Expression, Purification, and Initial Kinetic Characterization of the Large Subunit of the Human Mitochondrial DNA Polymerase†

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Received October 29, 1997; Revised Manuscript Received February 23, 1998

ABSTRACT: Faulty replication of the human mitochondrial genome is thought to be the cause of many diseases; moreover, the low selectivity of the mitochondrial DNA polymerase has been implicated as the cause of many side effects observed in the treatment of viral infections such as HIV. To better understand how the mitochondrial genome is replicated, we cloned a cDNA encoding the large subunit of human DNA polymerase γ, the enzyme that replicates the mitochondrial genome. The large subunit was recombinantly expressed and purified to near homogeneity. The purified enzyme demonstrated both polymerase and 3′-5′ exonuclease activity. The purified protein was examined in single nucleotide incorporation assays, demonstrating that the enzyme had a maximum polymerization rate of 3.5 s^{-1} and a dissociation rate from the DNA substrate of 0.03 s^{-1}, affording a calculated processivity of 116. The dissociation constants for the enzyme binding to DNA and nucleoside triphosphate were 39 nM and 14 μM, respectively. The 3′-5′ exonuclease rate was measured at 0.18 s^{-1}. Though the slow rate of polymerization suggests that the large subunit of human DNA polymerase γ may require accessory factors to increase its processivity of polymerization, the kinetic parameters indicate that the large subunit of DNA polymerase γ could replicate the mitochondrial genome in a physiologically relevant time frame. This study provides the initial characterization of the large subunit of DNA polymerase γ and establishes the baseline for examination of the effects of accessory proteins such as the putative small subunit.

Mitochondria are the only organelles in animal cells that contain their own extranuclear genome. The mitochondrial genome encodes for 13 essential proteins, none of which are involved in DNA transcription or replication. The genes responsible for these activities are encoded in the nuclear genome (*1*). Inhibition of the replication of the mitochondrial genome by antiviral nucleotide analogues results in impaired mitochondrial function and causes toxic side effects in treatments for AIDS and Hepatitis B infection (*2*).

DNA polymerase γ, which replicates the mitochondrial genome, has been purified from many different organisms including *Homo sapiens* (*3*−*6*). Human DNA polymerase γ purifies as a heterodimer of a 140-kDa subunit and a smaller subunit of 54 kDa (*4*). The large subunit of *Xenopus laevis* DNA polymerase γ has been shown to have both polymerase activity and 3′-5′ exonuclease activity (*7*). Recently, the cDNAs encoding the large subunit for many different species, including human, have been cloned (*8*−*13*), and sequence analysis shows that the large subunit has regions of significant homology to the DNA polymerase A family (*10*, *14*). Antisera to the human large subunit has been shown to deplete human mitochondrial lysates of polymerase activity (*10*). Recent studies of the recombinant *Drosophila melanogaster* large subunit have shown that it is capable of both polymerase activity and 3′-5′ exonuclease activity, but further characterization of the large subunit from *Drosophila* has been hampered by the low solubility of the recombinant protein (*11*). Purified *D. melanogaster* DNA polymerase γ can exhibit low processivity (defined as the number of nucleotides incorporated per binding event), and homogeneous fractions of DNA polymerase γ are less active than less pure preparations of the enzyme (*15*), suggesting the possible loss of an accessory protein during purification.

In this paper we describe our cloning of the large subunit of human DNA polymerase γ, and we also present a method for the expression and purification of a significant amount of soluble large subunit for human DNA polymerase γ and provide an initial kinetic characterization of its activities.

EXPERIMENTAL PROCEDURES

Identification of Partial Human cDNAs. The full-length peptide sequence of MIP1 was used in a tBLASTn (*16*) search of the dbEST[1] database at the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/). This returned several human cDNAs (Accession numbers: T86774, T98759, and R20417) with strong homology to MIP1. The three largest cDNAs were obtained from the IMAGE Consortium (*17*).

Cloning of the Full-Length cDNA. The cDNA insert was isolated from the human cDNA T98759 and used to probe

† This research was supported by grants to K.A.J. (GM44613 from the National Institutes of Health) and S.W.G. (Predoctoral training fellowship from the National Institutes of Health).

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[1] The abbreviations used are as follows: dbEST, database of expressed sequence tags; EDTA, (ethylenedinitrilo)tetraacetic acid; dNTP, deoxynucleoside triphosphate; dTTP, deoxythymidine triphosphate.

S0006-2960(97)02685-8 CCC: $15.00
Published on Web 04/11/1998

a human teratocarcinoma cDNA library that was packaged in λgt10 (*18*), and this revealed two independent positive clones. The phage DNA for both clones was isolated. The cDNA inserts were isolated from the phage genomic DNA via an *Eco*RI digestion, isolated by gel electrophoresis, and then ligated into the vector pBlueScript II SK- (Stratagene, La Jolla, CA) and sequenced. The first clone (3.6 kb) contained the carboxyl terminus of the protein including the poly-A tail. The second clone (2.1 kb) was aligned to the first clone, and it extended the first clone's sequence for 1.2 kb on its 5′ end. The sequence of the second clone overlapped that of the first identically for 900 bp on its 3′ terminus. Analysis of the two sequences showed that they encoded for an open reading frame for a 145-kDa protein. The complete cDNA was made by digesting the vector containing the second clone with *Eco*RV and *Afl*III and isolating a 1.5-kb fragment of the cDNA insert for the second clone. The construct containing the first clone was identically digested, and the vector along with a large portion of the cDNA insert for the first clone was isolated. These two fragments were ligated together, making a full-length cDNA clone for the catalytic subunit.

Peptide Sequence Alignment of DNA Polymerase γs. All of the currently cloned DNA polymerase γ peptide sequences from several different species were aligned using the MACAW program (*19*). The alignments were completed using the segment pair overlap method.

