Accessory Subunit of Mitochondrial DNA Polymerase from Drosophila Embryos

CLONING, MOLECULAR ANALYSIS, AND ASSOCIATION IN THE NATIVE ENZYME*

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A full-length cDNA of the accessory (β) 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 361 residues and a predicted molecular mass of 41 kDa. The gene encoding the β subunit lies within 4 kilobase pairs of that for the catalytic subunit in the Drosophila genome, on the left arm of chromosome 2. The two genes have similar structural features and share several common DNA sequence elements in their upstream regions, suggesting the possibility of coordinate regulation. A human cDNA homolog of the accessory subunit was identified, and its nucleotide sequence was determined. The human sequence encodes a polypeptide with a predicted molecular mass of 43 kDa that shows a high degree of amino acid sequence similarity to the Drosophila β subunit. Subunit-specific rabbit antisera, directed against the recombinant catalytic and accessory subunit polypeptides overexpressed and purified from Escherichia coli, recognize specifically and immunoprecipitate the native enzyme from Drosophila embryos. Demonstration of the physical association of the two subunits in the Drosophila enzyme and identification of a human accessory subunit homolog provide evidence for a common heterodimeric structure for animal mitochondrial DNA polymerases.

Animal mitochondria are essential energy-producing organelles that contain multiple copies of their double-stranded circular DNA genome. Accumulating evidence documents the involvement of specific mitochondrial DNA (mtDNA)* mutations in the pathogenesis of genetic and aging-related degenerative diseases in humans that involve the central nervous system, heart, muscle, endocrine system, kidney, and liver (1). The mitochondrial genome encodes proteins required for oxidative diseases in humans that involve the central nervous system, heart, muscle, endocrine system, kidney, and liver (1). The mitochondrial genome encodes proteins required for oxidative phosphorylation; it does not, however, encode any of the proteins that are required for its faithful duplication. A single nuclear encoded DNA polymerase (pol γ) is involved in the replication of animal mtDNA (2). Because pol γ and all of the other proteins required for mitochondrial DNA replication are encoded by nuclear genes and imported into mitochondria from the cytoplasm, mutations in nuclear genes can also affect the integrity of mtDNA. In fact, human diseases resulting from multiple mtDNA deletions have been shown to be caused by alterations in nuclear encoded genes (3). At the same time, antiviral drugs (such as zidovudine) administered in long term therapy have been shown to induce mitochondrial dysfunction resembling that in mitochondrial genetic disease as a result of their inhibitory effects on pol γ (4).

We are studying Drosophila embryos as an animal model of mitochondrial function and have shown that Drosophila pol γ in its native form is a heterodimer of 125- and 35-kDa subunits (5, 6). The large (α) subunit contains both 5′→3′ DNA polymerase and 3′→5′ exonuclease activities (5, 7). Although we have been unable to assign a specific biochemical function to the accessory (β) subunit, we have shown that it is important in maintaining the catalytic efficiency and/or the structural integrity of the holoenzyme (6). We have recently cloned and characterized biochemically the catalytic subunit of Drosophila pol γ (7). The catalytic subunit has also been cloned from several yeasts, Xenopus, man, and mouse (8–11). However, the subunit structure of native pol γ from these sources remains an unresolved issue.

We report here the cloning and molecular analysis of the β subunit of Drosophila pol γ and demonstrate its association in the native enzyme. We also describe the identification of a human accessory subunit homolog, providing evidence for a common heterodimeric structure in animal mitochondrial DNA polymerases.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. [γ-32P]ATP was purchased from ICN. Plasmid pUC119, pET-11a, and agt11 DNAs were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides as indicated below were synthesized in an Applied Biosystems Model 477 digoxigenidolide synthesizer.

Enzymes and Proteins—Drosophila pol γ Fraction VI was prepared as described by Wernette and Kaguni (5). T4 polynucleotide kinase, T4 DNA ligase, and S1 nuclease were purchased from Boehringer Mannheim. Tag DNA polymerase and exonuclease III were from Life Technologies, Inc.

