Characterization of the Native and Recombinant Catalytic Subunit of Human DNA Polymerase γ: Identification of Residues Critical for Exonuclease Activity and Dideoxynucleotide Sensitivity

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ABSTRACT: The human DNA polymerase γ catalytic subunit was overexpressed in recombinant baculovirus-infected insect cells, and the 136 000 Da protein was purified to homogeneity. Application of the same purification protocol to HeLa mitochondrial lysates permitted isolation of native DNA polymerase γ as a single subunit, allowing direct comparison of the native and recombinant enzymes without interference of other polypeptides. Both forms exhibited identical properties, and the DNA polymerase and $3' \rightarrow 5'$ exonuclease activities were shown unambiguously to reside in the catalytic polypeptide. The salt sensitivity and moderate processivity of the isolated catalytic subunit suggest other factors could be required to restore the salt tolerance and highly processive DNA synthesis typical of γ polymerases. To facilitate our understanding of mitochondrial DNA replication and mutagenesis as well as cytotoxicity mediated by antiviral nucleotide analogues, we also constructed two site-directed mutant proteins of the human DNA polymerase γ. Substituting alanine for two essential acidic residues in the exonuclease motif selectively eliminated the $3' \rightarrow 5'$ exonucleolytic function of the purified mutant polymerase γ. Replacement of a tyrosine residue critical for sugar recognition with phenylalanine in polymerase motif B reduced dideoxynucleotide inhibition by a factor of 5000 with only minor effects on overall polymerase function.

Mitochondrial DNA (mtDNA)[1] is one of the two essential genomes in human cells, and mutations within mtDNA are responsible for a wide range of degenerative diseases (for review, see refs *1* and *2*). The human mitochondrial genome, a 16 569 bp closed circular DNA molecule, is replicated by nuclear encoded proteins, including DNA polymerase γ (*3*). DNA polymerase γ is the only replicative eukaryotic DNA polymerase that is sensitive to antiviral nucleotide analogues (*4*−*10*). Consequently, long-term treatment of patients with antiviral nucleotide analogues such as zidovudine (AZT), zalcitabine (ddC), and didanosine (ddI) induces mitochondrial dysfunctions that mimic mitochondrial genetic diseases (*11*−*14*).

Originally cloned from *Saccharomyces cerevisiae* (*15*), the coding sequences for the catalytic subunit of DNA polymerase γ have been isolated from humans, mice, chickens, *Xenopus laevis*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, and *Pychia pastoris* (*16*−*19*). The predicted sizes for these proteins range from 115 kDa for *S. pombe* to 143 kDa for *Sa. cerevisiae*. Besides containing motifs for polymerase function, all contain sequence motifs predicting a $3' \rightarrow 5'$ exonuclease function, implying this activity is intrinsic to the catalytic subunit. The $3' \rightarrow 5'$ exonuclease activity of DNA polymerase γ has been shown to enhance the high-fidelity DNA synthesis exhibited in vitro by purified γ polymerases (*20*−*23*). Alteration of the conserved exonuclease motif in yeast results in a mutator phenotype (*24*), and exonuclease activity is associated with a partially purified form of the recombinant *Drosophila* catalytic subunit (*19*).

The role of DNA polymerase γ in determining the mutation rate of mitochondrial DNA and the mechanism behind the sensitivity to antiviral nucleotide analogues is poorly understood. Thus, a thorough investigation into the structure−function relationships of this protein and its interaction with nucleotides and nucleotide analogues is needed. We have previously cloned the cDNA for the human, chicken, *Drosophila*, and *S. pombe* DNA polymerase γ catalytic subunit (*16*, *17*). Here, we report on overexpression of the human DNA pol γ gene in baculovirus, purification to homogeneity of the catalytic subunit, and characterization of the enzyme relative to native HeLa cell DNA polymerase γ. We have also constructed and characterized two mutant forms of the human DNA polymerase γ rendered exonuclease-deficient or resistant to dideoxynucleotide triphosphates by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials. Poly(rA)·oligo(dT)$_{12-18}$, poly(dC)$_{300}$, oligo(dG)$_{10}$, NTPs, and dNTPs were purchased from Pharmacia. Radioisotopes [α-^{32}P]dTTP, [α-^{32}P]dGTP, and [γ-^{32}P]ATP were from Amersham. Oligonucleotides were made on an Applied Biosystems 392 DNA synthesizer or purchased from

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[1] Abbreviations: bp, base pair; BSA, bovine serum albumin; Sf9, *Spodoptera frugiperda*; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; BuPdGTP, butylphenyl-dGTP; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; mtDNA, mitochondrial DNA; Exo$^-$, exonuclease-deficient; ddR, dideoxynucleotide-resistant.

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Oligos Etc. Sf9 cells and the pVL1393 baculovirus transfer vector were from M. Summers (Texas A&M University, College Station, TX). The recombinant catalytic subunit of human DNA polymerase α was purified as described (*25*, *26*).

Full-Length Human DNA Polymerase γ Constructs. The full-length cDNA of human DNA polymerase γ was assembled from one genomic clone and two cDNAs isolated during our initial cloning of human pol γ (*17*). The *Ssp*I−*Nde*I fragment of the HeLa cDNA clone K12 and an *Sst*II−*Nde*I fragment of heart cDNA clone R were inserted into *Eco*RV−*Sst*II-digested pBluescript II KS (Stratagene) to make plasmid pΔγBSKS. The 1.1 kb *Sst*II fragment from the chromosome 15 G1 genomic clone was inserted into the *Sst*II site of pΔγBSKS to give plasmid pHuγBSKS. Resequencing confirmed the proper assembly of the full-length clone (GenBank accession number U60325). Baculovirus transfer vectors harboring the full-length gene with (H102/H103) or without (H100/H103) the putative mitochondrial targeting sequence were generated by PCR. The 5′-primers were CCA GCG AAG GGG TTC ATG GTC TCC AGC TCC GTC (H100) or CCA AAG GAA GGT GGT CTG ACT CCC AGC GT (H102), and the 3′-primer was AAT ACG CGG CCG CCA GGA GTG CTA TGG TCC A (H103). The amplified DNAs were gel purified, digested with *Xmn*I and *Not*I, and inserted into *Sma*I- and *Not*I-digested pVL1393. The sequences of the resulting plasmids 102/103HuγpVL (encoding amino acids 1−1240) and 100/103HuγpVL (encoding amino acids 26−1240) were confirmed.

The His-tagged recombinant pol γ cDNA baculovirus transfer vector was also constructed. The *Bam*HI−*Bgl*II pol γ cDNA insert from 100/103HuγpVL was excised and inserted into *Bam*HI-digested pQE9 (Qiagen) to make pHuγpQE9. The N-terminal 1256 bp of DNA pol γ cDNA with the N-terminal His tag was generated from pHuγpQE9 by amplification using the primers ACA GAA TTC GAG GAG TTA ACC ATG GGA GGA TCG CAT CAC (forward) and CAG AGT CAC TGG GTG GGG ACA CCT CTC CAA GAA GAG (reverse) to introduce eukaryotic translation signal sequences. This new *Eco*RI−*Dra*III fragment was ligated into *Eco*RI−*Dra*III-digested pHuγpQE9. The *Eco*RI−*Not*I fragment containing the full-length 3647 bp pol γ was excised from this plasmid and inserted into pVL1393 to generate pHuγpQVL1393SL.