Creation of the Full-Length cDNA. A tBLASTn search of dbEST, using the peptide sequence from residue 900 to residue 1100, indicated that the human cDNA clone with the accession number of R66338 contained a cDNA that encoded for this region of the peptide. The vector containing this cDNA was obtained from the IMAGE consortium and sequenced on both strands. Our original clone contained a 9 amino acid deletion and a point mutation of D999H. The changes were located between a unique *Hind*III site and a unique *Eco*R47III site. The corresponding region of R66338 encoded for an open reading frame with a peptide sequence identical to that encoded by the cDNA clones submitted to GenBank (*9*, *10*, *12*). The region between the *Hind*III site and the *Eco*R47III site was excised from the vector containing R66338 and ligated into the same region of our cDNA. This created a construct containing a full-length cDNA identical to those previously submitted to GenBank. All further experiments in this study utilized this cDNA.

Bacterial Overexpression of Protein. PCR was used to create an insert with a *Nhe*I site and a Methionine codon immediately prior to the 29th amino acid from the amino terminus along with a *Not*I site on the carboxyl terminus of the cDNA (*20*). The insert was introduced into pET24a (Novagen, Madison, WI) to create a construct that was tagged with six histidines on its carboxyl terminus. The expression construct was transformed into BL21(DE3) cells. The transformed BL21(DE3) cells were grown to an OD_{600} of 0.6 in LB media including kanamycin at 50 μg/mL. Protein production was then induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.4 mM. After 1 h of induction at 37 °C, the cells were harvested by centrifugation for 5 min at 10000 rpm in a Sorvall GSA rotor at 4 °C. Cell pellets were then frozen in liquid nitrogen.

SDS−PAGE Analysis. All SDS−polyacrylamide gels were performed according to the method of Laemmli (*20*). A separating gel of 7.5% was used for all gels, and proteins were visualized with Coomassie blue R-250 (*21*).

Purification of the Bacterially Expressed Protein. All procedures were carried out at 4 °C unless otherwise specified. The bacterial cell pellets were thawed and resuspended in 10 volumes (mass to volume) of lysis buffer (20 mM Tris-Cl pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 60 μg/mL lysozyme, 0.2 mM phenylmethylsulfonyl fluoride, and 0.7 μg/mL leupeptin). The suspension was stirred at room temperature for 30 min to lyse the cells. The lysate was sonicated on ice until it lost viscosity and then centrifuged for 1 h at 40000 rpm in a Beckman 60Ti rotor at 4 °C. The pellet and supernatant were analyzed via 7.5% SDS−PAGE, and the protein was wholly contained within the insoluble pellet. The pellet was washed once with 40 mL of lysis buffer containing 0.5% Triton X-100, and then it was centrifuged as above. The pellet was retained and washed with 40 mL of lysis buffer containing 0.5 M urea. The supernatant was centrifuged as above, and the resulting pellet was retained and resuspended in 40 mL of denaturing buffer (20 mM Tris-Cl pH 7.9, 250 mM NaCl, 5 mM imidazole, and 6 M urea). The resuspension was centrifuged as before, and the supernatant was loaded onto a 10 cm in length by 1 cm in diameter chelating sepharose (Pharmacia, Uppsala, Sweden) column that had been charged with 400 mM $NiSO_4$ and equilibrated in denaturing buffer. All chromatography steps in the bacterial purification were performed at room temperature. The sample was loaded at 1 mL/min and washed with 10 column volumes of denaturing buffer. The column was washed with 6 column volumes of denaturing buffer containing 40 mM imidazole. The protein was eluted with 6 volumes of denaturing buffer containing 1 M imidazole.

Production of Polyclonal Antisera to the Bacterially Expressed Protein. The purified histidine tagged protein was separated on a preparative 7.5% SDS−polyacrylamide gel (*20*). The protein running at 138 kDa was excised from the gel and homogenized in a 1:1 mix with ddH_2O. The preparation was sent to Centralized Biological Laboratories on the Pennsylvania State University campus (University Park, PA) where rabbit polyclonal antisera were generated. The antisera were further purified over protein-A agarose (*20*).

Expression of Protein in Baculovirus and Sf9 Cells. PCR was used to create an insert with a consensus Kozak sequence, a *Bam*HI site, and a start codon just prior to the 30th residue from the amino terminus along with a *Xba*I site just after the stop codon within the cDNA. The insert was digested with *Bam*HI and *Xba*I and ligated into a pFastBac1 vector (Gibco-BRL, Gaithersburg, MA) that had been identically digested. Recombinant high titer baculovirus was produced according to the protocols included in the Bac to Bac system (Gibco-Brl, Gaithersburg, MA). Viral titers were optimized for protein expression, and then the viral stocks were used to infect SF9 cells at a multiplicity of infection >10 for a time course of between 36 and 96 h. Optimal expression, as determined by SDS−PAGE analysis, occurred at 72 h post infection.

Large-Scale Growth of Sf9 Cells and Infection with Recombinant Baculovirus. Two liters of SF9 cells were grown to $(1-2) \times 10^6$ cells/mL in SF900 serum free media

(Gibco-BRL, Gaithersburg, MA) in stirrer flasks at 27 °C. Cells were infected with viral stock at a multiplicity of infection >10 for protein production. The cells were grown for 72 h and then harvested at 4000 rpm in a Sorvall SLH-3000 rotor for 10 min at 4 °C. The cell pellet was washed with PBS and centrifuged again as above. The cell pellet was frozen in liquid nitrogen.

Western Blot Analysis. The fractions were first separated by SDS−PAGE and then blotted to nitrocellulose (*20*). The blot was incubated with a 1:5000 dilution of primary antibody followed by a 1:2000 dilution of antirabbit secondary antibody conjugated to alkaline phosphatase (*20*).

