Baculovirus Expression Reconstitutes Drosophila Mitochondrial DNA Polymerase*

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Drosophila mitochondrial DNA polymerase has been reconstituted and purified from baculovirus-infected insect cells. Baculoviruses encoding full-length and mature forms of the catalytic and accessory subunits were generated and used in single and co-infection studies. Recombinant heterodimeric holoenzyme was reconstituted in both the mitochondria and cytoplasm of SF9 cells and required the mitochondrial presequences in both subunits. The recombinant holoenzyme contains DNA polymerase and 3'-5' exonuclease that are stimulated substantially by both salt and mitochondrial single-stranded DNA-binding protein. Thus, the recombinant enzyme exhibits biochemical properties indistinguishable from those of the native enzyme from Drosophila embryos. Production of the catalytic subunit alone yielded soluble protein with the chromatographic properties of the heterodimeric holoenzyme. However, the purified catalytic core has a 50-fold lower specific activity. This provides evidence of a critical role for the accessory subunit in the catalytic efficiency of Drosophila mitochondrial DNA polymerase.

Recent progress in genetic, biochemical, and structural studies of DNA polymerases (pol I-III) has expanded our understanding of their structure and mechanism. Eight distinct DNA polymerases have been identified in eukaryotic cells, and they are all encoded by nuclear genes (1-4). Although all perform the same basic enzymatic reaction of nucleotide addition to the 3'-end of a primer, eukaryotic cells have evolved a varied set of DNA polymerases to carry out DNA synthesis on different substrates in replication, recombination, and repair. Seven of the eight DNA pols have been demonstrated to be nuclear enzymes involved in either nuclear DNA replication or DNA repair. In contrast, pol γ is the sole DNA polymerase implicated in the replication of animal mitochondrial DNA (5), and it may also carry out base excision repair because it contains a 5' deoxyribosyl phosphate lyase activity in the catalytic subunit and participates in DNA repair at abasic sites in vitro (6, 7).

Although the subunit structure of DNA polymerases is generally conserved within a DNA polymerase class, that of mitochondrial DNA polymerase has been an unresolved issue. pol γ from Drosophila is a heterodimer of 125- and 35-kDa subunits (8), whereas in budding yeast it appears to be a single polypeptide encoded by the MIP1 gene (9). We have identified human, mouse, and rat homologs of the small accessory subunit of Drosophila pol γ (10), but none has been identified in yeast or nematodes. The very low abundance of pol γ in animal cells, where it represents ~1% of the total DNA polymerase activity (11), has limited studies of its structure and mechanism. Nonetheless, the discovery of both mitochondrial DNA diseases (12), and the severe inhibitory effects on pol γ of antiviral and antitumor drugs (13, 14) emphasizes the critical need for these studies.

The baculovirus expression system provides a eukaryotic environment for recombinant protein production. We have taken advantage of its features of high level expression and capacity for simultaneous expression of multiple genes to achieve the reconstitution, purification, and biochemical characterization of a recombinant form of Drosophila pol γ from insect cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Enzymes and Proteins—**Drosophila pol γ Fraction VI was prepared from embryonic mitochondria as described by Wernette and Kaguni (8). mitochondrial single-stranded DNA-binding protein (mtSSB) was purified as described by Farr et al. (15). Polyclonal antisera raised against bacterially produced recombinant α- or β-subunit were as described by Wang et al. (10).

**Nucleotides and Nucleic Acids—**Baculovirus transfer vector pVL1392/pVL1393 and linearized wild type baculovirus AcMNPV DNA (BaculoGold) were purchased from PharMingen. Wild type baculovirus AcMNPV was the gift of Dr. Suzanne Thieme (Department of Entomology, Michigan State University). Synthetic oligodeoxynucleotides as indicated below were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer.

**Insect Cells and Tissue Culture Medium—**SF9 (Spodoptera frugiperda) cells were the gift of Dr. Suzanne Thieme. TC-100 insect cell culture medium and fetal bovine serum were from Life Technologies, Inc. Insect cell transfection buffer and Grace’s medium were from PharMingen.

**Chemicals—**SeaKem ME low melting agarose was purchased from FMC BioProducts. Amphotericin, penicillin-G, streptomycin, tryptose broth, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Sodium metabisulfite and leupeptin were purchased from J. T. Baker Chemical Co. and the Peptide Institute (Minoh-Shi, Japan), respectively.