Construction of Mutant Derivatives of Human DNA Polymerase γ. A putative exonuclease-deficient DNA pol γ baculovirus transfer vector was constructed by utilizing PCR to replace two amino acid residues, $D_{198} \rightarrow A$ and $E_{200} \rightarrow A$, in the ExoI motif of 100/103HuγpVL. Separate primary amplification reactions utilized either CGC GGA TCC CGG TTC ATG GTC TCC (H110) and the mutagenic primer CAA GCA GAC CGC CAC GGC GAA CAC CAG (H113) or the mutagenic primer CTG GTG TTC GCC GTG GCG GTC TGC TTG (H112) and TGT ACA GGG ACT CGA TGG (H73) as primer pairs. Reaction mixtures (50 μL) contained 10 ng of 100/103HuγpVL, 5 μL of 10× Vent buffer, each dNTP at 0.2 mM, 100 pmol of each primer (H110/H113 or H112/H73), and 1 unit of Vent DNA polymerase (New England Biolabs). The reaction mixtures were heated at 95 °C for 1 min followed by 30 of the following cycles: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. After a final incubation at 72 °C for 10 min and gel purification, the H110/H113 and H112/H73 products were mixed and re-amplified as they were before with H110 and H73 as the primer pair to join the two products. Substituting this mutant *Bam*HI−*Xma*I fragment for the wild type *Bam*HI−*Xma*I segment of 100/103HuγpVL generated Exo⁻ 100/103HuγpVL. Similarly, a putative dideoxynucleotide-resistant DNA pol γ baculovirus transfer vector was constructed by making the single amino acid substitution $Y_{951} \rightarrow F$ in the pol B motif of 100/103HuγpVL. Primary amplifications utilized either ATC AGG CAC CCC GAC TAT GAT GAG GAA (H122) and the mutagenic primer ACC ATA GAT GCG GCC GAA GTT GAA GAT (H123) or the mutagenic primer ATC TTC AAC TTC GGC CGC ATC TAT GGT (H124) and GGT CCC AGG AAA GGA TCA GAT CTG CAG (H125) as primer pairs. The first- and second-round amplifications were performed as described above, and substituting the mutant *Stu*I−*Not*I fragment of the H122/H125 product for the *Stu*I−*Not*I fragment of 100/103HuγpVL produced ddR 100/103HuγpVL. The resulting plasmid inserts were sequenced to confirm these mutations.

Expression of Recombinant DNA Polymerase γ. Recombinant baculoviruses were generated by cotransfection of transfer plasmids with BaculoGold Linearized Baculovirus DNA (Pharmingen) by standard baculovirus techniques (*27*). Sf9 cells were maintained at 27 °C in T_{175} flasks in Grace's Insect Media (Life Technologies) supplemented with 10% fetal calf serum (Gemini). Cultures were split 1:2 into Sf900 II serum-free medium (Life Technologies) and grown to ∼80% confluence prior to infection with recombinant baculovirus at a multiplicity of infection of approximately 10. Cells were dislodged and harvested 48−54 h later by centrifugation at 1000*g*, washed once in SF900 II SFM, and frozen in liquid nitrogen.

Purification of Recombinant DNA Polymerase γ. All chromatographic steps were performed at 4 °C. A thawed cell pellet (6.8 g) derived from 26 T_{175} flasks infected with 100/103HuγpVL was resuspended in 3 volumes of lysis buffer consisting of 0.05 M KPO_4 (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, and 1 μg/mL leupeptin (buffer A) also containing 0.5% Nonidet P-40 (NP-40) and 0.1 M NaCl. Lysed cells were centrifuged at 20000*g* for 10 min, and the supernatant (fraction I, 24.5 mL, 11.7 mg/mL) was diluted to 40 mL with buffer A also containing 0.05 M NaCl prior to the addition of 20 mL of phosphocellulose P-11 (Whatman) equilibrated in this buffer. Following 45 min of end-over-end mixing, the resin was collected by vacuum filtration, washed three times with 50 mL of buffer A also containing 0.05 M NaCl, and placed in a glass column (4.1 cm × 4.9 cm^2). The column was developed with a 200 mL linear gradient of buffer A also containing 0.05 M NaCl to buffer B, which is composed of 0.3 M KPO_4 (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 1 μg/mL leupeptin, and 0.05 M NaCl. Fractions possessing reverse transcriptase activity (see below) eluted at ∼0.12 M KPO_4 (fraction II, 60 mL, 0.22 mg/mL). Fraction II was adjusted to 0.3 M KPO_4 (pH 7.5) and applied to a phenyl-Sepharose HP column (Pharmacia, 5.1 cm × 0.79 cm^2) equilibrated in buffer B. The column was washed with 10 mL of buffer B and developed with a 4.0 mL linear

gradient from buffer B to buffer A containing 0.05 M NaCl. Reverse transcriptase activity eluted very late in the gradient, and fraction III (5.6 mL, 0.24 mg/mL) was applied directly to a 1 mL column of single-stranded DNA−cellulose (Sigma) equilibrated in 0.025 M Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer C) also containing 0.05 M NaCl. The column was washed with 9 mL of equilibration buffer and developed with a 15 mL linear gradient of NaCl (0.05−0.6 M) in buffer C. Fractions containing DNA polymerase activity eluted at ~0.30 M NaCl (fraction IV, 2.9 mL, 0.17 mg/mL). Fraction IV was applied directly to a 1 mL column of ceramic hydroxylapatite (CHT-II, Bio-Rad) equilibrated in 0.05 M Tris-HCl (pH 7.5), 10% glycerol, 1 mM 2-mercaptoethanol, 0.01% NP-40, and 0.1 M KCl (buffer D). The column was washed with 4 mL of buffer D and developed with a 20 mL linear gradient of buffer D containing 0 to 0.36 M KPO_4 (pH 7.5). DNA pol γ activity eluted at ~0.27 M KPO_4, and fraction V (2.8 mL, 0.060 mg/mL) was diluted with 0.05 M Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.01% NP-40 (buffer E) until the conductivity was equivalent to that of buffer E also containing 0.1 M KCl. The enzyme was applied to a 1.0 mL HR 5/5 Mono Q (Pharmacia) FPLC column equilibrated in buffer E containing 0.1 M KCl. The column was washed with 3 mL of equilibration buffer and eluted with a 20 mL linear gradient of KCl (0.1 to 0.5 M) in buffer E. Pol γ activity eluted at ~0.22 M KCl, and fraction VI (1.3 mL, 0.022 mg/mL) was frozen in small aliquots with liquid nitrogen and stored at −80 °C.

Native DNA polymerase γ was purified from HeLa cell mitochondria by the above method with the following additions and exceptions. HeLa S_3 cells were grown at 37 °C in 15−30 L batches in spinner cultures to ~5×10^5 cells/mL in DMEM supplemented with 10% calf serum prior to harvest by centrifugation. Cells were lysed by hypotonic Dounce homogenization, and mitochondria were isolated by the two-step discontinuous sucrose gradient method (*28*). Mitochondria were lysed in 3 volumes of 0.02 M Tris-HCl (pH 8.0), 10% glycerol, 0.1% 2-mercaptoethanol, 0.3 M NaCl, 0.5% Triton X-100, 0.1 mM PMSF, and 1 μg/mL leupeptin. Lysates were centrifuged at 20000*g* for 10 min, and the supernatants from some batches were stored at −80 °C after freezing in liquid nitrogen. Fresh and thawed lysates derived from 95 L of HeLa cells (490 mg of protein) were pooled and adjusted by dilution with buffer A until the conductivity was equivalent to that of buffer A containing 0.05 M NaCl. All subsequent purification steps were the same as those for the recombinant protein, except 50 mL of phosphocellulose P-11 was utilized and the volumes of the three washes and the gradient were doubled for this first column.

Purification of His-Tagged Recombinant DNA Polymerase γ. Sf9 cells infected with pHuγpQVL1393SL baculovirus were thawed on ice and lysed by the addition of 4 volumes of 0.1 M Tris-HCl (pH 7.5), 8% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5% NP-40, and 0.1 M NaCl. The lysate was centrifuged at 30000*g* for 15 min at 4 °C, and the supernatant (fraction I) was mixed for 30 min at 4 °C with 1 mL of a Ni−NTA agarose slurry (Qiagen) equilibrated in the same buffer. The resin was washed three times with 50 mL of a solution composed of 0.5 M NaCl, 0.05 M Tris-HCl (pH 8.0), and 1% Triton X-100. The washed beads were placed in a 1 mL disposable column, and protein was eluted with 0.25 M imidazole and 0.05 M Tris-HCl (pH 9.0) followed by a 2 h dialysis against 0.025 M Tris-HCl (pH 8.0), 10% glycerol, 0.05% NP-40, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.05 M NaCl. The enzyme (fraction II) was applied to a 1.0 mL HR 5/5 Mono Q column equilibrated in 0.05 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 M KCl. Protein was eluted with a linear KCl gradient (0.1 to 0.5 M) in this buffer, and fractions containing His-pol γ were dialyzed into 0.025 M Tris-HCl (pH 8.0), 0.05% NP-40, 1 mM 2-mercaptoethanol, 1 mM EDTA, 50% glycerol, and 0.05 M NaCl (fraction III).

