The Mitochondrial p55 Accessory Subunit of Human DNA Polymerase γ Enhances DNA Binding, Promotes Processive DNA Synthesis, and Confers N-Ethylmaleimide Resistance*

(Received for publication, July 27, 1999, and in revised form, September 28, 1999)

Susan E. Lim‡, Matthew J. Longley‡, and William C. Copeland§

From the Laboratory of Molecular Genetics, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Human DNA polymerase γ is composed of a 140-kDa catalytic subunit and a smaller accessory protein variously reported to be 45–54 kDa. Immunoblot analysis of the purified, heterodimeric native human polymerase γ complex identified the accessory subunit as 55 kDa. We isolated the full-length cDNA encoding a 55-kDa polypeptide, expressed the cDNA in Escherichia coli and purified the 55-kDa protein to homogeneity. Recombinant Hp55 forms a high affinity, salt-stable complex with Hp140 during protein affinity chromatography. Immunoprecipitation, gel filtration, and sedimentation analyses revealed a 190-kDa complex indicative of a native heterodimer. Reconstitution of Hp140-Hp55 raises the salt optimum of Hp140, stimulates the polymerase and exonuclease activities, and increases the processivity of the enzyme by several 100-fold. Similar to Hp140, isolated Hp55 binds DNA with moderate strength and was a specificity for double-stranded primer-template DNA. However, Hp140-Hp55 has a surprisingly high affinity for DNA, and kinetic analyses indicate Hp55 enhances the affinity of Hp140 for primer termini by 2 orders of magnitude. Thus the enhanced DNA binding caused by Hp55 is the basis for the salt tolerance and high processivity characteristic of DNA polymerase γ. Observation of native DNA polymerase γ both as an Hp140 monomer and as a heterodimer with Hp55 supports the notion that the two forms act in mitochondrial DNA repair and replication. Additionally, association of Hp55 with Hp140 protects the polymerase from inhibition by N-ethylmaleimide.

Human mitochondrial DNA is a 16,569-base pair closed circular molecule encoding 13 polypeptides required for oxidative phosphorylation and 24 specialized tRNA and rRNAs needed for translation within the organelle (for review see Refs. 1 and 2). Point mutations or deletions in mitochondrial DNA cause of a wide range of neurological and cardiopathological diseases (3). The human mitochondrial genome is replicated by the nuclear encoded DNA polymerase γ (4). DNA polymerase γ has been characterized as a processive, salt-tolerant, dideoxynucleotide-sensitive, aphidicolin-resistant, Family A type DNA polymerase that can utilize a wide variety of DNA substrates including poly(rA)oligo(dT) (5). Animal cell DNA polymerase γ from Drosophila melanogaster was first shown unequivocally by Wernette and Kaguni (6) to consist of two subunits of 125 and 35 kDa in the highly purified fraction. Highly purified DNA polymerase γ from Xenopus laevis contains two subunit of 140 and 50 kDa (7). An initial report on human HeLa cell DNA polymerase γ identified a 140-kDa polypeptide and a 54-kDa polypeptide in the most purified fraction (8).

We have previously cloned the cDNAs for the human, chicken, Drosophila, and Schizosaccharomyces pombe DNA polymerase γ catalytic subunit (9, 10). The overexpressed human 140-kDa catalytic subunit contains DNA polymerase, 3′ → 5′ exonuclease, and 5′ dRP lyase activities in the absence of the accessory subunit (11, 12). Native heterodimeric Drosophila polymerase γ is highly processive and capable of copying M13 within 60 min (13). However, the isolated human DNA polymerase γ catalytic subunit displays salt sensitivity and only modest processivity (11).

The accessory subunit for the Drosophila mitochondrial DNA polymerase has been cloned and shown to encode a polypeptide of 41 kDa (14). A BLAST search with the Drosophila accessory subunit sequence identified a human homolog encoding a 43-kDa polypeptide in the IMAGE Consortium cDNA collection (14). Recently, the X. laevis accessory subunit was cloned, overexpressed, and purified from baculoviral infected insect cells (15). We demonstrated that the recombinant X. laevis accessory subunit both confers processive synthesis to the human DNA polymerase γ catalytic subunit and stimulates polymerase activity on certain substrates (15). In this report, through the combined efforts of protein purification and identification of full-length cDNA clones, we identify the HeLa cell accessory subunit to be a 55-kDa polypeptide instead of the reported 43 kDa. Characterization of the human DNA polymerase γ accessory subunit, the influence of this accessory subunit on DNA replication by DNA polymerase γ, and identification of the accessory subunit as the cause of processive DNA synthesis are presented.