Lysis of Infected Cell Pellet and Ammonium Sulfate Precipitation of Protein from the Soluble Fraction. All procedures were carried out at 4 °C unless otherwise specified. The cell pellet was resuspended in 10 volumes (mass to volume) of ammonium sulfate lysis buffer (600 mM $(NH_4)_2SO_4$, 250 mM NaCl, 50 mM Tris-Cl pH 8.0, 0.2 mM phenylmethylsulfonyl fluoride, 0.7 μg/mL leupeptin, 0.5 μg/mL pepstatin-A) and stirred on ice for 30 min. The lysate was sonicated until it lost viscosity and centrifuged for 30 min at 40000 rpm in a Beckman 45Ti rotor. The supernatant was retained and brought to 35% saturation by the addition of solid $(NH_4)_2SO_4$ while stirring for 45 min and was then centrifuged in a Sorvall SS-34 rotor at 18000 rpm for 15 min. The pellet was resuspended into 70 mL of TDEG buffer at pH 7.9 (50 mM Tris-Cl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol) containing 50 mM NaCl by stirring for 1 h followed by 5 strokes in a dounce homogenizer.

DEAE-Sepharose Chromatography. All procedures were carried out at 4 °C. A 200-mL DEAE-sepharose column was poured and equilibrated in TDEG buffer at pH 7.9 containing 50 mM NaCl. The resuspended pellet from above was loaded onto the column at 2 mL/min. Protein elution was monitored with a continuous flow UV detector, and the column was washed with TDEG buffer at pH 7.9 containing 50 mM NaCl until the A_{280} returned to baseline. The protein was eluted with a 2-L gradient from 50 mM NaCl to 500 mM NaCl in TDEG buffer at pH 7.9 at 2 mL/min. Fractions were analyzed for polymerase activity by incorporation of [α-^{32}P]-labeled dTTP (see below). Active fractions also containing DNA polymerase γ, as judged by western blot and SDS−PAGE analysis, were pooled and dialyzed for 4 h against TDEG buffer at pH 7.9 containing 50 mM NaCl using a 50000-Da MWCO membrane.

Single-Stranded DNA Cellulose Chromatography. All procedures were carried out at 4 °C. A 40-mL single-stranded DNA cellulose column was poured and equilibrated with TDEG buffer at pH 7.9 containing 50 mM NaCl. The dialysate from above was loaded onto this column at 1 mL/min. This column was washed with TDEG buffer at pH 7.9 containing 50 mM NaCl until the A_{280} returned to baseline. The protein was eluted with a 500-mL gradient from 50 mM NaCl to 1 M NaCl in TDEG buffer at pH 7.9 at 1 mL/min. The fractions were assayed for DNA polymerase activity. Active fractions that also contained DNA polymerase γ, as judged by western blot and SDS−PAGE analysis, were pooled and dialyzed against TDEG at pH 7.2 containing 50 mM NaCl for 4 h in a 50000-Da MWCO membrane.

Mono-S Chromatography. All procedures were carried out at 4 °C. The dialysate from above was loaded at 0.5 mL/min onto a HR5/5 Mono-S column (Pharmacia, Uppsala, Sweden) that had been equilibrated in TDEG at pH 7.2 containing 50 mM NaCl. The column was washed with equilibration buffer until the A_{280} returned to baseline. The protein was eluted with a 15-mL gradient from 50 mM NaCl to 1 M NaCl in TDEG at pH 7.2 at 0.5 mL/min. The eluted fractions were analyzed for polymerase activity. Active fractions containing DNA polymerase γ, as judged by western blot and SDS−PAGE analysis, were pooled. The pooled fractions were dialyzed against TDEG at pH 8.4 containing 100 mM NaCl for 4 h. The dialyzed fractions were then frozen in liquid nitrogen and stored at −80 °C. The protein concentration was measured by A_{280} using an extinction coefficient, determined by the amino acid content of the expressed protein, of 234420 M^{-1} cm^{-1} (*22*). The protein concentration was also determined by Bradford analysis (*20*).

DNA Polymerase Activity Assay by Incorporation of [α-^{32}P]-Labeled dTTP. An aliquot (5 μL) from the purification was rapidly mixed with 20 μL of assay cocktail (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 10 μg/mL aphidicolin, 1 μM poly dA-oligo dT_{12-18} (Pharmacia, Uppsala, Sweden), and 5 μL of 3000 Ci/mmol of [α-^{32}P]-dTTP). The reaction mixture was incubated for 15 min at room temperature, and then 10 μL of each reaction was spotted onto DE81 filter paper (Whatman, Maldstone, England). Polymerase activity was determined by washing filters of unincorporated nucleotide followed by counting in a scintillation counter (*23*).

Single-Incorporation DNA Polymerase Assay. The enzyme·DNA complex (70 nM DNA polymerase γ, 100 mM NaCl, 50 mM Tris-Cl pH 7.5, and 2000 nM 25/45-mer primer template) was mixed with an equal volume of nucleotide mix (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 500 μM dTTP) at 37 °C using a Rapid Chemical Quench Flow instrument produced by KinTek Corporation (State College, PA). The final reaction mixture contained 1000 nM 25/45-mer primer/template, 250 μM dTTP, and 2.5 mM $MgCl_2$. The reactions were quenched at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 10, and 30 s with an excess of 500 mM EDTA. Product formation was followed as in the active site titration experiment.

Active Site Titration. The enzyme·DNA complex (DNA polymerase γ, 100 mM NaCl, 50 mM Tris-Cl pH 7.5, and varying concentrations of 25/45-mer primer template) was mixed using a Rapid Chemical Quench Flow instrument produced by KinTek Corporation (State College, PA) with an equal volume of nucleotide mix (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 500 μM dTTP) at 37 °C. The 25-mer was 5′ labeled with [γ-^{32}P] ATP and annealed to the 45-mer. For each of the primer/template concentrations, the reaction mixture was quenched with an excess of 500 mM EDTA at 2.5 s. The reaction products were then separated on a sequencing gel and analyzed on a Phosphorimager model 445SI and ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

K_d for dTTP Binding and Maximum Burst Rate. This experiment was performed as described for the single-incorporation assay, except the concentration of dTTP was varied in the nucleotide mix. Final concentrations of 10,

25, 50, 100, and 250 μM of dTTP were used, and the reactions were quenched at 0, 0.25, 0.5, 0.75, 1, 1.5, and 2 s.