Bacterial Strains and Bacteriophage—Escherichia coli LE392 (hidR514, hasA, supE44, supF58, lac Y1 or ΔlacZYA6, galK2, galT22, metB1, trpR55) was used for screening a S11 ovarian cDNA library from Drosophila melanogaster (the generous gift of Dr. Chuen-Shue Chiang, Stanford University). E. coli XL1-Blue (recA1, endA1, gyrA96, thi, lacI17, supE44, relA1, lacF- proAB, lacF-I ΔM15, Tn10(tet)) was used to subclone the β subunit cDNA for sequence analysis. E. coli BL21(DE3) (ompT, r b M )- was used for the expression of pET-11a constructs.

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Chemicals—Isopropyl-β-D-thiogalactopyranoside, nitro blue tetrazo-
lum, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma. Sodium metabisulfite and leupeptin were purchased from J. T. Baker Inc. 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 (5). The enzyme (255 pmol), purified from 1120 g of embryos with an average age of 9 h, was denatured for 3 min at 65 °C in 1 × Laemmli sample buffer (12) and was electrophoresed on a 5–15% linear gradient SDS-polyacrylamide gel (12). After electrophoresis, the gel was soaked for 10 min in transfer buffer (10 mM CAPS, pH 11.0, 10% (v/v) methanol, and 0.056% (v/v) SDS), and the 35-kDa β subunit polypeptide was transferred to polyvinylidene difluoride membrane (Westran, Schleicher & Schuell; pre-soaked for 10 min in metha-
ol and then for 15 min in transfer buffer) for 16 h at 150 mA using a Hoefer Transphor electrophoresis unit (Model TE22). The polyvinylidi-
dene difluoride membrane was then stained for 90 s in 0.5% Ponceau S (Sigma) and 1% (v/v) acetic acid and destained for 90 s in 1% (v/v) acetic acid, and the protein-containing membrane (80 mm²) was excised and rinsed with water. The membrane was submitted for protein sequence analysis to Harvard MicroChem (Harvard University), where the mem-
brane was digested with trypsin, and the tryptic polypeptides 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 phenylthiohydantoin-derivative analyzer. The amino-terminal se-
quencies of five tryptic peptides are indicated in Fig. 1.

Cloning of the β Subunit of D. melanogaster pol γ—We used the peptide sequence IVPHELDAEILNPNDYQADHR to generate two degenerate oligonucleotide primers, 5′-GATC/CTT/C/GAC/AGCTG/ (AG/GA/CA/CT/CT/T-3′ (forward) and 5′-AT/AG/TC/AG/AT/AGCTG/ GC/CT/TA/GA/TA-3′ (reverse), for use in PCR synthesis on a
Model 477A pulse liquid peptide sequenator derived from an on-line Model 120A phenylthiohydantoin-derivative analyzer. The amino-terminal se-
quencies of five tryptic peptides are indicated in Fig. 1.

Preparation of bacterial cell extracts was as described for the cata-
ytic subunit by Lewis et al. (7), except that the lysis buffer contained 0.1 mM NaCl, and detergents (0.5% (w/v) deoxycholic acid and 0.5% (v/v) Nonidet P-40) were added to the cell extract prior to centrifugation to recover the soluble fraction. The resulting pellet was resuspended in 0.5 volume of original cell culture fluid (0.1 mM EDTA; then the sample was re-frozen for 10 min, and the resulting supernatant fluid was removed and discarded. The previous step was repeated three times, and the final “detergent-washed, insoluble pellet” was extracted with 7 M urea as described by Lewis et al. (7).

For antibody production, the recombinant α- and β subunits obtained from an extracted insoluble fractions were electrophoresed on a 10% SDS-polyacrylamide gel, and the protein-containing bands were ex-
cised. The gel slices were minced in 10 mM NaPO₄, pH 7.0, and 154 mM NaCl (PBS) and emulsified with an equal volume of Freund’s complete adjuvant. Immunizations of virgin female New Zealand White rabbits were performed by subcutaneous injections of 15–30 μg of protein. Booster immunizations were administered in Freund’s incomplete adjuvant at 2–4-week intervals.