Polymerase Assays. Reverse transcriptase activity was determined in reaction mixtures (50 μL) containing 0.025 M HEPES-KOH (pH 8.0), 2.5 mM 2-mercaptoethanol, 10 μg of acetylated BSA, 0.5 mM $MnCl_2$, 10 μM [α-^{32}P]dTTP (600−3000 cpm/pmol), 2.5 μg of poly(rA)·oligo$(dT)_{12-18}$, and 0.1 M NaCl. Polymerase activity on poly$(dC)_{300}$·oligo$(dG)_{10}$ was measured in reaction mixtures (50 μL) containing 0.025 M HEPES-KOH (pH 8.0), 2.5 mM 2-mercaptoethanol, 5 μg of acetylated BSA, 1 mM $MgCl_2$, 10 μM [α-^{32}P]dGTP (6000 cpm/pmol), 1 μg of poly$(dC)_{300}$·oligo$(dG)_{10}$ (20:1 mass ratio), and 0.05 M NaCl. DNA polymerase activity on activated salmon sperm DNA was determined in reaction mixtures (50 μL) containing 0.02 M Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 5 μg of acetylated BSA, 10 mM $MgCl_2$, dCTP, dGTP, and dATP all at 50 μM, 50 μM [α-^{32}P]-dTTP (1200−1500 cpm/pmol), and 20 μg of DNase I-activated salmon sperm DNA. In all cases, TCA-insoluble radioactivity was determined by liquid scintillation counting following incubation at 37 °C for 15 min. One unit of enzyme incorporated 1 pmol of total dNMPs into acid-insoluble DNA in 1 h at 37 °C.

Exonuclease Assay. Exonuclease activity was determined in reaction mixtures (10 μL) containing 0.025 M HEPES-KOH (pH 7.6), 5 mM 2-mercaptoethanol, 1 μg of acetylated BSA, 5 mM $MgCl_2$, and 0.05 pmol of the 5′-^{32}P-labeled 22-mer TGC ATG CCT GCA GGT CGA CTC T. As indicated, double-stranded substrate was formed by hybridization of the labeled oligo to positions 6282−6261 of M13mp18 DNA. Following incubation at 37 °C for 15 min, reactions were terminated by the addition (10 μL) of 95% deionized formamide, 0.01 M EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples (2.5 μL) were analyzed by electrophoresis through denaturing 12% polyacrylamide gels, and reaction products were visualized and quantified with a Molecular Dynamics PhosphorImager and NIH Image 1.61 imaging software.

Processivity Assay. Processivity reaction mixtures (10 μL) contained 0.025 M HEPES-KOH (pH 7.6), 5 mM 2-mercaptoethanol, 0.5 μg of acetylated BSA, 5 mM $MgCl_2$, each dNTP at 25 μM, and 0.05 pmol of the 5′-^{32}P-labeled 22-mer hybridized to M13mp18 DNA. Limiting quantities of enzyme were preincubated with excess labeled substrate for 1 min at either 0 or 37 °C, as indicated. Following this preincubation, the remaining reaction components were added, including, as indicated, 2.1 μg of unlabeled, sonicated, heat-denatured calf thymus DNA harboring ~18 pmol of random 3′-ends acting as a polymerase "trap". Following incubation at 37 °C for 15 min, reactions were terminated and analyzed as described above for exonuclease reactions.

Sedimentation Analysis. Protein samples (60 μL) were layered onto linear 10 to 30% sucrose gradients (5 mL) containing 0.05 M Tris-HCl (pH 7.5), 5% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.01% NP-40, and 0.1 M KCl. Following centrifugation at 4 °C for 14 h at 55 000 rpm in a Beckman SW 55 Ti rotor, fractions (70 μL) were collected from the bottom of each gradient.

Gel Filtration Chromatography. A Superose 12 HR 10/30 FPLC column (Pharmacia) was equilibrated in and developed with 0.05 M Tris-HCl (pH 7.5), 5% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.01% NP-40, and 0.1 M KCl and calibrated with apoferritin (67.3 Å), catalase (52.2 Å), aldolase (48.1 Å), and ovalbumin (30.5 Å).

Production of Antibodies to DNA Polymerase γ. The quantities of antigen sufficient for antibody production required pol γ gene expression in *Escherichia coli* and purification of the insoluble polypeptide from inclusion bodies by a denaturation−renaturation protocol. The *Bam*HI−*Not*I segment of 102/103HuγpVL bearing the pol γ gene was inserted into *Bam*HI−*Not*I-digested pET32c (Novagen), and the 7 kb *Sst*I fragment of this new plasmid was religated to make D_{SstI}HuγpET32, encoding an inducible, histidine-tagged, N-terminal domain (residues 26−276) of the pol γ polypeptide, named DPg. The DPg protein was expressed in *E. coli* BL21(DE3) cells and purified as a denatured polypeptide using Ni−NTA agarose in the presence of 6 M urea. Judged to be 90% pure by SDS−PAGE, the eluted 28 kDa DPg protein was used as an antigen for the production of polyclonal antibodies in rabbits.

Other Methods. The protein concentration was determined according to Bradford (*29*) with bovine serum albumin as the standard. For immunoblots, proteins were resolved by SDS−PAGE, electrotransferred to Immobilon P membranes (Millipore) in 0.025 M Tris and 0.192 M glycine, and sequentially incubated with a DPg polyclonal antisera (1:150 dilution) and alkaline phosphatase-conjugated second antibody prior to visualization with the Western Blue reagent (Promega).

RESULTS

Overexpression of Human DNA Polymerase γ. Partial-length clones generated to determine the complete nucleotide sequence of the catalytic subunit of human DNA polymerase γ (*17*) were assembled into a full-length cDNA of pol γ, and plasmids encoding either the full-length polypeptide (pro-p140) or the protein lacking the putative mitochondrial targeting sequence (p140) were constructed. Although expression of pro-p140 in *E. coli* resulted in an entirely insoluble protein, almost 50% of the p140 protein was expressed in soluble form in *E. coli* (data not shown). Unfortunately, the soluble p140 protein was devoid of DNA polymerase activity, so prokaryotic expression was abandoned in favor of the eukaryotic baculoviral expression system proven so successful for other human replicative DNA polymerases (*25*, *30*). Baculovirus transfer vectors harboring pro-p140 or p140 cDNA were constructed, and recombinant baculoviruses were produced by cotransfection of Sf9 cells with the transfer vectors and linearized wild type baculovirus. Analysis of whole cell lysates of infected Sf9 cells indicated overexpression of pro-p140 and p140 at levels comparable to that observed for the β-galactosidase control

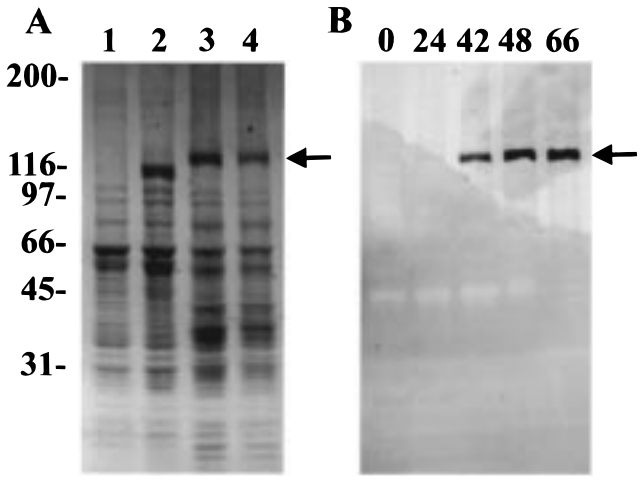

FIGURE 1: Overexpression of the catalytic subunit of human pol γ. (A) Whole cell lysates derived from 1×10^5 cells 3 days after baculoviral infection were separated on a 4 to 20% SDS−polyacrylamide protein gel and stained with Coomassie Blue. Polypeptides present in uninfected Sf9 cells (lane 1) and cells infected with baculoviruses encoding recombinant β-galactosidase (lane 2), recombinant full-length pro-p140 (102/103HuγpVL, lane 3), and recombinant p140 without a mitochondrial targeting sequence (100/103HuγpVL, lane 4) are shown. Protein molecular mass markers are indicated on the left. (B) Immunoblot of the time course of the infection by baculovirus 100/103HuγpVL. Cells were harvested at the indicated times (hours) postinfection. Whole cell extracts were resolved on a 4 to 20% SDS−polyacrylamide protein gel, electrotransferred to an Imobilon P membrane (Millipore), and probed with EF1 antisera (*17*) raised against the C-terminal portion of the human pol γ catalytic subunit as described in Experimental Procedures. The arrow indicates the position of the pol γ catalytic subunit.