**Methods**

**Construction of Recombinant Baculoviruses—**Baculovirus transfer vectors containing either the complete or modified coding sequences of the α- and β-subunits of Drosophila pol γ were prepared by standard DNA manipulations. Ndel fragments containing the complete coding sequence of the α-subunit with or without its mitochondrial presequence were released from the Escherichia coli expression vector pET (16) and subcloned into the EcoRI site of the transfer vector pVL1393 after the DNAs were rendered blunt ended with E. coli DNA pol I Klenow fragment (New England Biolabs) to generate pVL93α and pVL93αmt, respectively. A transfer plasmid containing the α-subunit with its mitochondrial presequence replaced at the N terminus with a hexa-histidine tag (pVL93αH6) was obtained by replacing the XbaI fragment of pVL93α (representing the N-terminal portion of the coding sequence) with an XbaI fragment from an E. coli expression vector.
Baculovirus Expression of Drosophila pol γ—Fraction I was prepared from 500 ml of cell culture, chromatographed on phosphocellulose, and precipitated with ammonium sulfate as described above. The pellet was resuspended in 5 ml of 10 mM potassium phosphate buffer containing 45% glycerol and stored at −20 °C (Fraction Ib). Fraction Ib (3–5 mg protein) was dialyzed in 10 mM potassium phosphate buffer in a collodion bag (molecular mass cut-off, 25,000 kDa) until an ionic equivalent of 85 mM KCl was reached and loaded onto a single-stranded DNA-cellulose column equilibrated with 20 mM potassium phosphate buffer at a flow rate of 1.3 ml/h. The column was washed with 2 volumes of potassium phosphate buffer containing 100 mM KCl at 2.7 ml/h followed by successive elutions at 4 ml/h with potassium phosphate buffer containing 250 mM KCl (8 ml), 600 mM KCl (6 ml), and 1 M KCl (4 ml). Active fractions were pooled (Fraction III), and solid ammonium sulfate (0.2 g/ml) was added to increase hydrophobic interactions. After stirring for 20 min on ice, the suspension was centrifuged for 10 min at 20,000 rpm. The supernatant was loaded onto an octyl-Sepharose column (0.5 ml) equilibrated with 20 mM potassium phosphate buffer at a flow rate of 0.5 ml/h. The octyl-Sepharose column was washed with 4 volumes of equilibration buffer at 2 ml/h and then eluted successively with 4 volumes of 20 mM potassium phosphate buffer containing 1 M NaCl and 2% Triton X-100 followed by two 8 ml fractions of 20% glycerol (Fraction IV) and loaded onto two 12–30% glycerol gradients as described (8). Active fractions were pooled, stabilized by addition of glycerol to 45%, and stored at −20 °C or frozen in liquid nitrogen and stored at −80 °C.

Nickel-Nitrioltriacetic Acid-Agarose Affinity Purification of Recombinant pol γ—Other Methods—DNase I-activated calf thymus DNA or singly primed M13 DNA was added as indicated in the figure legends. The beads were eluted successively with equilibration buffer containing 25 mM imidazole (0.5 ml), 250 mM imidazole (0.5 ml), and 500 mM imidazole (0.5 ml). Active fractions were pooled (Fraction IV, 0.9 ml) and loaded onto two 12–30% glycerol gradients as described (8). Active fractions were pooled (Fraction III, 0.9 ml) and loaded onto two 12–30% glycerol gradients as described (8). Active fractions were pooled (Fraction IV, ~8 μg of protein, 20,000–25,000 units/mg), stabilized by addition of glycerol to 45%, and stored at 20 °C or frozen in liquid nitrogen.

DNA Polymerase Activity—DNA polymerase activity was assayed on DNaSe I-activated calf thymus DNA or singly primed M13 DNA as described by Wernette and Kaguni (8) and Farr et al. (15), respectively. Specific modifications are indicated in the figure legends. One unit of activity is the amount that catalyzes the incorporation of 1 nmol of deoxyadenosine triphosphate into acid insoluble material in 60 min at 30 °C using DNaSe I-activated calf thymus DNA as the substrate.

3′-5′ Exonuclease Assay—Strategies were prepared as described by Farr et al. (15). Reaction mixtures (0.05 ml) contained 50 mM Tris-HCl, pH 8.5, 4 mM MgCl₂, 10 mM DTT, 0–180 mM KCl as indicated, 400 μg/ml bovine serum albumin, 4 μg 5′-end labeled singly primed recombinant M13 DNA containing a 3′ terminal mispair, and 0.05 unit of Fraction V enzyme. mTSSB (0.4 μg) was added as indicated in the figure legends. Incubation was for 30 min at 30 °C. Samples were then mixed in 1% SDS and 10 mM in EDTA, heated for 10 min at 65 °C, and precipitated with ethanol in the presence of 1 μg of sonicated salmon sperm DNA as carrier. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris borate. Aliquots were denatured for 2 min at 100 °C, loaded on a 7 M urea, 6% polyacrylamide gel (15 × 23 × 0.075 cm) containing 20 mM Mops and 25 mM EDTA. After electrophoresis, the gel was washed in 15% glycerol for 20 min and exposed to a PhosphorImager screen (Molecular Dynamics). The data were analyzed using the ImageQuant version 4.2a software.