RESULTS

Cloning of the cDNA Encoding Human DNA Polymerase γ. The complete peptide sequence of the yeast DNA polymerase γ, termed MIP1 (*8*), was used in a tBLASTn (*16*) search of dbEST using an online search engine (http://www.ncbi.nlm.nih.gov/). The search returned several human cDNAs with strong homology to MIP1. These cDNAs were used to probe a human teratocarcinoma cDNA library (*18*). The probe led to the isolation of two independent clones that were combined to create an open reading frame of 3.7 kb that encoded a protein of 145 kDa with a high degree of homology to MIP1. At the time of this publication, three other groups (*9, 10, 12*) have submitted complete cDNA sequences to GenBank encoding for the large subunit of human DNA polymerase γ. The peptide sequences for these cDNAs are identical. In comparison, the sequence we cloned had a deletion of 9 amino acids from position 987 to position 996. Interestingly, when the DNA polymerase γ family large subunits from all species cloned to date are aligned, this stretch of 9 amino acids is highly conserved, including the residues TKG, which are completely conserved (data not shown). The only other change between our sequence and the sequences from Genbank was a histidine to aspartate at position 999. Because three independent cDNAs for the large subunit have been isolated encoding identical peptide sequences, we presume these sequences will have greater physiological relevance. Therefore, in our expression, purification, and initial characterization of the enzyme, we expressed a protein identical to the peptide sequence predicted by the coding region of the cDNAs previously submitted to Genbank (*9, 10, 12*).

Bacterial Expression of DNA Polymerase γ and Production of Antisera. The open reading frame of the sequence of the large subunit of DNA polymerase γ was analyzed using the helical wheel program available in the Wisconsin Software package (Genetics Computer Group, Madison, WI) in order to estimate the end of the mitochondrial targeting sequence. The program predicted an amphipathic helix indicative of a mitochondrial targeting sequence occurring in the first 30 amino acids (*24*). The open reading frame was therefore truncated 29 amino acids from the amino terminus and subcloned into pET24a for expression in BL21-(DE3) cells with a histidine tag on its carboxyl terminus. Despite attempting many different expression conditions and lysis techniques, this protein had low solubility (data not shown). Therefore, for the purposes of generating antibodies, we expressed the histidine-tagged protein and purified the insoluble protein in 6 M urea over a chelating sepharose column. The eluted fractions were further purified by preparative SDS−PAGE. The protein migrating at 138 kDa was excised and used to generate polyclonal antisera in rabbits.

Expression and Purification of Soluble DNA Polymerase γ in Baculovirus. By using PCR methods we created an insert encoding the large subunit. The insert was used to express the protein in baculovirus according to the protocols included with the Bac to Bac system (Gibco-Brl). The

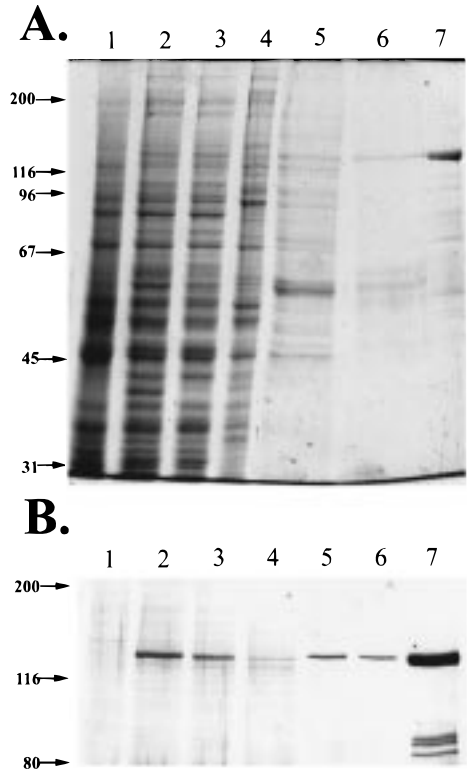

FIGURE 1: Protein purification. (A) A 7.5% SDS−polyacrylamide gel stained with Coomassie R-250 showing all steps in the purification of recombinant human DNA polymerase γ. (B) A western blot of all steps in the protein purification probed with anti human DNA polymerase γ antisera as described under Experimental Procedures. Lane 1: lysate of uninfected SF9 cells. Lane 2: lysate of SF9 cells infected for 72 h with recombinant baculovirus containing the cDNA for human DNA polymerase γ. Lane 3: the soluble protein fraction. Lane 4: 35% ammonium sulfate cut of the soluble protein fraction. Lane 5: fractions containing DNA polymerase γ after DEAE-sepharose chromatography. Lane 6: fractions containing DNA polymerase γ after single-stranded DNA cellulose chromatography. Lane 7: pooled DNA polymerase γ after Mono-S chromatography.

expression of the large subunit during a typical preparation can be seen by SDS−PAGE and western blot analysis (Figure 1A,B, lanes 1 and 2). Many different lysis conditions were examined (data not shown), but osmotic lysis with $(NH_2)_4SO_4$ was the only condition that resulted in a significant amount of soluble protein. The lysate was clarified by ultracentrifugation, and the supernatant was retained (Figure 1A,B, lane 3). DNA polymerase γ was further purified by ammonium sulfate precipitation (Figure 1A,B, lane 4), DEAE-sepharose chromatography (Figure 1A,B, lane 5), single-stranded DNA cellulose chromatography (Figure 1A,B, lane 6), and Mono-S chromatography (Figure 1A,B, lane 7). The final step served to concentrate as well as purify the protein, resulting in a nearly homogeneous protein. The purity of the protein varied between 90% and 95% for different preparations. The protein had a molecular mass of 138 kDa by SDS−PAGE analysis, which corresponds with the molecular mass predicted for the amino acid sequence. The protein concentration was determined both by A_{280} analysis and Bradford assay and resulted in protein concentration estimates ranging between 421 nM and 2.3 μM, depending on the preparation (*20*, *22*). This preparation technique yields 200−500 μg of nearly homogeneous human DNA polymerase γ large subunit.