Protein Gel Electrophoresis, Transfer, and Immunoblotting—SDS-
polyacrylamide gel electrophoresis was performed according to
Laemmli (12). Proteins were transferred to nitrocellulose membranes (BA85, Schleicher & Schuell) and probed by immunoblotting using the goat anti-rabbit IgG-alkaline phosphatase (Bio-Rad) method as de-
scribed.

Immunoprecipitation of Drosophila DNA Polymerase γ—Pol γ Fraction VI (~1 μg) was diluted 1:1 with PBS, incubated for 10 min at 65 °C, and then incubated with preimmun serum or subunit-specific rabbit antiserum overnight on ice. Immune complexes were precipitated by incubation with preswollen protein A-agarose (50 μl of a 50% slurry) for 2 h with gentle rotation. The precipitates were collected by centrifuga-
tion, washed three times in PBS, suspended in Laemmli sample buffer, heated for 10 min at 85 °C, and re-centrifuged. The supernatant frac-
tions were then subjected to immunoblotting analysis as described by Olson et al. (6).

RESULTS

Cloning of the β Subunit of D. melanogaster DNA Polymerase γ—D. melanogaster mitochondrial DNA polymerase holo-
zyme was purified to near homogeneity from embryonic mito-
chondria as described by Wernette and Kaguni (5). The enzyme
derived from 1120 g of embryos was subjected to SDS-poly-
acrylamide gel electrophoresis, and the β subunit polypeptide
was transferred to polyvinylidene difluoride membrane. Fol-
derowing tryptic digestion and fractionation of the resulting pep-
tides by microbore HPLC, five amino-terminal sequences were obtained. Degenerate oligonucleotides were constructed that corresponded to amino- and carboxyl-terminal amino acid res-
dues of one of the peptides and were used to amplify by PCR D. melano-
gaster cDNA fragments as described under “Methods.” A correctly sized PCR product was recovered by gel purifica-
tion, and DNA sequence analysis confirmed that it corre-
sponded to the amino acid sequence of the tryptic peptide. A 23-nucleotide DNA probe was then synthesized and used to screen a βgt11 cDNA library derived from D. melanogaster ovarian mRNA. Tertiary-screen positive clones (seven) were obtained. Based on the nucleotide sequence, a cDNA insert was amplified by PCR and subcloned into plasmid pUC119 at its unique
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An autoradiograph of 1083 nucleotides was identified, encoding a β subunit polypeptide of 361 amino acid residues with a predicted molecular mass of 41 kDa (Fig. 1). This is consistent with the size of 35 kDa determined for the small subunit in purified pol γ (5). The amino-terminal sequences of all five tryptic peptides derived from the β subunit of the native enzyme are located within the deduced amino acid sequence of
the β subunit cDNA. Because of the limited amount of β subunit polypeptide derived from native pol γ, we did not obtain the amino-terminal sequence of the mature β subunit. However, because the amino terminus of one of the tryptic peptides is located at position 119 in the deduced amino acid sequence, the mitochondrial presequence peptide is no longer than 18 amino acid residues. No other in-frame translational initiation codon is present between the 5′-end of the cDNA and the ATG codon at position 11, although there are multiple in-frame stop codons.

Identification of a Human Homolog of the β Subunit—No homolog of the β subunit was identified upon searching the latest releases of the GenBank™ and EMBL databases (Releases 98 and 49, respectively) or the Saccharomyces genome database. However, we detected a partial human cDNA sequence (GenBank™ accession number H05453) in the dbEST (expressed sequence tag) database of the National Center for Biotechnology Information in a BLAST search using the deduced amino acid sequence of the Drosophila β subunit as query. We obtained the human cDNA (I.M.A.G.E. Consortium LLNL) Clone ID number 44673), determined the nucleotide sequence in its entirety on both DNA strands (1606 bp), and found it to contain a complete open reading frame.