Other Methods—Protein concentration was determined by the method of Bradford (18) with bovine serum albumin as the standard.
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**RESULTS**

**Overexpression of Recombinant Subunits of Drosophila Mitochondrial DNA Polymerase in Baculovirus-infected Insect Cells**—Baculoviruses were constructed as described under "Experimental Procedures" to encode the catalytic (α) and accessory (β) subunits of Drosophila pol γ, with or without their mitochondrial presequences, and with or without a hexa-histidine or T7 antigen tag at the N or C terminus (Fig. 1). Protein analysis of whole cell extracts of infected Sf9 cells by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining shows that the expression levels of the recombinant α- and β-subunit polypeptides are similar to that of the viral polyhedron protein (Fig. 2A). Cells infected with various α-subunit baculoviruses all produce a polypeptide of 125 kDa that is identified as the recombinant catalytic subunit by immunoblot analysis with subunit-specific rabbit antisera (10) (Fig. 2B). Likewise, cells infected with various β-subunit baculoviruses produce a polypeptide of ~35 kDa that reacts with accessory subunit-specific antibody. At the same time, extracts from uninfected Sf9 cells and cells infected with wild type virus do not exhibit any cross-reactive polypeptides. Production of total recombinant protein was calculated to be ~2 μg/ml of cell culture. Proteolytic degradation was observed, and subcellular fractionation experiments indicated that only about 10–20% of the recombinant protein was recovered in the soluble fraction. Overexpression in other insect cell lines (Sf21 or High Five) neither improved solubility nor limited proteolysis; there was also no apparent effect of varying the multiplicity of infection or time course of infection on these problems (data not shown).

**Presequences Target Mitochondrial Import of Recombinant pol γ Subunits, but the Process Is Inefficient**—To evaluate the requirement for mitochondrial targeting sequences in import of the recombinant pol γ subunits, Sf9 cells were infected individually or in combination with baculoviruses encoding the recombinant subunits with or without their mitochondrial presequ
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Fig. 3. Mitochondrial import of recombinant pol γ subunits. Sf9 cells were infected with recombinant baculoviruses expressing the α- and/or β-subunits as full-length proteins (α or β) or lacking their mitochondrial presequences (αNL or βNL) at a multiplicity of infection of 5 and harvested 48 h after infection. Soluble mitochondrial (Mt) and cytoplasmic (Cy) fractions were prepared as described under "Experimental Procedures," and aliquots were denatured and electrophoresed in a 10% SDS-polyacrylamide gel. Recombinant polypeptides were detected by immunoblotting using the goat anti-rabbit IgG-alkaline phosphatase method with combined subunit-specific rabbit antisera. Lanes 1 and 2 represent fractions derived from co-expression of full-length α- and β-subunits. Lanes 3 and 4 represent fractions derived from co-expression of the α- and β-subunits lacking their mitochondrial presequences. Lanes 5–8 represent fractions derived from individual expression of the α- or β-subunits lacking their mitochondrial presequences.

Fig. 4. Reconstitution of recombinant Drosophila pol γ in mitochondria of Sf9 cells. Soluble mitochondrial extract derived from Sf9 cells co-infected with recombinant baculoviruses expressing the α- and β-subunits was sedimented in a 12–30% glycerol gradient. Aliquots of the resulting fractions were analyzed in a 10% SDS-polyacrylamide gel, and recombinant polypeptides were detected by immunoblotting. Lane numbers represent the gradient fractions.

Fig. 5. Reconstitution of recombinant Drosophila pol γ in the cytoplasm of Sf9 cells requires mitochondrial presequences. Soluble cytoplasmic fractions prepared from Sf9 cells infected with recombinant baculoviruses expressing the α- and β-subunits were chromatographed on phosphocellulose as described under "Experimental Procedures." The peak fractions were analyzed in a 10% SDS-polyacrylamide gel, and recombinant polypeptides were detected by immunoblotting. Lane 1 represents a fraction derived from co-expression of full-length α- and β-subunits. Lane 2 represents a fraction derived from co-expression of the α- and β-subunits lacking their mitochondrial presequences. Lane 3 represents a fraction derived from co-expression of the α-subunit lacking its mitochondrial presequence with a full-length β-subunit. Lane 4 represents a fraction derived from individual expression of the α-subunit lacking its mitochondrial presequence.

from 200 g of Drosophila embryos. Although the yields are similar, the baculovirus system offers two advantages. First, the procedure is amenable to an ~10-fold increase in scale, whereas enzyme production from Drosophila embryos is near its limit. Second, the baculovirus system can be used to produce mutant enzyme derivatives.