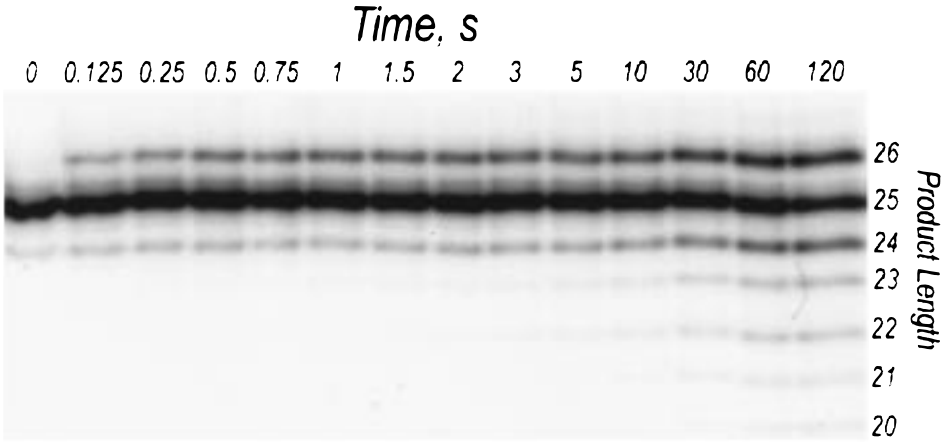

FIGURE 2: Product formation in a single-nucleotide incorporation assay. The polymerase reaction was carried out with 80 nM DNA polymerase γ large subunit incubated with 1000 nM 25/45-mer DNA primer/template in 50 mM Tris-Cl at pH 7.5 and 100 mM NaCl. The DNA primer was 5′ labeled with [γ-^{32}P] ATP. To start the reaction, an equal volume of solution containing 50 mM Tris-Cl at pH 7.5, 100 mM NaCl, 500 μM dTTP, and 5 mM $MgCl_2$ was rapidly mixed utilizing a rapid chemical quench-flow from KinTek Corporation (State College, PA) with the above enzyme DNA solution at 37 °C. The final concentrations of the DNA, enzyme, and dTTP were 500 nM, 40 nM, and 250 μM, respectively. The reaction was allowed to proceed for the times shown and then quenched with an excess of 500 mM EDTA. The time points were then analyzed on a 16% sequencing gel. Product formation was visualized on a Molecular Dynamics Phosphorimager model 445SI.

DNA Polymerase and Exonuclease Assays. The polymerase activity was measured in single-nucleotide incorporation assays by incubating the large subunit in the absence of Mg^{2+} with a 25-nucleotide primer DNA that had been annealed to a 45-nucleotide template DNA.

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GCCTCGCAGCCGTCCAACCAACTCA

CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG
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DNA 25/45-mer primer/template

The reaction was initiated by the addition of Mg^{2+} and dTTP to the enzyme·DNA complex. A typical time course of this reaction obtained by the analysis of products on a sequencing gel is shown in Figure 2, where both polymerase activity and exonuclease activity are occurring simultaneously. The exonuclease products (24-mer and below) and polymerase products (26-mer) were quantified separately for each time point.

When an excess of 25/45-mer primer/template was preincubated with a low concentration of enzyme and the reaction was initiated with the addition of Mg^{2+} and dTTP, product was formed as shown in Figure 3A. The steady-state rate constant (k_{cat}) was calculated from the slope of the line divided by the concentration of the enzyme·DNA complex, estimated by the *y*-intercept, to give a value of 0.03 s^{-1}. This rate represents the rate of dissociation of the enzyme from the DNA after each single-nucleotide incorporation event. The nonzero *y* intercept indicates that there was product formation occurring at a higher rate at early time points such that the intercept approximates the concentration of the enzyme−product complex formed in the first turnover.

When the active enzyme concentration (see below) was increased to 38 nM and the product formation was monitored at shorter times, we resolved a burst of product formation followed by a linear phase as shown in Figure 3B. The data were fitted to a burst equation, a single-exponential plus a linear term ($A^*[1 - \exp(-kt)] + mt$), giving a fast exponential rate of 3.14 s^{-1} followed by a slow linear rate of 0.03 s^{-1}, calculated as the slope, *m*, divided by the amplitude of the burst, *A*.

This enzyme also showed significant exonuclease activity on correctly paired DNA, as seen in Figure 2, and so the rate of this activity was also measured. This was accomplished by measuring the rate of formation of the exonuclease products in the absence of dTTP. A time course for exonuclease product formation is shown in Figure 3C. Exonuclease products were formed in a single linear phase. When the slope of this line was divided by the active enzyme concentration in the reaction, a k_{cat} for exonuclease activity of 0.18 s^{-1} was obtained.

Active Site Titration. Because the dissociation of the enzyme from the DNA was slow relative to the rate of polymerization, it was possible to titrate the enzyme active sites by examining the DNA concentration dependence of the burst amplitude (*25*). The active site titration was performed by preincubating a fixed concentration of enzyme (63 nM, determined by A_{280}) with increasing concentrations of DNA 25/45-mer primer/template, and the reaction was initiated by the addition of Mg^{2+} and dTTP. Reactions were quenched at 2.5 s, which allowed for full burst amplitude to be attained with negligible contributions from subsequent turnovers. The burst amplitude as a function of DNA concentration is shown in Figure 4. The data were fitted to the quadratic equation ($[E\cdot D] = 0.5(K_d + E_t + D_t) - [0.25(K_d + E_t + D_t)^2 - (E_tD_t)]^{1/2}$) which gave a K_d value for an active complex between the polymerase γ large subunit and the primer/template of 39 ± 10 nM. The fit also indicated the maximum active site concentration was 38 ± 3 nM. This value is only 60% of the expected amplitude of the active site titration based on absorbance measurements. We are currently pursuing investigations into the basis for the reduced apparent amplitude of the active site titration for our preparations. All concentrations of the large subunit of DNA polymerase γ reported in this study, unless otherwise noted, have been determined by active site concentration.