(Figure 1A), but only ~10% of each protein was expressed in soluble form (data not shown). Subcellular fractionation of Sf9 cells infected with baculovirus bearing pro-p140 indicated abundant, catalytically active pol γ in the cytoplasmic fraction, whereas ~5% of the available pro-p140 was associated with the soluble mitochondrial fraction. In a parallel baculoviral infection, catalytically active p140 protein lacking the mitochondrial targeting sequence was localized entirely within the cytoplasmic fraction (data not shown). Therefore, we chose to characterize the mature form of the recombinant catalytic subunit to avoid potential heterogeneity from partial processing of pro-p140 by the protein importation machinery of the insect mitochondria. Immunoblot analysis (Figure 1B) indicated optimal expression and minimal proteolysis of p140 48−66 h postinfection.

Purification of the Recombinant Catalytic Subunit of DNA Polymerase γ. The soluble mature form of the recombinant catalytic subunit of pol γ was purified to apparent homogeneity from baculovirus-infected Sf9 cell extracts by sequential chromatography over phosphocellulose, phenyl-Sepharose, single-stranded DNA−cellulose, hydroxylapatite, and MonoQ FPLC columns as described in Experimental Procedures (Table 1). The final fraction from this purification is presented in lane 1 of Figure 3. The purification generally took less than 36 h to complete, and purified p140 was stable at 4 °C for several days despite a relatively low protein concentration. Fraction VI was stored at −80 °C, and a single freeze−thaw cycle reduced activity by ~50%.

Mutant Derivatives of Human Pol γ p140. Three distinguishing characteristics of the γ polymerases are highly

Table 1: Purification of the Recombinant Catalytic Subunit of Human DNA Polymerase γ

fraction	total protein[a] (mg)	total activity[b] (units × 10^{-6})	specific activity (units/ng)	yield (%)
I. lysate	290	30	0.10	100
II. phosphocellulose	13	13	1.0	45
III. phenyl-Sepharose	1.4	10	7.5	34
IV. DNA−cellulose	0.49	6.7	14	23
V. hydroxylapatite	0.17	6.1	36	20
VI. MonoQ	0.029	3.5	120	12

[a] Protein was determined by the method of Bradford (*29*) with bovine serum albumin as the standard. [b] Activity was determined as described in Experimental Procedures in 15 min reactions utilizing poly(rA)•oligo-(dT). One unit of enzyme incorporates 1 pmol of dTTP in 1 h at 37 °C.

A. Exonuclease motif I

Human DNA pol γ	195-lVF**D**V**E**vcla
Xenopus DNA pol γ	169-lVF**D**V**E**vcla
Drosophila DNA pol γ	182-lVF**D**V**E**vcvs
S. pombe DNA pol γ	168-mVF**D**V**E**vlyk
S. cerevisiae DNA pol γ	168-vVF**D**V**E**tlyn
T7 DNA pol	2-lvs**D**I**E**anal
T5 DNA pol	135-vaF**D**S**E**tsal
E. coli pol I	352-faF**D**T**E**tdsl

B. Polymerase motif B

Human DNA pol γ	943-RehAKIFN**Y**GRIYG
Xenopus DNA pol γ	913-RehAKVFN**Y**GRIYG
Drosophila DNA pol γ	861-RdhAKViN**Y**aRIYG
S. pombe DNA pol γ	732-RdsAKVFN**Y**GRlYG
S. cerevisiae DNA pol γ	744-RneAKIFN**Y**GRIYG
T7 DNA pol	518-RdnAKtFI**Y**GFLYG
T5 DNA pol	562-RqaAKait**F**GiLYG
E. coli pol I	754-RrsAKaiN**F**GLIYG
Taq DNA pol	659-RraAKtIN**F**GvLYG

FIGURE 2: Amino acids targeted for mutagenesis. (A) Amino acid alignments of exonuclease motif I for DNA polymerase γ sequences and bacterial DNA polymerases. Bold and boxed amino acids signify the conserved, catalytically essential Asp and Glu residues changed to Ala in the human Exo⁻ pol γ. (B) Amino acid alignments of polymerase motif B for DNA polymerase γ sequences and bacterial DNA polymerases. The bold and boxed amino acid signifies the conserved Tyr changed to Phe in the human ddR pol γ. Sequences were derived from GenBank accession numbers U60325 (human), U60298 (*Drosophila*), U49509 (*Xenopus*), Z47976 (*S. pombe*), J05117 (*Sa. cerevisiae*), V01146 (T7 DNA polymerase), V00317 (*E. coli* pol I), M24354 (T5 DNA polymerase), and X66105 (*Thermus aquaticus flavus* DNA polymerase).

processive DNA synthesis (*31*), high-fidelity DNA synthesis partially afforded by an associated proofreading 3′ → 5′ exonuclease activity (*19*−*24*), and extreme sensitivity to dideoxyribonucleotides (*5*, *10*). We sought to identify amino acid residues critical to exonuclease function and dideoxynucleotide discrimination. Alignment of the γ polymerases with bacterial DNA polymerases revealed extensive similarity within exonuclease motifs (Figure 2A). Mutagenesis studies of *E. coli* pol I identified two acidic side chains in exonuclease motif I that were essential for metal binding and catalysis (*32*). In the hopes of producing an exonuclease-deficient pol γ, we utilized site-directed mutagenesis to convert these essential Asp and Glu residues to Ala, and the new protein was designated Exo⁻ p140. Tabor and Richardson identified a Tyr residue in polymerase motif B of T7 DNA polymerase that, when changed to Phe, rendered the

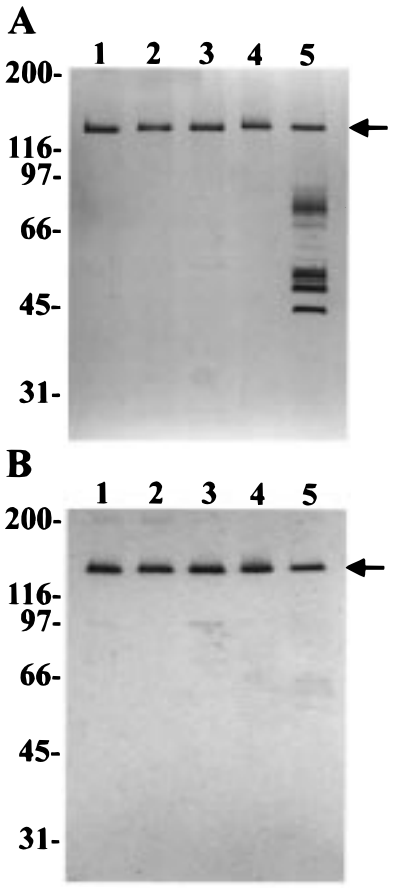

FIGURE 3: Purity of various pol γ proteins. Samples containing 50 ng of each purified catalytic pol γ polypeptide were resolved on 4 to 20% SDS−polyacrylamide gels and detected by (A) silver staining or (B) electrotransfer to charged nylon and probing with DPg antisera as described in Experimental Procedures. Fraction VI proteins were as follows: lane 1, wild type p140; lane 2, Exo⁻ p140; lane 3, ddR-p140; lane 4, His-p140 (fraction III); and lane 5, HeLa pol γ. Mobilities of molecular mass markers (kilodaltons) are indicated. The arrow indicates the position of the pol γ catalytic subunit.

resulting mutant protein insensitive to inhibition by dideoxynucleotides (*33*). Inspection of this region of the human pol γ sequence indicated that Tyr_{951} likely was aligned with the critical Tyr residue in T7 pol (Figure 2B). To determine whether this residue was responsible for the dideoxynucleotide sensitivity of the γ polymerases, we changed this residue to Phe and designated the new protein ddR-p140. Recombinant baculoviruses encoding mature Exo⁻ p140 and mature ddR-p140 were expressed and purified as described above for the wild type recombinant p140. The chromatographic behavior of both proteins was indistinguishable from that of wild type p140. Exo⁻ p140 (9 μg) was purified from 7.3 g of infected Sf9 cells for an overall yield of 4%, whereas ddR-p140 (23 μg) was derived from 8.3 g of cells for an overall yield of 15%. Detection of the proteins by silver staining following SDS−polyacrylamide gel electrophoresis indicated both were homogeneous (Figure 3A).

