-washing procedure of O'Donnell (20) to obtain inclusion body fractions.

1 MOPHILIRTEVSIVSEVDEVHVSSTKSVFRRKPKQQQVQFVEEPPKPKPEENVGHPTE
51 YAEKLVEQONHRLHGLQFPPQPSQIQEQVQAFYKDLERHENVDEVI
101 SGAPVDSLQLKLPRLGANIEEHFMHKEQVQYYFRELPLPQVCOELFK
151 RPKNWAFFHTWAIYDLPDTATMTYVPELKLQYQFDEVCLFSBDQFAYFLAT
201 AVSTKBHYNQVESLTKILVSPKLAEVDOVPSHPEPHPTPSFELPLOTS
251 GGPLVQVHNNYDARLKKEFYIYDDSTFVYVTMLKNVCMSVFTYSYRAM
301 LKSMKFPAARDDLGKSMQSLMBGMVHLLCDGTDLSKEFINVTLEEE
351 QYQVQFQSFLLNYCASDUEVHLRSLVQLYVARFPFASLNGMLSEA
401 VLPVFVHYMDIYQLAQTYDSLSEAKHLQGLAEACCSLLDDQYQRNL
451 WLKEDESLQVQKLEQPFRKPLFVTVEIPGSSFPERFQISHYQLYQ
501 QALLPAKLRLGPQPLYRKLRCXKEPAARDEILDESDEEPVSWGAQEST
551 GNQPIAESLKNWQRYLPYKRFQPPFPEDFSDQVDNLQMLQER
601 CPYFPEARSLASBSADAMLQILQGKEKXKQDOQILSTQ
651 YQGQGVYXKVLQCCFFKLPDLPNSFPRPVNLQDEKLFQNEFVLNM
701 GDPQCAQARRAVIMHMRMNIDGLQQLQWQDSMQGGLPMQGKTC
751 QPYAGIYCPQVACOTLSRANSEPHATGSDRDSLQGSLYMQAPP
801 GYRLVGADVQSELWATLQGYQQ-YAATPTGLMTLSGKHSNQG
851 KSTSSVQGSLNQVASTGDQYQTLTMLHPFOLPEFLGLTQAQGKXK
901 WIFPSITGRXKVRPLNEEPDELEASAYVSXVATXQRTMLAEVHVR
951 PHQGQESTSAVPSLVRAGATLQQPFPFPLGRMLKALGTAQPQFPFPL
1001 LTVPSLQLRTPLTFQVQTVQLSAKPAQSLQSLAKQHQTQTLQ
1051 PKALASSHTHMNTSURCVSVSGLQDLMRSMVAVPSQEVDTVLKKCEMTD
1101 CKTVSNLNLQGQDQLQQVSLASEZLQQGSGNQVQDIWXS

FIG. 1. Deduced amino acid sequence of the catalytic subunit of Drosophila pol γ. Amino acid residues enclosed in boxes correspond to those for which the amino acid sequence was determined by peptide sequencing as described under "Methods."
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Fig. 2. Alignment of the 3′→5′ exonuclease and DNA polymerase domains in family A DNA polymerases. The three conserved 3′→5′ exonuclease and three DNA polymerase domains in the catalytic subunit of pol γ from several sources were aligned with those of E. coli DNA polymerase I. Klenow fragment using the PileUp program of the GCG program package. Amino acid residues shown to be critical for catalytic activity in the Klenow fragment (17, 31–32) and in the exonuclease domains in S. cerevisiae MPI1 (18) are indicated by asterisks. An amino acid residue shown to be involved in nucleotide selectivity (33, 34) is indicated by the closed circle (see the text). Dmpolγ, D. melanogaster pol γ; Scpolγ, S. cerevisiae pol γ (13); Sppolγ, S. pombe pol γ (14, 15); Xlpolγ, X. laevis pol γ (15).

were then extracted with 2 M urea.

The soluble and extracted, insoluble fractions were assayed for DNA polymerase activity on DNase I-activated calf thymus DNA under standard conditions (2). The recombinant catalytic subunit exhibits DNA polymerase activity characteristic of that of pol γ from Drosophila embryos; DNA synthesis is stimulated severalfold by 200 mM KCl and is inhibited by d3TTP. Protein analysis by SDS-polyacrylamide gel electrophoresis, followed either by silver staining or by immunoblot analysis with rabbit antiserum against native pol γ from Drosophila embryos, identifies the overexpressed polypeptide of 125 kDa as the intact catalytic subunit (Fig. 3, A and B). Smaller overexpressed and immunoreactive polypeptides are also present. These likely result from proteolysis in vivo, because their relative abundance in cells extracted with SDS is comparable with that observed in purified fractions. Notably, however, the insoluble fractions generally exhibit a higher ratio of full-length versus truncated polypeptides relative to the soluble fractions, where the intact catalytic subunit represents up to 50% of the total protein, with α-subunit related polypeptides together constituting up to 80% of the total protein.