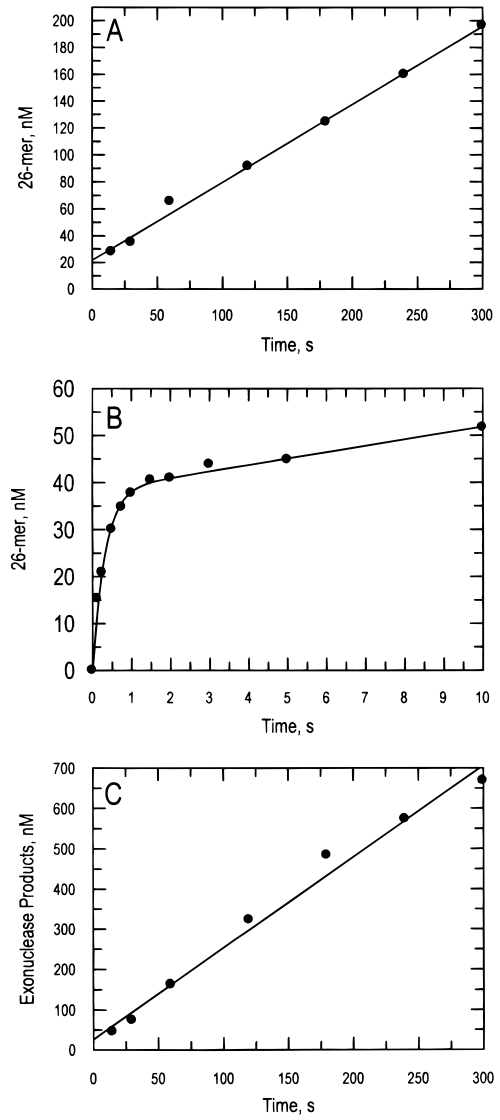


FIGURE 3: Polymerase and exonuclease assays. (A) A steady-state single-nucleotide incorporation assay was carried out by incubating 26.2 nM enzyme with 2000 nM 25/45-mer DNA primer/template, where the primer had been 5′ labeled as above, in 50 mM Tris-Cl at pH 7.5 and 100 mM NaCl. To start the reaction, an equal volume of buffer containing 50 mM Tris-Cl at pH 7.5, 500 μM dTTP, 100 mM NaCl, and 5 mM $MgCl_2$ was rapidly mixed with the above solution at 37 °C. This gave final concentrations of DNA, enzyme, dTTP, and $MgCl_2$ of 1000 nM, 13.1 nM, 250 μM, and 2.5 mM, respectively. The reaction was quenched with an excess of 500 mM EDTA at the time points shown, and the time points were separated by gel electrophoresis as in Figure 2. The products were quantified on a Molecular Dynamics Phosphorimager model 445, and the data were plotted and fitted to a line. (B) A transient-state polymerase assay. The reaction was the same as in A, except that the starting concentration of the enzyme was 80 nM, giving a final enzyme concentration of 40 nM. The reaction was quenched at the time points shown and analyzed as in A. The data were fitted to a burst equation plus linear term ($A[1 - \exp(-kt)] + mt$). (C) An exonuclease assay. The reaction was the same as in A except the solution that was mixed with the enzyme·DNA solution did not contain any dTTP. This allowed for the measurement of the exonuclease rate individually. The data were quenched and analyzed as in A. The data were fitted to a line.

K_d for dTTP and Maximum Burst Rate. The equilibrium dissociation constant, K_d, for the interaction of dTTP with the enzyme·DNA complex was measured by reacting a preincubated solution of the large subunit and primer/template with varying Mg·dTTP concentrations and monitoring the product formation over time. The rate of the burst phase as a function of dTTP concentration is plotted in Figure 5. These data were fitted to a hyperbola (burst rate = k_{max}·[dTTP]/(K_d + [dTTP])) with a K_d of 14 ± 1 μM and a maximum rate of incorporation of 3.5 ± 0.1 s^{-1}.

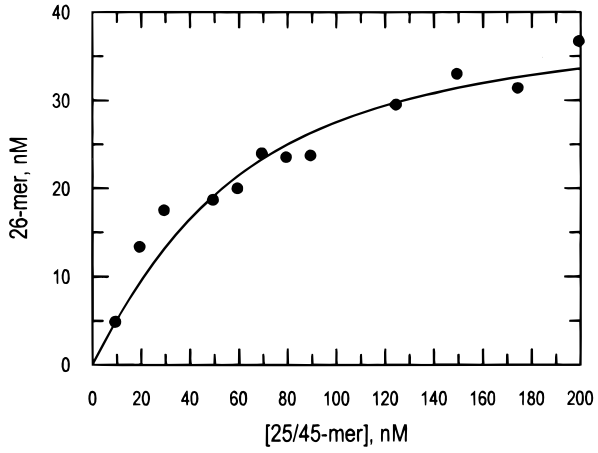


FIGURE 4: Active site titration with DNA 25/45-mer. The large subunit of DNA polymerase γ (63 nM, by A_{280}) was preincubated with increasing concentrations of 25/45-mer primer/template. The reaction was initiated by the addition of dTTP and Mg^{2+} and then quenched after 2.5 s. The data represent a plot of the burst amplitudes at varying final concentrations of 25/45-mer primer/template. The solid line is the fit of the data to the quadratic equation $[E{\cdot}D] = 0.5(K_d + E_t + D_t) - [0.25(K_d + E_t + D_t)^2 - (E_tD_t)]^{1/2}$, which gave an E·25/45-mer K_d of 39 ± 10 nM and a maximum active enzyme·DNA complex of 38 ± 3 nM.