His-Tagged Recombinant DNA Polymerase γ. In an effort to obtain greater quantities of recombinant p140, we expressed the catalytic subunit in baculovirus as a histidine-tagged protein. As described in Experimental Procedures, the cDNA for mature p140 was fused with the histidine tag from pQE9 and subcloned into a baculovirus transfer vector pVL1393. Expression of His-p140 was equivalent to that

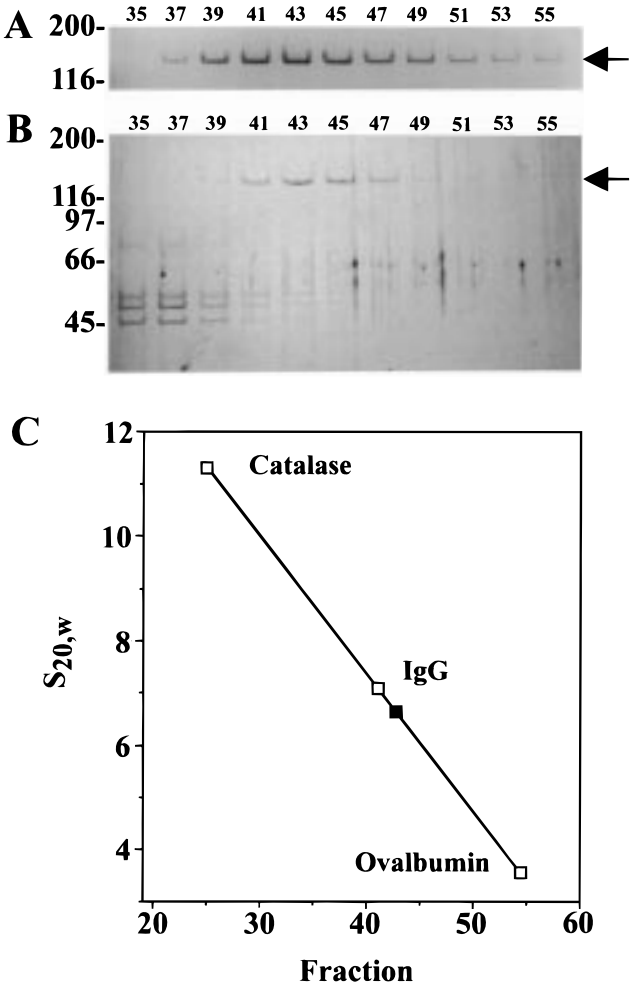

FIGURE 4: Sedimentation analysis. Wild type recombinant pol γ (fraction VI, 1.3 μg) and native HeLa pol γ (fraction VI, 1.0 μg) were subjected to band sedimentation with 10 to 30% sucrose gradients as described in Experimental Procedures. Analysis of the indicated gradient fractions (20 μL) by electrophoresis with 4 to 20% SDS−polyacrylamide gels followed by silver staining revealed the sedimentation profiles of (A) recombinant pol γ and (B) HeLa pol γ. Positions of molecular mass markers (kilodaltons) are indicated. (C) The peak position of each pol γ (■) was determined by reverse transcriptase assay utilizing poly(rA)·oligo(dT), and sedimentation coefficients were estimated relative to the positions of standard proteins (□; catalase, $s_{20,w}$ = 11.3 S; γ-globulin, $s_{20,w}$ = 7.1 S; ovalbumin, $s_{20,w}$ = 3.55 S) sedimented in a parallel gradient. The arrow indicates the position of the pol γ catalytic subunit.

of untagged p140 (data not shown), and His-p140 was easily purified (Experimental Procedures) to homogeneity by chromatography on a Ni-chelating column followed by MonoQ FPLC resulting in a greater than 10-fold higher yield of catalytic subunit compared to that from the conventional preparation (Figure 3A, lane 4).

Comparison of Native Pol γ and Recombinant Wild Type p140. Proper characterization of the recombinant proteins required comparison to a naturally derived enzyme, so DNA polymerase γ was also purified by the established scheme from HeLa cell mitochondria (Experimental Procedures). The chromatographic behavior of native HeLa pol γ was indistinguishable from that observed for wild type p140, and the enzyme eluted from the final column (MonoQ) as a single, well-isolated peak of UV-absorbing material. The native enzyme (fraction VI, 17 μg) was purified approximately

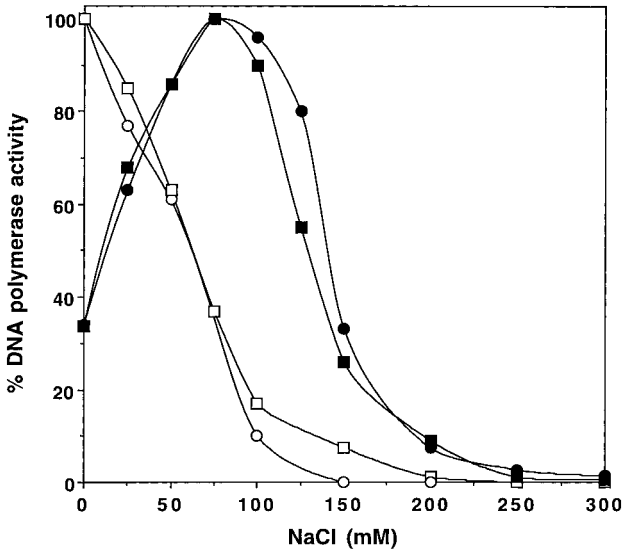

FIGURE 5: Effects of salt on DNA polymerase and reverse transcriptase activities of native and recombinant p140. DNA polymerase activity (open symbols) on activated salmon sperm DNA and reverse transcriptase activity (filled symbols) on poly(rA)·oligo(dT) were measured as described in Experimental Procedures. Reaction mixtures contained 1.0 ng of WT-p140, fraction VI (squares), or 0.5 ng of HeLa pol γ, fraction VI (circles), and contained salt as indicated.

13500-fold more than that in mitochondrial lysates, and the overall yield of activity was 6.3%. Analysis by 4 to 20% SDS−PAGE and silver staining revealed five polypeptides with molecular masses of 135, 77, 52, 49, and 45 kDa (Figure 3A, lane 5). Discounting possible differential staining by the silver reagent, the five polypeptides were judged to be present in near stoichiometric amounts. Immunoblot analysis with antibodies raised against the N-terminal 28 kDa of the human pol γ catalytic subunit (DPg polypeptide) indicated the four smaller proteins were not derived by proteolysis of the largest protein (Figure 3B). Additionally, antibodies raised against a 40 kDa polypeptide from the C-terminal region of the human pol γ catalytic subunit [CD7 and EF1 polypeptides (*17*)] also did not recognize these lower-molecular mass protein bands (data not shown). Therefore, band sedimentation analysis was performed to determine whether the smaller proteins were physically associated with the catalytic subunit. Interestingly, sedimentation cleanly resolved the catalytic polypeptide ($s_{20,w}$ = 6.6 S) from the other four proteins, which appeared to cosediment as a larger, 8.5 S complex (Figure 4B,C). Purified recombinant WT-p140 sedimented as a single, symmetrical peak with an $s_{20,w}$ value of 6.6 S (Figure 4A,C). DNA polymerase and 3′ → 5′ exonuclease activities cosedimented for both the recombinant WT-p140 and the HeLa pol γ (data not shown). Additionally, the salt preferences of isolated HeLa-p140 (fraction VII) and WT-p140 (fraction VII) both on poly(rA)·oligo$(dT)_{12-18}$ and activated salmon sperm DNA were identical to those observed for each fraction VI (see Figure 5 below). Although DNA polymerase activity of purified WT-p140 and HeLa pol γ could not be detected following gel filtration (Experimental Procedures), active His-p140 (fraction I) eluted with a Stoke's radius of 44.2 Å (data not shown). This value, together with the sedimentation coefficient and an assumed typical partial specific volume of 0.725 cm^3/g, was an indication of a native molecular mass