The human β subunit homolog has a deduced amino acid sequence of 372 residues, comparable to that of 361 residues for the Drosophila polypeptide. Alignment of the Drosophila and human sequences using the Gap program of the Genetics Computer Group program package (Version 7) shows 31% amino acid sequence identity and 60% sequence similarity overall. The C-terminal regions contain a leucine zipper motif with the sequences L(\(X\))6L(\(X\))6L(\(X\))6L(\(D\), positions 1321–342) and V(\(X\))6L(\(X\))6L(\(X\))6M (human, positions 1328–349) (Fig. 2). This 22-amino acid stretch represents the most highly conserved sequence in the polypeptide, with 55% sequence identity and 86% similarity. The Drosophila β subunit also contains a zinc finger motif of the C2H2 class on the amino-terminal side of the leucine zipper motif (positions 257–285). Although the human homolog lacks this motif, it contains a zinc-binding signature sequence (LGDFELHMY) found in neutral zinc
metallopeptidases (16), near the middle of the polypeptide at positions +193–202.

**Genomic DNA Structure of the β Subunit of Drosophila pol γ**—The data base search revealed an unidentified genomic sequence corresponding to the β subunit cDNA that was derived from a *D. melanogaster* P1 genomic DNA library (GenBank™ accession numbers L39626 and L39627). Remarkably, the same P1 clone contains the catalytic subunit gene of *D. melanogaster* mitochondrial DNA polymerase (mtPolA) (7). Furthermore, the genes encoding the two subunits of the native holoenzyme are separated only by an interval sequence of 3.8 kilobase pairs, with the β subunit gene located 5′ to the catalytic subunit gene (Fig. 3).

Comparison of the genomic and cDNA sequences indicates that the β subunit gene contains only one small intron of 54 nucleotides that splits the codon specifying Ala231 in the de-duced amino acid sequence. The intron/exon boundaries are GTAAGT (donor site) and TAG (acceptor site), respectively. The coding sequence of the exons in the genomic clone is 99% identical to that of the corresponding regions of the cDNA. The intron/exon boundaries are separated only by an interval sequence of 3.8 kilobase pairs, with the β subunit gene located 5′ to the catalytic subunit gene (Fig. 3).

A search for RNA polymerase II promoter elements in the genomic sequence 16 bp upstream from the 5′-end of the cDNA sequence, and the translational initiation codon is located at a position 459 bp downstream (Fig. 3). Two additional consensus transcriptional initiator sequences are located within 500 bp in the upstream region. Interestingly, a *Drosophila* promoter-activating element, DRE, previously identified in a number of genes involved in nuclear DNA replication (18), is located 230 bp upstream from the putative translational start site. Furthermore, potential binding sites for nuclear respiratory factor 1 (NRF-1) (19) and the transcription factor E2F (position 2028) and potential NRF-1 (positions −53, −494, and −860)- and E2F (position −899)-binding sites are also present in the upstream region of the catalytic subunit gene of *Drosophila* pol γ.

The 3′-end of the cDNA clone contains an 18-nucleotide poly(A) sequence. Surprisingly, the poly(A) sequence begins within the translational termination codon (TAATAAAA). As with the comparison of the cDNA and genomic sequences, the polyadenylation site is located no more than six nucleotides downstream from the termination codon. This feature is common in mitochondrially encoded genes and may be of evolutionary significance. As with the catalytic subunit cDNA (7), no match to the consensus poly(A) signal sequence (AAATAAA) is present in the β subunit cDNA, although 23 out of 24 of the nucleotides immediately upstream of the termination codon are deoxyadenylate or thymidylate residues. Interestingly, both the catalytic and β subunit cDNAs contain the sequence AAATAA at positions −51 and −16, respectively, relative to the polycadenylation site, which might serve as the poly(A) signal. This alternative signal has been implicated in directing polyadenylation of developmentally regulated transcripts in several *Drosophila* genes (21–24). Taken together, the presence and locations of common transcriptional and post-transcriptional regulatory signals in the catalytic and accessory subunit genes of *Drosophila* pol γ suggest the possibility of coordinate gene regulation of the heterodimeric mitochondrial DNA polymerase.