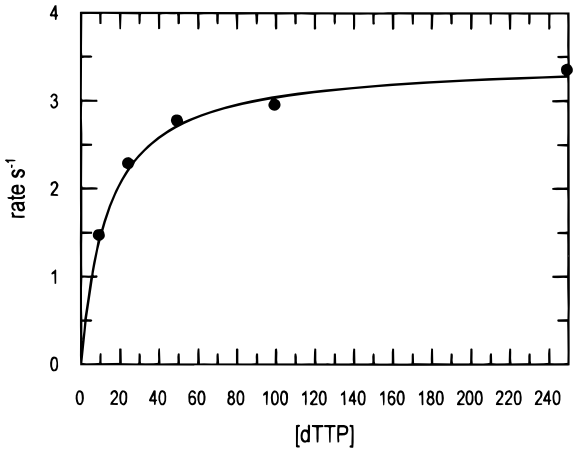
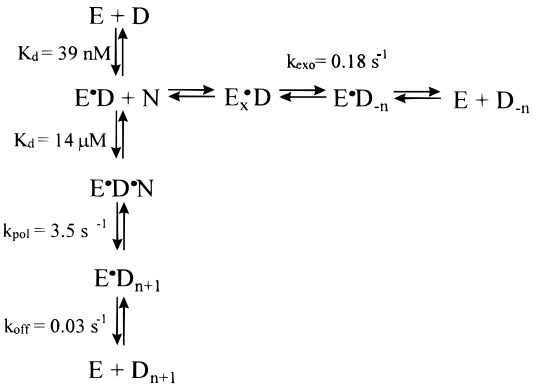


FIGURE 5: K_d for nucleotide and estimation of maximum burst rate. γ polymerase (56 nM) was preincubated with 2000 nM 25/45-mer primer/template. This was mixed with an equal volume of buffer containing 5 mM $MgCl_2$ and varying concentrations of dTTP in μM. The burst rate was plotted as a function of dTTP concentration. This data were fitted to a hyperbola with a K_d for dTTP of 14 ± 1 μM and a maximum burst rate of 3.5 ± 0.13 s^{-1}.

DISCUSSION

We expressed the large subunit of human DNA polymerase γ and purified the protein to near homogeneity (Figure 1A). The purification yielded 200−500 μg of DNA polymerase γ, between 90% and 95% pure. Western blot analysis (Figure 1B) indicated that some, if not all, of the contamination found in the final fraction could be attributed to slight proteolysis of the 138-kDa peptide. This purification represents the first isolation of the large subunit of DNA

Scheme 1



polymerase γ from recombinant sources in soluble form. Purification of the large subunit of DNA polymerase γ from the recombinant *Drosophila* gene resulted in a largely insoluble protein (*11*). Purification of human DNA polymerase γ from tissue culture only yields 10 μg of protein from 40 L of HeLa cells (*4*). Therefore, the purification of the soluble large subunit of DNA polymerase γ from recombinant sources was critical to further characterization of the polymerase by transient state kinetic analysis.

The large subunit of human DNA polymerase γ was obtained in amounts of purity sufficient to pursue an initial characterization of the enzyme's activities. Figure 2 demonstrates that the large subunit of human DNA polymerase γ possesses both polymerase and exonuclease activity in the absence of any accessory factors, which is consistent with the findings for the large subunit of *Drosophila* (*11*).

A minimal mechanism of correct nucleotide incorporation by the large subunit of DNA polymerase γ is shown in Scheme 1, which is a simplified version of a mechanism that was completely solved for the related T7 DNA polymerase (*25*). Because human DNA polymerase γ and T7 DNA polymerase both belong to the polymerase A family (*10*, *14*), and because of the commonality of the T7 mechanism for other DNA polymerases (*26*), we expect the large subunit of polymerase γ to follow a similar mechanism, although with altered rates and selectivity.

Nucleotide incorporation occurred with a burst followed by a steady-state rate of incorporation (Figure 3A,B), indicating that a fast polymerization step was followed by a slower step, which is consistent with Scheme 1. The slower step can be interpreted as the dissociation of the enzyme from the bound primer/template to allow binding to another primer/template, where it begins polymerization anew (*25*). The maximum rate of the forward polymerization step occurred when the rate of dNTP binding was not limiting. Figure 5 allowed us to extrapolate a maximum polymerization rate of 3.5 s^{-1} by comparing the burst rate versus increasing concentrations of dTTP. Figure 5 also allowed for the dissociation constant for the binding of the dNTP to the E·D complex to be calculated as 14 μM.

The proposed exonuclease domains between all DNA polymerases containing a 3′-5′ exonuclease are very highly conserved (*27*). Therefore it is highly probable that T7 DNA polymerase and DNA polymerase γ follow very similar exonuclease mechanisms. The steady-state exonuclease activity on correctly paired double-stranded DNA occurs at 0.18 s^{-1} (Figure 3C). The apparent exonuclease rate

Table 1. Parameters That Affect Processivity for Several DNA Polymerases[a]

	γ	RT	T7	Klenow
K_d, DNA (nM)	39	4.7	18	5
K_d, dNTP (μM)	14	14	18	17
k_{pol} (s^{-1})	3.5	30	300	50
k_{off} (s^{-1})	0.03	0.16	0.2	0.2
processivity	116	200	1500	250

[a] The abbreviations are as follows: γ, the large subunit of human DNA polymerase γ; RT, HIV-1 reverse transcriptase; T7, T7 DNA polymerase; Klenow, Klenow fragment. The values for polymerases other than DNA polymerase γ are taken from previous work (*25*, *29*, *30*). Processivity was calculated as k_{pol}/k_{off}.

represents a composite rate between the rate the DNA substrate is transferred to the exonuclease site, the actual rate of exonuclease activity, and the dissociation of the DNA from the enzyme (E·D $\rightleftharpoons$ E_x·D $\rightleftharpoons$ E·D_{-n} $\rightleftharpoons$ E + D_{-n}, Scheme 1). Studies on T7 DNA polymerase have shown that for correctly paired DNA the transfer of the DNA substrate to the exonuclease site is the rate-limiting step in the analysis of 3′-5′ exonuclease activity (*28*). Consequently we expect that the observed rate of 0.18 s^{-1} is largely a measure of the single-nucleotide excision rate limited by the rate of transfer of the DNA to the exonuclease site. The rate of 0.18 s^{-1} is comparable to that seen with T7 DNA polymerase and correctly base-paired substrates (*31*).