Table 2: Effects of Inhibitors on the Native and Recombinant Catalytic Subunit of Human DNA Polymerase γ[a]

inhibitor	concentration	HeLa Pol γ (%)	WT-p140 (%)	recombinant pol α (%)
none		100	100	100
NEM	0.01 mM	67	65	90
	0.04 mM	40	37	81
	0.10 mM	20	14	42
	0.50 mM	6	5	25
aphidicolin	0.01 mM	100	99	78
	0.10 mM	99	96	75
	0.30 mM	99	95	44
BuPdGTP	0.25 μM	106	100	24
	1.0 μM	81	91	5.8
	5.0 μM	64	65	1.3
	10.0 μM	40	38	0.4
DMSO	1%	100	100	
	5%	99	99	
heat for 5 min at 37 °C		40	61	

[a] DNA polymerase reactions were performed as described in Experimental Procedures and included 1.0 ng of WT-p140 (fraction VI), 0.5 ng of HeLa pol γ (fraction VI), or 6 ng of recombinant pol α and the indicated concentrations of inhibitors. Poly(dC)$_{300}$·oligo(dG)$_{10}$ was used as the substrate for analysis with NEM, aphidicolin, and BuPdGTP, whereas poly(rA)·oligo(dT)$_{12-18}$ was used for DMSO and thermostability analyses. Inhibition with NEM was performed in the absence of sulfyhdryl-reducing agents. All values are the average of duplicate determinations.

of 138 000 Da and a frictional ratio of 1.30 for p140. A calculated partial specific volume of 0.721 cm^3/g based on the predicted amino acid sequence of p140 was an indication of a native molecular mass of 136 000 Da with a frictional ratio of 1.31 (*34*).

The apparent identity of the native and recombinant catalytic polypeptides led us to compare their responses to various substrates and inhibitors. First, the effects of salt on the DNA polymerase and reverse transcriptase activities of the two enzymes were measured. The DNA polymerase activity on natural DNA of both WT-p140 and HeLa pol γ was highest in reaction mixtures that contained no salt, and increasing concentrations of salt equally inhibited both enzymes (Figure 5). Reverse transcriptase activities were optimal at approximately 75 mM NaCl, and the inhibitory effects of higher and lower salt concentrations were indistinguishable for the two enzymes (Figure 5). Second, the responses of the enzymes to chemical inhibitors and heat inactivation were measured (Table 2). Both enzymes demonstrated extreme sensitivity to NEM, as evidenced by a ~60% reduction in polymerase activity by only 40 μM NEM. Relative to the pol α control, both enzymes were resistant to aphidicolin and butylphenyl-dGTP. Neither enzyme was affected by 5% DMSO, and rates of heat inactivation were similar for the two enzymes.

Characterization of the Mutant Recombinant Proteins. The 3′ → 5′ exonuclease activities of the native, recombinant wild type, histidine-tagged, and putative exonuclease-deficient and dideoxynucleotide-resistant forms of DNA polymerase γ were measured and quantitated as described in Experimental Procedures (Figure 6). Although the nuclease activity of the Exo$^-$ p140 enzyme was undetectable, the other four enzymes exhibited robust exonuclease activity that functioned with nearly equal efficiency on both single-stranded and double-stranded DNA substrates. The inclusion of 75 mM NaCl in the reaction mixtures reduced exonuclease

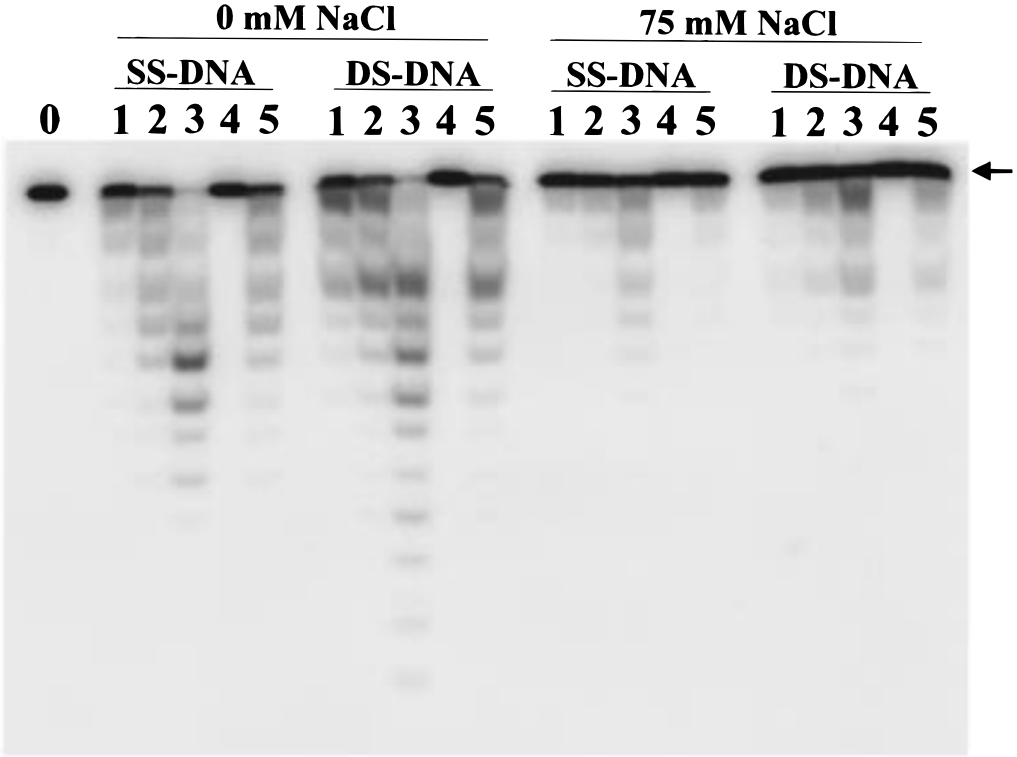

FIGURE 6: Exonuclease activity of pol γ polypeptides. The products of 3′ → 5′ exonuclease reactions were resolved on denaturing polyacrylamide gels as described in Experimental Procedures. Reactions with 0 mM NaCl or 75 mM NaCl were performed with both single-stranded and double-stranded DNA substrates, as indicated. The position of the 22-mer oligonucleotide substrate (arrow) is shown: lane 0, no enzyme; lane 1, 1 ng of HeLa pol γ (fraction VI); lane 2, 1 ng of wild type p140 (fraction VI); lane 3, 2.6 ng of His-p140 (fraction III); lane 4, 1 ng of Exo$^-$ p140 (fraction VI); and lane 5, 1 ng of ddR-p140 (fraction VI).