Stimulation of Both DNA Polymerase and 3'-5' Exonuclease in Recombinant Drosophila pol γ Salt and by Mitochondrial Single-stranded DNA-binding Protein—The recombinant pol γ holoenzyme exhibits very similar biochemical properties as compared with native pol γ from Drosophila embryos (Table II). It is an active DNA polymerase with 3'-5' exonuclease activity. Furthermore, the specific activity of the recombinant enzyme is 20,000 units/mg, which is similar to that of native pol γ. Its DNA polymerase activity is stimulated 8-fold by elevated salt (200 mM KCl) on DNase I-activated calf thymus DNA and 30-fold by mtSSB on singly primed M13 DNA. Its 3'-5' exonuclease activity is mispair-specific (Fig. 7) and is also stimulated by salt and by mtSSB (Fig. 7 and Table II). Mispair specificity was observed at both low and elevated salt and in the presence or absence of mtSSB; under each condition, less than 10% of the paired termini generated by 3'-terminal mispair hydrolysis were hydrolyzed.

Role of the Accessory Subunit in pol γ Function—We sought to compare the reconstituted holoenzyme with the individually expressed catalytic core to evaluate the biochemical role of the accessory subunit in pol γ function. Upon expression of the catalytic subunit alone, we found that its expression level, solubility, and chromatographic properties are very similar to the holoenzyme, suggesting its structural integrity (Fig. 5 and data not shown). Similarly, both the reconstituted holoenzyme and the catalytic core can be purified by an alternate scheme using a baculovirus encoding the catalytic subunit with a C-terminal hexa-histidine tag. Here the purification scheme involves phosphocellulose and metal chelation affinity chromatography followed by glycerol gradient sedimentation, as described under "Experimental Procedures." In either of the standard or affinity purification schemes, we obtained a similar purity and specific activity for the reconstituted holoenzyme (Fig. 6, Table I, and "Experimental Procedures"), but the specific activity of the purified catalytic core alone was greatly reduced, maximally 2% of that of the holoenzyme. This corroborates our previous dissociation studies of the native enzyme (19), and taken together these results indicate that the accessory subunit in Drosophila pol γ is required primarily for the catalytic efficiency of the holoenzyme. By comparison, the accessory subunit of bacteriophage T7 DNA polymerase, E. coli...
specific activity and KCl stimulation of DNA polymerase were performed in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 2% SDS-polyacrylamide gels, and the proteins were stained with silver nitrate. Nearly homogeneous fractions of native and recombinant Drosophila pol γ were denatured and electrophoresed in 10% SDS-polyacrylamide gels, and the proteins were stained with silver nitrate.

**Table I**

| Fraction | Volume | Protein | DNA pol activity* | Specific activity | Yield |
|----------|--------|---------|-------------------|------------------|-------|
| I. Soluble cytoplasmic extracta | 15 ml | 71.4 mg | 8092 units | 113 units/mg | 100% |
| II. Phosphocellulose and ammonium sulfate | 4.5 ml | 1.8 mg | 974 units | 529 units/mg | 12% |
| III. ssDNA-cellulose | 11.2 ml | 0.7 mg | 748 units | 1084 units/mg | 9% |
| IV. Octyl-Sepharose | 3.0 ml | NDa | 159 units | ND | 2% |
| V. Glycerol gradient | 4.0 ml | 0.0075 mg | 149 units | 20,000 units/mg | 2% |

a Activity was measured in the standard assay on DNase I-activated calf thymus DNA in the presence of 200 mM KCl. Under these conditions, Fraction I varies in its resistance to 10 μg/ml aphidicolin from 15–40%, whereas Fraction V is >90% resistant. Thus, the indicated overall yield of 2% represents a severalfold underestimate.

b Fraction I was prepared from 0.5 liter of infected Sf9 cells.

c ND, not determined.