As shown in Scheme 1, for any DNA polymerase that contains a 3′-5′ exonuclease, the measurement of the forward polymerase activity is complicated by having to account for the competing exonuclease activity (*25*). To a first approximation, the observed rate of polymerization will approach the sum of the polymerization and exonuclease rates. Thus, at most, the 0.18 s^{-1} exonuclease rate makes an insignificant contribution to the observed rate of 3.5 s^{-1}. Moreover, binding of the nucleotide may stabilize the DNA bound to the polymerization site and further inhibit the exonuclease during polymerization. Therefore, we can confidently estimate the forward polymerization rate of the large subunit of human DNA polymerase γ to be 3.5 s^{-1}.

The exonuclease activity may also alter the observed rate of dissociation of the E·D_{n+1} complex. After polymerization, the enzyme either dissociates or proceeds down the exonuclease path, E·D_{n+1} $\rightleftharpoons$ E_x·D_{n+1} $\rightleftharpoons$ E·D. The excision of a base to form E·D from E·D_{+1} is expected to occur at the steady-state exonuclease rate of 0.18 s^{-1}. In the measurement of the forward polymerization reaction, the next correct nucleotide is present at saturating concentrations. Therefore when the enzyme proceeds via the exonuclease reaction to E·D, it immediately binds another nucleotide and again polymerizes to form E·D_{n+1}. Because all of the steps involved in cycling from E·D_{n+1} to E·D and back to E·D_{+1} are predicted to be at least 6-fold faster than the dissociation rate from the polymerase, there is little net effect of exonuclease on this rate. Therefore, 0.03 s^{-1} is an accurate estimation of the off rate of the enzyme from the DNA in the forward polymerase reaction.

Table 1 lists several key kinetic parameters for DNA polymerases that have had their kinetic mechanism of nucleotide incorporation determined (*25*, *29*, *30*). Comparing between polymerases, it is interesting to note that the large subunit of DNA polymerase γ binds DNA with the lowest

affinity of any of the other DNA polymerases. T7 DNA polymerase has a 20−80-fold reduced affinity for DNA when it lacks its accessory protein thioredoxin (*31*). The large subunit of human DNA polymerase γ has also been proposed to have a small subunit (*4*); therefore it is possible that the small subunit or another accessory factor performs a similar function as thioredoxin and its presence may enhance the interactions between polymerase γ and DNA. The use of an accessory factor to increase binding to DNA is common for several DNA polymerases (*32*), and it would not be unexpected that the large subunit of DNA polymerase γ might also make use of an accessory factor in a similar function. It is also possible that the duplex region of the 25/45-mer DNA primer/template utilized in these experiments is too small for optimal binding, which would also account for the slightly lower affinity of the large subunit for DNA. Experiments testing longer template/primers are currently under way.

When the processivity of the large subunit of DNA polymerase γ was calculated by dividing the polymerization rate of the enzyme by its rate of dissociation from the DNA, a value of 116 was obtained (Table 1). An enzyme that is responsible for the replication of a 16-kb genome might be expected to have high processivity. The low processivity of the large subunit is again reminiscent of the relationship between T7 DNA polymerase and thioredoxin. T7 preparations lacking thioredoxin have a processivity of less than 50 (*33*), which is similar to the value calculated for the large subunit. It might be expected that the small subunit partly enhances the processivity of polymerase γ as thioredoxin partly increases the processivity of T7, by increasing the rate of polymerization (Patel and Johnson, unpublished observations).

A processivity of 50, which is similar in magnitude to the processivity of 116 for the large subunit of human DNA polymerase γ, has been calculated for purified *D. melanogaster* polymerase γ by other methods (*15*). The purified *Drosophila* polymerase γ still has the small subunit present although highly purified fractions of DNA polymerase γ have lower activity than less pure fractions (*15*). Because the small subunit was present in the highly purified fraction of polymerase γ, which has low processivity, it is possible that it does not confer processivity on polymerase γ.

While the data in Table 1 suggest that the large subunit is an inefficient polymerase and the above arguments outline how a possible accessory protein could improve its efficacy, one must examine if the kinetic parameters could account for the in vivo replication of the mitochondrial genome. A typical mammalian cell divides every 15−40 h (*34*), and it has been shown that mitochondrial DNA synthesis occurs in the S and G2 phases of the cell cycle (*35*). Because these phases will take many hours to complete, it is anticipated that human DNA polymerase γ will have minimally several hours to replicate the genome. A mammalian mitochondrion contains from 5 to 10 copies of the mitochondrial genome per organelle, and the mammalian mitochondrial genome is 16 kb in length (*36*). Therefore the mitochondrial population of DNA polymerase γ must maximally replicate 3.2×10^5 bases in a cell cycle to completely replicate the double-stranded genome. On the basis of its forward polymerization rate of 3.5 s^{-1}, in a 5-h period a single molecule of the large subunit of DNA polymerase γ could replicate 6×10^4 bases. Each mitochondrion would require as few as 6 copies of the large subunit of DNA polymerase (one per genome copy) to replicate the entire genomic DNA in a 5-h period. Much shorter replication periods could be achieved by a modest increase in the amount of DNA polymerase γ. Though the large subunit of human DNA polymerase γ is a comparatively poor polymerase, as shown by Table 1, these simple calculations suggest it is possible that the large subunit could function without the aid of accessory proteins.

In summary, we have cloned, expressed, purified, and kinetically characterized the large subunit of DNA polymerase γ. The kinetic data suggests that the large subunit of DNA polymerase γ may require an accessory protein to increase its polymerization rate and processivity and possibly stabilize its binding to DNA. On the basis of the kinetic data and a common polymerase mechanism, the small subunit of DNA polymerase γ might assist in DNA binding and processivity. However, calculations suggest that the forward polymerization rate of the large subunit, though slow, could account for the replication of the mitochondrial genome in a physiologically relevant time period.

ACKNOWLEDGMENT

We would like to thank Billie C. Cartwright for technical assistance and John J. Chicca II for assistance in the cloning of the cDNA. The authors also thank Dr. Smita S. Patel, Dr. Susan P. Gilbert and Dr. B. Franklin Pugh for critical reading of this manuscript. Supported by grant GM44613 from the National Institutes of Health (to K.A.J.).

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BI972685U