Biochemical Characterization of the Catalytic Subunit—Because the purity, integrity, and specificity of catalytic subunit derived from the urea-extracted insoluble fraction were greater than those of that derived from the soluble fraction, we used the former fraction for further biochemical characterization.

In a DNA binding analysis by gel electrophoretic mobility shift assay, we found that as with native pol γ (4), the catalytic subunit forms a stable and discrete complex with a 40-nucleotide template-primed DNA (Fig. 4A). Further, photochemical cross-linking of enzyme-DNA complexes in the presence of UV light demonstrates a specific association of the catalytic subunit with template-primed DNA that can be competed nearly completely by a 10-fold excess of unlabeled DNA substrate (Fig. 4B). Interestingly, although the specific activity of the isolated subunit in terms of its DNA polymerase function is about 20-fold lower than that of native pol γ, its DNA binding activity appears similar. This discrepancy might be explained by the possibility that truncated forms are capable of DNA binding but do not exhibit DNA polymerase activity.

To demonstrate the association of 3′→5′ exonuclease in the catalytic subunit, we subjected the urea-extracted insoluble fraction to sedimentation in a glycerol gradient. A single peak of DNA polymerase activity was observed, sedimenting at a position precisely coincident with a peak of 3′→5′ exonuclease activity (Fig. 5). Further, the ratio of DNA polymerase to 3′→5′ exonuclease across the peak fractions was invariant: the DNA polymerase-to-exonuclease ratios for the indicated fractions between 11 and 16 were 0.90, 0.96, 1.0, 1.0, 0.91, and 1.1, respectively, where the peak fraction (fraction 13) was assigned an arbitrary value of 1.0. The 3′→5′ exonuclease co-sedimenting quantitatively with DNA polymerase was well separated from a second broad peak of nonspecific nuclease activity that sedimented more slowly. The DNA polymerase- and 3′→5′ exonuclease-containing fractions also corresponded to fractions in which the 125-kDa catalytic subunit polypeptide was identified upon photochemical cross-linking of enzyme-DNA complexes. These data provide the first biochemical demonstration of 3′→5′ exonuclease in the catalytic subunit of animal mitochondrial DNA polymerase.

DISCUSSION

Mitochondrial DNA polymerase is the key enzyme involved in replication of the mtDNA genome. Inasmuch as pol γ is the sole DNA polymerase found in animal mitochondria (21), it serves a critical role in the maintenance of the genetic integrity of mtDNA, both in its replicative function and apparently in excision repair of some types of DNA lesions (22). We and others have shown previously that animal pol γ is highly accurate in nucleotide polymerization (23–27) and that it contains a mispair-specific 3′→5′ exonuclease that proofreads errors...
Bacterially expressed catalytic subunit (urea-extracted insoluble fraction) binds to template-primer DNA by the recombinant catalytic subunit. The reaction products were electrophoresed in a 4.5% native polyacrylamide gel, and the gel was autoradiographed. Lane 1 represents a no protein control. Lane 2 represents a sample containing pol γ Fraction VI (0.09 units, 7 fmol) and radiolabeled substrate DNA (0.11 pmol). Lanes 3–5 represent samples containing 0.06 units (96 fmol) of recombinant catalytic subunit and labeled substrate DNA (0.22 pmol) in the absence (lane 3) and the presence of unlabeled competitor DNA (lane 4, 1.1 pmol; lane 5, 2.2 pmol). B, photochemical cross-linking of the recombinant catalytic subunit to template-primer DNA. The samples were incubated with labeled substrate DNA as in A and irradiated with UV light for 15 min at 0°C. The DNA-protein complexes were processed and electrophoresed in a 7.5% SDS-polyacrylamide gel, and the gel was autoradiographed. Protein, labeled substrate DNA, and unlabeled competitor DNA were as in A. Lane 1 represents a no protein control. Lane 2 represents a sample containing pol γ Fraction VI. Lanes 3–5 represent samples containing the recombinant catalytic subunit. C, template-primer DNA.

during in vitro DNA synthesis (3, 27–29). Further, Foury and co-workers have shown that the 3′ → 5′ exonuclease in yeast pol γ contributes substantially to replication fidelity in vivo (18, 30). Nonetheless, in biochemical studies to date, a subunit assignment has not been made in an animal mitochondrial DNA polymerase. The cloning, bacterial overexpression, and biochemical characterization of the 125-kDa subunit of Drosophila pol γ demonstrates that the 3′ → 5′ exonuclease function resides in the polymerase catalytic subunit.