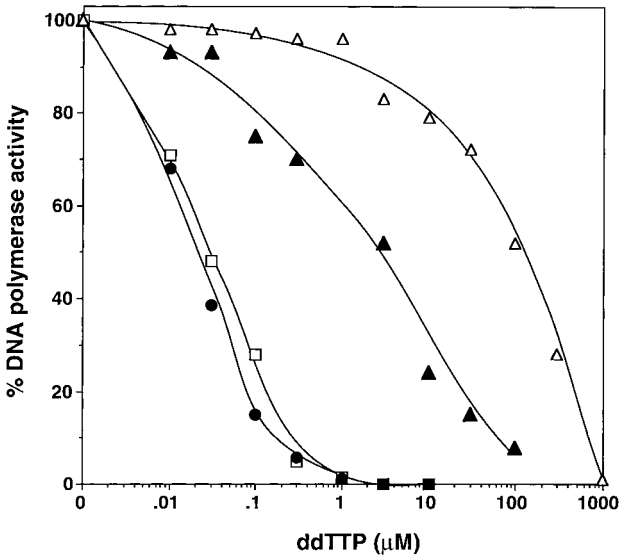

FIGURE 7: Effects of ddTTP on DNA synthesis by WT-p140, HeLa pol γ, and ddR-p140. Reverse transcriptase activity was determined as described in Experimental Procedures on poly(rA)·oligo(dT)$_{12-18}$ with 10 μM dTTP, the indicated amounts of ddTTP, and approximately 1 ng each of WT-p140 (□), HeLa pol γ (●), ddR-p140 (△), or the large fragment of *E. coli* DNA polymerase I (▲). The percent activity is expressed relative to no ddTTP addition.

activity of the four enzymes by 70% (Figure 6), and 100 mM NaCl completely inhibited nuclease function (data not shown). These results prove the 3′ → 5′ exonuclease function is intrinsic to the human p140 polypeptide and identify at least one of two amino acids critical to this function. In addition, the conservation of the 5′-label among oligonucleotide substrates and products verified that the preparations lacked 5′ → 3′ exonuclease activity.

The ability of dideoxynucleotides to inhibit the reverse transcriptase activities of wild type recombinant p140 and the ddR-p140 mutant protein was also measured. With poly(rA)·oligo(dT) as the substrate, the ddR-p140 mutant protein was 5000-fold less sensitive to ddTTP than WT-p140 (Figure 7). Similarly, with poly(dC)·oligo(dG), WT-p140 was markedly inhibited by ddGTP concentrations as low as 0.5 μM (20:1 dGTP:ddGTP ratio), but comparable inhibition of ddR-p140 was not achieved until ddGTP concentrations exceeded 9−10 μM, presenting an ∼1:1 dGTP:ddGTP ratio (data not shown). The differences in these two observations likely involve the template preferences of the enzymes. Taken together, these results demonstrate that Tyr$_{951}$ is critical to dideoxynucleotide incorporation and inhibition.

Processivity Measurements. To help delineate any differences between the native and recombinant forms of pol γ p140, we performed primer extension reactions under conditions in which the enzymes were constrained to single or multiple binding events (Figure 8). Examination of these reaction products revealed several interesting observations. First, little or no radiolabeled primer was extended when the enzyme was preincubated with the "polymerase trap" prior to the substrate, indicating the effectiveness of the trap (Figure 8, lane 5). Second, when constrained to a single, productive binding event, HeLa pol γ, WT-p140, His-p140, and Exo$^-$ p140 could extend primers by up to 52 nucleotides, but only when preincubated with the substrate at 0 °C (Figure 8, lane 1). Preincubation at 37 °C resulted in the vast majority of the enzyme migrating to the trap (Figure 8, lane 3). Third, in the absence of the trap, multiple binding events permitted primer extension by more than 100 nucleotides, and generation of such products was independent of preincubation temperature (Figure 8, lanes 2 and 4). Products longer than ∼150 nucleotides were not observed, as expected from reactions with excess substrate and limiting enzyme. Fourth, significant 3′ → 5′ exonuclease activity was only observed in reaction mixtures not containing trap, suggesting that initial binding of pol γ to DNA occurs at the polymerase active site and that excision occurs by an intermolecular mechanism. Fifth, the maximum processivity of ddR-p140 was only ∼14 nucleotides, and distinct pausing was observed within short polypyrimidine and polypurine tracts immediately downstream of the primer. With the exception of the ddR-p140, the overall processivity and spectrum of pause sites of the enzymes appeared to be the same.

Table 3: Specific Activities of HeLa and Recombinant Pol γ Proteins on Various Substrates[a]

	DNA polymerase activity[b] (units/ng)			exonuclease activity[c] (units/ng)
enzyme	poly(rA)·oligo(dT)$_{12}$	activated DNA	poly(dC)$_{300}$·oligo(dG)$_{10}$	[^{32}P]22-mer
HeLa pol γ	78	14	102	0.078
WT-p140	61	14	100	0.14
His-p140	88	11	92	0.13
Exo$^-$ p140	19	4.4	22	<0.001
ddR-p140	9.7	7.2	2.9	0.15

[a] DNA polymerase and exonuclease activities were determined as described in Experimental Procedures and included 0.1−40 ng of purified enzyme where possible. Specific activities were derived from at least two independent determinations. [b] One unit of polymerase activity incorporates 1 pmol of total dNMP in 1 h at 37 °C. [c] One unit of exonuclease activity degrades 1 pmol of single-stranded ^{32}P-labeled 22-mer in 1 h at 37 °C.

Substrate Utilization. DNA polymerase γ is known to utilize different substrates with widely varying efficiencies. We assayed the ability of each form of native and recombinant pol γ to utilize poly(rA)·oligo(dT), DNaseI-activated salmon sperm DNA, and poly(dC)·oligo(dG). A range of enzyme concentrations was tested in the standard reactions (Experimental Procedures), and specific activities were derived from values for which activity was proportional to enzyme. Typical of the γ polymerases, the native, wild type, histidine-tagged, and exonuclease-deficient forms exhibited a 4−8-fold preference for the homopolymeric templates over the salmon sperm DNA (Table 3). This differential was reduced for ddR-p140 due to a 10−35-fold decrease in utilization of the homopolymers relative to the wild type. When the lower processivity and/or pausing observed for ddR-p140 on primed M13 DNA was considered (Figure 8), this result was not unexpected. Tabor and Richardson observed a similar reduction in overall activity as the cost for high dideoxynucleotide discrimination with the corresponding mutation in T7 DNA polymerase (*33*). Overall DNA polymerase activity of Exo$^-$ p140 was approximately 25% of the wild type activity on all three polymerase substrates. This reduction of polymerase activity has been observed with single alanine changes of these amino acids in bacterial DNA polymerases (*35*). In addition, utilization of all of the substrates by His-p140 was equivalent to that of native and recombinant wild type forms, indicating the