**Bacterial Overexpression and Purification of the β Subunit and Production of Subunit-specific Antiserum**—To elucidate structure-function relationships in *Drosophila* pol γ, we pursued bacterial overexpression and purification of the β subunit and prepared rabbit antisera to both subunits. To do so, we engineered by PCR an NdeI site surrounding the ATG codon at position +1 of the β subunit and at a site distal to the termination codon. We then inserted the coding region fragment into the NdeI site of the bacteriophage T7 promoter-based expres-
The accessory subunit of mitochondrial DNA polymerase remains unresolved. The redundant nature of mitochondrial DNA does not eliminate the requirement for high fidelity DNA replication because the accumulation of mutations in mtDNA is linked to degenerative diseases in humans (1). The key replicative enzyme in mitochondria is pol γ. Mitochondrial DNA polymerases from several species including chicken, pig, fly, frog, yeast, and man have been demonstrated to contain dual enzymatic activities (27–32). They possess both highly accurate DNA polymerase and mispair-specific 3' → 5' exonuclease activities. While we have shown that Drosophila pol γ is a heterodimer of 125- and 35-kDa subunits (5), the structure of other mitochondrial DNA polymerases remains unresolved.

In a recent report, we presented physical and immunological evidence that the 35-kDa subunit of Drosophila pol γ is intact and structurally distinct from the 125-kDa catalytic subunit (6). Here we demonstrated that the β subunit is encoded by a distinct nuclear gene and bears no similarity to the catalytic β subunit in its amino acid sequence. Furthermore, we found that rabbit antisera directed against recombinant polypeptides recognize specifically and immunoprecipitate the native enzyme and that the catalytic and β subunit polypeptides form a physical complex in native pol γ. These data are consistent with subunit dissociation studies of native pol γ that showed that subunit separation by gel filtration or velocity sedimentation in the presence of denaturants likely occurs only upon partial denaturation of the enzyme (6).

**DISCUSSION**

The redundant nature of mitochondrial DNA does not eliminate the requirement for high fidelity DNA replication because the accumulation of mutations in mtDNA is linked to degenerative diseases in humans (1). The key replicative enzyme in mitochondria is pol γ. Mitochondrial DNA polymerases from several species including chicken, pig, fly, frog, yeast, and man have been demonstrated to contain dual enzymatic activities (27–32). They possess both highly accurate DNA polymerase and mispair-specific 3' → 5' exonuclease activities. While we have shown that Drosophila pol γ is a heterodimer of 125- and 35-kDa subunits (5), the structure of other mitochondrial DNA polymerases remains unresolved.

In a recent report, we presented physical and immunological evidence that the 35-kDa subunit of Drosophila pol γ is intact and structurally distinct from the 125-kDa catalytic subunit (6). Here we demonstrated that the β subunit is encoded by a distinct nuclear gene and bears no similarity to the catalytic β subunit in its amino acid sequence. Furthermore, we found that rabbit antisera directed against recombinant polypeptides recognize specifically and immunoprecipitate the native enzyme. We conclude that the 35-kDa polypeptide is a bona fide subunit of Drosophila pol γ.

The nuclear gene encoding the β subunit is located at a distance only 3.8 kilobase pairs from the catalytic subunit gene, on the left arm of chromosome 2. That the genes encoding the two subunits of the mitochondrial holoenzyme are linked in the nuclear genome may be of evolutionary significance given current hypotheses regarding the bacterial origin of mitochondria. At the same time, the phenomenon may relate to the present day expression of the genes. The genomic location, similar gene
structure (i.e. TATA-less promoter, presence of consensus transcriptional initiator and alternative poly(A) signal sequences, small introns, and short 3′-untranslated region), and the presence of the DRE and potential binding sites for transcription factors NRF-1 and E2F in the 5′-upstream region suggest that the two genes share common regulatory mechanisms and the possibility that they are coordinately regulated. The DRE is the recognition sequence for an 80-kDa DNA-binding protein, DREB (18, 33), that is responsible for activating the promoters of nuclear replication genes in Drosophila, including proliferating cell nuclear antigen-, cyclin A-, and DNA polymerase α-encoding genes (34, 35). Functional NRF-1 sites have been identified in many mammalian nuclear genes that encode mitochondrial proteins. These include cytochrome c, mitochondrial transcription factor A, and at least one subunit of each of the respiratory complexes III, IV, and V (19, 36). Eight members of the E2F family in mammals have been shown to form heterodimeric complexes that are crucial for transcriptional activation of genes regulating S phase entry (e-myC and cyclin E) and genes functioning to engage DNA synthesis (dihydrofolate reductase, thymidine kinase, proliferating cell nuclear antigen, and pol α) (20). Drosophila E2F-1 is essential for activation of pol α gene expression (37) and is required for the G1 to S phase transition during embryogenesis (38). The DRE, NRF-1, and E2F sequence elements are found in the Drosophila pol γ genes and is consistent with the facts that mitochondrial DNA polymerase is required for mtDNA replication and hence for cell proliferation and that pol γ activity varies greatly during development and is highest in embryos (5).