**Table II**

| Biochemical properties of native and recombinant Drosophila pol γ | Native | Recombinant |
|--------------------|--------|-------------|
| DNA polymerase Specific activity | 26,900–80,000 | 20,000 |
| (units/mg)a | | |
| Stimulation by KCl | 20 | 9 |
| (200 vs. 30 mM; fold) | | |
| Stimulation by mtSSB | 15–20 | 22 |
| (30 mM KCl; fold)a | | |
| 3'-5' exonuclease Stimulation by KCl | 5 | 4 |
| (120 vs. 30 mM; fold) | | |
| Stimulation by mtSSB | 7 | 5 |
| (30 mM KCl; fold) | | |

a Specific activity and KCl stimulation of DNA polymerase were measured in the standard assay on DNase I-activated calf thymus DNA.

b 3'-5' exonuclease activity and mtSSB stimulation of DNA polymerase and 3'-5' exonuclease were measured on singly primed M13 DNA.

**Fig. 6.** SDS-polyacrylamide gel electrophoresis of recombinant Drosophila pol γ. Nearly homogeneous fractions of native and recombinant Drosophila pol γ were denatured and electrophoresed in 10% SDS-polyacrylamide gels, and the proteins were stained with silver nitrate.

**Fig. 7.** Mispair-specific 3'-5' exonuclease in recombinant Drosophila pol γ is stimulated by salt and by mtSSB. Recombinant Drosophila pol γ (Fraction V) was assayed for exonuclease activity on singly primed M13 DNA as described under ‘Experimental Procedures’ in the presence of 30 mM (lanes 1 and 2) or 120 mM KCl (lanes 3 and 4) and in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of saturating levels of mtSSB.

**Discussion**

We have established a reproducible method for recombinant production and reconstitution of Drosophila mitochondrial DNA polymerase using the baculovirus expression system. Although we have pursued a variety of approaches since we isolated cDNAs for its two subunits, we failed to achieve reconstitution of the heterodimeric holoenzyme by bacterial expression (10). In the baculovirus system, the recombinant subunits of pol γ are targeted and imported into mitochondria of Sf9 cells whether they are expressed individually or coordinately, suggesting that the cellular machinery for mitochondrial localization and processing is generally conserved between Drosophila and S. frugiperda. At the same time, the low yield of recombinant pol γ in Sf9 cell mitochondria likely results from the lytic nature of baculovirus infection. It is recognized that host protein synthesis is reduced greatly 24 h after infection, when viral late genes dominate mRNA transcription. Nonetheless, that the recombinant holoenzyme can be purified from the soluble cytoplasmic fraction, and shown to exhibit both nearly identical chromatographic behavior and the biochemical and physical properties of native Drosophila pol γ, argues strongly that proper folding and subunit assembly occurs in the cytoplasm of the cultured insect cells.

In examining cytoplasmic reconstitution of mitochondrial DNA polymerase, we constructed recombinant baculoviruses encoding the mature forms of both subunits that lack their mitochondrial presequences. Although recombinant protein production was unaffected, holoenzyme reconstitution was eliminated when the catalytic and accessory subunit polypeptides were co-expressed without their presequences. Although the precise role of the presequence in promoting holoenzyme assembly is not clear, it seems plausible that the targeting of nascent recombinant protein to the mitochondrion itself brings the two subunits in close proximity, thus aiding heterodimer formation. In this regard then, the recombinant pol γ purified and characterized in this study is slightly different structurally from native Drosophila pol γ, because it contains the mitochondrial presequences in both subunits. However, the presequence in the catalytic subunit constitutes only 9 of its 1145 amino acid residues, and the predicted presequence in the accessory subunit is only 11 residues. We detected no significant alternations in the catalytic properties of our recombinant pol γ, including the form containing a C-terminal histidine-tag on the catalytic polypeptide, as compared with the native heterodimeric enzyme from Drosophila embryonic mitochondria.

The human pol γ catalytic subunit has been purified from cultured HeLa cells and as recombinant enzyme from baculovirus-infected Sf9 cells (21). These enzymes exhibit similar catalytic properties yet differ from native pol γ from a variety of sources because the DNA polymerase activity is salt-sensitive. Whether or not mtSSB stimulates either DNA polymerase or 3'-5' exonuclease activity is not known. Our biochemical characterization of recombinant Drosophila pol γ shows clearly that both its DNA polymerase and 3'-5' exonuclease activities are stimulated by elevated salt and by mtSSB. Whether the difference observed in our study reflects the functional significance...
of the accessory subunit remains to be determined. At the same time, our finding that the isolated Drosophila catalytic core has a specific activity at least 20-fold lower than that of the reconstituted heterodimer demonstrates the critical role of the accessory subunit in Drosophila pol γ.

In sum, we have established an effective eukaryotic expression system to produce recombinant mitochondrial DNA polymerase. To our knowledge, this work represents the first example of reconstitution of a multi-subunit mitochondrial enzyme using the baculovirus system. This now provides us with a powerful approach to study mutant forms of holoenzyme and to examine subunit interactions in Drosophila pol γ.

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