We have shown previously that Drosophila pol γ is a heterodimer and that its DNA polymerase function resides in the 125-kDa polypeptide (3, 4). Despite extensive efforts to dissociate and reconstitute the two subunits in order to make a subunit assignment for the 3′ → 5′ exonuclease and to examine the role of the 35-kDa polypeptide in enzyme function, we were unable to achieve subunit separation with retention of exonuclease activity (4). However, alignment of the catalytic subunit sequence with the sequence of E. coli DNA polymerase I Klenow fragment allows the identification of the three 3′ → 5′ exonuclease domains conserved in family A DNA polymerases, including yeast γ polymerases (Fig. 2). During the preparation of this manuscript, Bogenhagen and co-workers reported the sequence of the polymerase catalytic polypeptide in X. laevis pol γ (15), which also contains the conserved exonuclease domains (Fig. 2). Thus, although the subunit structures of other mitochondrial DNA polymerases have not been well defined, it is now apparent that both DNA polymerase and 3′ → 5′ exonuclease reside in a large polypeptide in fungal and both vertebrate and invertebrate animal forms.

A number of amino acid residues in the three conserved 3′ → 5′ exonuclease domains are identical in the γ polymerase subfamily and in E. coli DNA polymerase I, the prototype of the family A DNA polymerase group (16, Fig. 2). These include three aspartate residues shown in biochemical studies to be critical for 3′ → 5′ exonuclease activity in E. coli pol I Klenow fragment (17) and S. cerevisiae MIP1 (18), and by Beece et al. (19) in the crystal structure of Klenow to anchor two divalent metal ions. Likewise, in the DNA polymerase domains, four acidic residues (Asp709, Glu710 Asp882, and Glu 883) identified in Klenow as critical for catalysis (31, 32) are invariant in the γ pols. The γ pols also contain a second invariant aspartate in the first conserved polymerase domain at a position corresponding to Ser797 in Klenow. It will be interesting to explore through mutagenesis studies the possibility that the additional residue also plays a role in catalysis by pol γ and/or that it could substitute for the roles of either Asp709 or Glu710 in Klenow.

The γ pols contain two invariant amino acids in the conserved Pol II domain that were shown to be involved directly in dNTP binding by Klenow (Arg754 and Lys758; Ref. 33). Notably, in the same region, the γ pols all contain a tyrosine residue at a position corresponding to Phe762 in Klenow. Joyce and co-workers showed that Phe762 is also involved in dNTP binding and postulated that deoxyribose would be a plausible contact (33). Tabor and Richardson (34) demonstrated subsequently that this residue is responsible for determining the moderate sensitivity of Klenow to ddNTPs and that the high ddNTP sensitivity of bacteriophage T7 DNA polymerase, another member of the family A group, results from it containing a tyrosine at this position. Thus it seems likely that the ddNTP sensitivity of mitochondrial DNA polymerase also involves flexible nucleotide selectivity at this site and that the mitochondrial toxicity observed in long term antiviral therapy involving dideoxynucleoside analogs is a direct consequence of it.

A significant difference between animal γ pols and E. coli pol I Klenow is the spacing between the 3′ → 5′ exonuclease and...
the DNA polymerase domains. Whereas the separation is 196 amino acid residues in Klenow, it is 434 and 479 residues in the fly and frog sequences, respectively. In the Klenow structure determined with enzyme-DNA co-crystals, this region is involved in template-primer DNA binding (19). Notably, both the Drosophila and Xenopus catalytic polypeptides contain a putative leucine zipper domain in comparable locations within this region. In the Drosophila catalytic subunit, the putative leucine zipper involves amino acid residues 487–514. Inasmuch as we have demonstrated Drosophila pol γ to be a heterodimer, it is possible that the subunit interaction site is located within this region, allowing the interesting speculation that the small subunit is involved in enzyme processivity.

Through bacterial overexpression, purification, and partial biochemical characterization of the polymerase catalytic subunit, we have demonstrated 3′ → 5′ exonuclease catalytic activity. The general biochemical features of both activities are similar to those in Drosophila pol γ; the DNA polymerase is salt-stimulated and sensitive to ddNTPs, and the 3′ → 5′ exonuclease is highly mispair-specific. Further, the ratio of DNA polymerase to exonuclease activity in the recombinant catalytic subunit is similar to that in native pol γ. Thus, it will be of substantial interest to reexamine the more subtle biochemical features of the enzyme, including processivity and fidelity of nucleotide polymerization, to discern the role of the small subunit in holoenzyme function.

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Catalytic Subunit of Mitochondrial DNA Polymerase from *Drosophila* Embryos: CLONING, BACTERIAL OVEREXPRESSION, AND BIOCHEMICAL CHARACTERIZATION

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