Molecular cloning, bacterial overexpression, and biochemical analysis of the catalytic subunit allowed us to assign both 5′→3′ DNA polymerase and 3′→5′ exonuclease functions to the large subunit of Drosophila pol γ (7). Likewise, Foury (31) had shown, via its overexpression in mitochondrial extracts, that the 140-kDa product of the MIP1 gene contains both activities in Saccharomyces cerevisiae pol γ. Cloning and sequence alignments have also shown that a large polypeptide of 115–140 kDa contains both the conserved DNA polymerase and exonuclease domains in pol γ from several yeasts, Xeunopus, man, and mouse (8–11). However, pol γ purified from Xenopus, pig, and human cells has been shown to contain a large polymerase catalytic subunit and several smaller polypeptides, leaving unresolved the issue of the native structure of these enzymes (28, 32, 39). Our identification of a human homolog of the accessory subunit of mitochondrial DNA polymerase provides evidence for a common structure in animal mitochondrial DNA polymerases.

The accessory subunit of Drosophila pol γ and its human homolog represent novel proteins of 41 and 43 kDa, respectively. They contain a single highly conserved amino acid sequence, a leucine zipper motif in the C terminus of the polypeptides. Notably, a single leucine zipper motif is also present in the catalytic subunit sequences of Drosophila, Xenopus, man, and mouse (7, 10, 11). In each case, the leucine zipper motif lies between the conserved DNA polymerase and exonuclease domains. We suggest that the putative leucine zipper domains in the catalytic and accessory subunits represent the subunit interaction domain. If so, we would propose that both the heterodimeric composition and the structural basis for subunit interaction are conserved among animal pol γ holoenzymes.

What then is the role of the accessory subunit in Drosophila pol γ? To date, we have been unable to assign it a biochemical function. We have shown that Drosophila pol γ is both highly processive and highly accurate in nucleotide polymerization (40, 41), but have been unable to achieve subunit separation without nearly complete loss of catalytic activity (6). We have, however, demonstrated DNA polymerase and 3′→5′ exonuclease activities in the bacterially expressed catalytic subunit, albeit at ~20-fold reduced specific activity (7). Because the leucine zipper motif lies between the DNA polymerase and exonuclease domains in the catalytic subunit and structural studies on enzyme-DNA cocrystals identified a role for this region in template-primer DNA binding by E. coli DNA polymerase I Klenow fragment (42), we speculated that the small subunit may be involved in pol γ processivity (7). Remarkably, Richardson and co-workers (43) have recently shown that the processivity subunit of bacteriophage T7 DNA polymerase, E. coli thioredoxin, can also confer high processivity on an E. coli DNA polymerase I recombinant that was engineered to contain, between its exonuclease and DNA polymerase domains, the thioredoxin-binding domain of the T7 catalytic polypeptide. Notably, although each of these DNA polymerases is a member of the family A DNA polymerase group (44), a heterodimeric holoenzyme structure extends beyond this group. For example, herpes simplex virus DNA polymerase and nuclear pol α are members of the family B group (44). In herpesvirus replication, the virus-encoded UL42 protein functions as a processivity factor for the herpes DNA polymerase (45). The small subunit of animal pol α apparently functions to recruit its processivity factor, proliferating cell nuclear antigen (46–49). Inasmuch as Drosophila pol γ is highly processive in its heterodimeric form, we would suggest that the β subunit is itself the processivity factor. Further biochemical characterization of the recombinant catalytic subunit and reconstitution studies of the holoenzyme form should allow us to resolve this issue and to determine the structural basis for holoenzyme assembly.

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