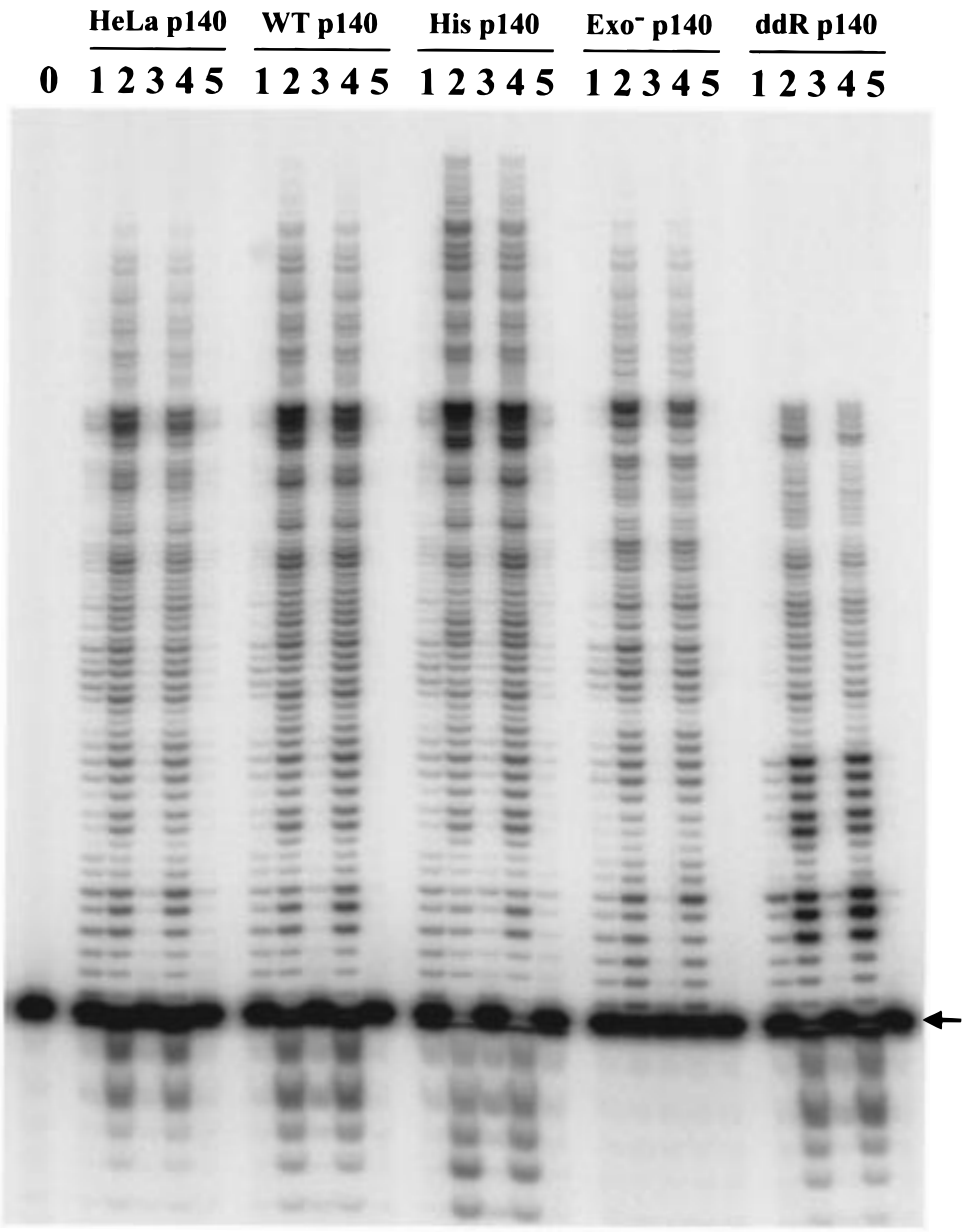

FIGURE 8: Processivity of pol γ p140s on primed M13. Processivity reactions were performed as described in Experimental Procedures using 3 ng of HeLa pol γ (fraction VI), 3 ng of wild type p140 (fraction VI), 7.9 ng of His-p140 (fraction III), 3 ng of Exo$^-$ p140 (fraction VI), or 3 ng of ddR-p140 (fraction VI). Products were resolved by denaturing PAGE. Within each set, the first two reaction mixtures were preincubated at 0 °C and the last three reaction mixtures were preincubated at 37 °C. Reactions depicted in lanes 1, 3, and 5 received heat-denatured DNA trap, and the fifth reaction in each set received trap prior to substrate The position of the unextended primer (arrow) is shown. In lane 0, there is no enzyme.

N-terminal addition of this metal binding domain did not substantially interfere with p140 activities.

DISCUSSION

This work represents both the first biochemical characterization of a native, isolated catalytic subunit of DNA polymerase γ from any organism and the first successful expression and purification of a recombinant DNA polymerase γ in a homogeneous, active form from higher eukaryotes. Initial experiments with baculovirally expressed, full-length pol γ indicated there was poor targeting to insect mitochondria, suggesting possible saturation of the insect protein importation machinery or suboptimal recognition of the human sequence. We chose to express the mature form of the recombinant protein lacking the putative mitochondrial targeting sequence to minimize entanglement with insect mitochondria and to ease purification. When minor differences in assay conditions are discounted, the DNA polymerase and exonuclease activities of the recombinant protein are comparable to values reported for the native human heterodimeric form of DNA polymerase γ (*36*). Also, the specific activities for both the exonuclease and DNA polymerase functions of the homogeneous recombinant catalytic subunit of human DNA polymerase γ were equivalent to those of the native catalytic subunit purified by the same method (Table 3), strongly implying proper folding of the recombinant enzyme in the insect cytosol. In fact, having demonstrated identical chromatographic and hydrodynamic behavior, substrate specificity, processivity, and responses to inhibitors, we conclude that the native and recombinant

catalytic subunits are physically and functionally equivalent. Nevertheless, it is noteworthy that the salt optima for DNA polymerase (Figure 5) and 3′ → 5′ exonuclease (Figure 6) activities of both the native and recombinant forms of isolated human p140 were significantly lower than salt optima reported for pol γ activities derived from other natural sources. For example, the DNA polymerase activity on natural DNA of heterodimeric pol γ from *Drosophila* preferred ~200 mM KCl (*37*), and the salt optimum for both the polymerase and the exonuclease activities of highly purified porcine pol γ was 150−200 mM NaCl (*38*, *39*). Furthermore, the reverse transcriptase activity of heterodimeric human pol γ was reported to have a salt optimum of ~150 mM NaCl, and the exonuclease activity of this enzyme preparation was not significantly inhibited until salt concentrations exceeded ~75 mM (*36*). We speculate that loosely associated subunits and/or accessory factors that were resolved from the catalytic subunit during purification are needed to confer salt tolerance to human DNA polymerase γ.

The purification of the native human DNA polymerase γ as an isolated catalytic subunit (6.6 S) was unexpected, because all previous reports on animal cell γ polymerases described association of the catalytic subunit with other polypeptides with varied compositions (for review, see ref *40*). The highly purified human enzyme is a 7.8 S heterodimer of 140 and 54 kDa subunits (*36*), whereas preparations from *Drosophila melanogaster* contain 135 and 35 kDa polypeptides in a 7.6 S dimer (*37*). The purified porcine liver DNA polymerase γ contains four polypeptides of 120, 55, 50, and 48 kDa (*21*), and preparations of DNA polymerase γ from *Xenopus* possess a 140 kDa catalytic polypeptide associated with several polypeptides of 100, 85, 55, 40, and 31 kDa (*41*). Although the latter two reports do not discount proteolysis, our immunoblot results do not support proteolysis being the source of the 77, 52, 49, and 45 kDa proteins that are copurified with HeLa p140. Recently, the second subunit from *Drosophila* was cloned and shown to encode a 41 kDa polypeptide (*42*). Interestingly, preliminary studies indicate that none of the four smaller HeLa proteins cross-react with antibodies raised against the recombinant 43 kDa human homologue of the *Drosophila* second subunit.[2] The four smaller proteins may simply be contaminants, but they cosediment as an 8.5 S complex, making it tempting to speculate they may participate with the missing 43 kDa subunit in a more processive, salt-tolerant, multimeric human polymerase γ.

Typically representing less than 1% of the DNA polymerase activity in the cell (*5*), DNA polymerase γ is the least abundant of the eukaryotic polymerases. Overexpression of the catalytic subunit of human DNA polymerase γ opens the door to previously unavailable lines of investigation, such as detailed kinetic, drug interaction, and structure−function studies. To this end, we constructed the dideoxynucleotide-resistant and exonuclease-deficient mutant forms of the human p140. The inability of DNA polymerase γ to discriminate against dideoxynucleotides and other antiviral nucleotide analogues may help explain the general cytotoxicity and mitochondrial degenerative disease states induced by these compounds (*8*, *9*, *11*−*14*, *43*−*45*). Through site-directed mutagenesis, we have identified an amino acid residue responsible for pol γ's sensitivity to ddNTPs. Within motif B of the polymerase domain, the Y951F enzyme is more like *E. coli* DNA polymerase I, the prototypical dideoxynucleotide-resistant DNA polymerase (refer to Figure 2B). This site appears to be unique to the bacterial type DNA polymerases since other dideoxy-sensitive polymerases such as DNA polymerase β and HIV reverse transcriptase do not share this homology within motif B (*46*). Additionally, dideoxy-resistant mutations within HIV reverse transcriptase have been identified in other regions of the polymerase, suggesting another mode of dideoxy discrimination for these mutant proteins (*47*). This demonstrated ability to enhance ddNTP discrimination by pol γ in vitro is a first step toward rational design of agents that do not inhibit mitochondrial function while retaining their antiviral properties.

The fidelity of DNA replication is high for the γ family of DNA polymerases as evidenced by a base substitution error rate of less than one error for every 500 000 bases polymerized (*21*). Such high fidelity is presumably due to the combined actions of base selectivity by the polymerase function and proofreading by the exonuclease function (*20*, *22*, *48*). Through mutagenesis, we eliminated the exonuclease activity (D198A and E200A) while largely retaining the polymerase function. Possession of the exonuclease-deficient mutant will permit us to measure the accuracy of the polymerase function uncoupled from the proofreading function, providing greater insight into the mechanisms by which the cell maintains the integrity of the mitochondrial genome.

[2] S. E. Lim, M. J. Longley, and W. C. Copeland, unpublished observation.

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