EXPERIMENTAL PROCEDURES

Materials—Poly[rA]oligo(dT)12–18, poly[dA]200, oligo(dT)12–18, NTPs, and dNTPs were purchased from Amersham Pharmacia Biotech. Radioisotopes [α-32P]dTTP and [γ-32P]ATP were from Amersham Pharmacia Biotech. Oligonucleotides were purchased from Oligos Etc. or Life Technologies, Inc. The human HeLa cDNA library was from Stratagene, and the human cerebellum library was from CLONTECH. Sequence analyses of cDNA was performed on an ABI377 PE sequencer with dRhodamine-labeled dideoxy NTPs. Human recombinant mito-
protein was expressed in Escherichia coli grown for 3 h at 25°C before harvesting the cells by centrifugation. The processivity of polymerase 9.0), 0.25 M imidazole (Fraction II) was passed through a Fast Desalting activity was determined by monitoring degradation of a 5'-X-100, 3 mg/ml lysozyme, 1 M EDTA, 1 M 2-mercaptoethanol treated with digested with BamHI- and NotI-digested PHughQE9 (11). The p43Δ15 protein was expressed in Escherichia coli DH15a and purified as a denatured polypeptide using Ni-NTA-agarose in the presence of 6 M urea.

To express the Hp55 without a mitochondrial targeting sequence but with a histidine tag, the cDNA was amplified using the primers 5'-AAA TGG TCC ACC AGT GGA AAA CCA AGC CCT TTG CTA CCC (forward) and 5'-AAA AAA GGA GCC GGC GCC GCT ATA CAT TCT TAG ATG ATA T (reverse) primers. The polymerase chain reaction product was digested with BamHI and SalI and inserted into pQE9 vector. The resulting plasmid, pQESL-Hp55, was transformed into E. coli JM105, and the DNA sequence was confirmed.

Expression of a Recombinant Hp55—All chromatographic steps were performed at 4°C. A 200-ml overnight culture of E. coli JM105 transformed with pQESL-Hp55 was added to 2 liters of 2xYT broth containing 100 µg/ml ampicillin. The culture was grown for 3 h at 37°C before induction with 1 mM isopropyl β-D-1-thiogalactoside. The culture was then grown for 3 h at 37°C before harvesting the cells by centrifugation. The E. coli cell pellet (~9 g of wet cells) was resuspended and lysed in 80 ml of 0.05 M Tri-HCI (pH 7.5), 0.15 mM NaCl, 25 mM imidazole, 1% Triton X-100, 3 mg/ml lysozyme, 1 µg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml α-macroglobulin, and 1 µg/ml leupeptin. The lysate was sedimented at 30,000 × g for 15 min at 4°C, and the supernatant (Fraction I) was mixed for 30 min at 4°C with 2 ml of Ni-NTA agarose slurry (Qiagen) equilibrated in the same buffer. The column was washed three times with a 50-ml solution composed of 0.5 M NaCl, 0.05 M Tris-HCl (pH 7.5), 25 mM imidazole, and 1% Triton X-100, placed into a disposable column, and further washed with the same buffer. Protein eluted from the Ni-NTA agarose by 0.05 M Tris-HCl (pH 9.0), 0.25 M imidazole (Fraction II) was passed through a Fast Desalting column (Amersham Pharmacia Biotech) equilibrated in 25 mM KPO4 (pH 7.0), 10% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Protein samples of high purity (Fraction III) were applied to a 1 ml Mono S HR 5/5 fast protein liquid chromatography column (Amersham Pharmacia Biotech) equilibrated in the same buffer. The column was washed with 4 ml of equilibration buffer and developed with a 20-ml linear gradient of NaCl (0.1–1.0 M) in Buffer A. Homogenous Hp55 eluted at ~0.54 M NaCl, and Fraction IV (0.22 mg of total protein) was frozen with liquid nitrogen in small aliquots and stored at ~80°C.

Production of Antibodies to the Mitochondrial DNA Polymerase γ Accessory Protein—The insoluble p43 protein was purified on Ni-NTA by a deuterium protocol, and the partially purified protein was injected into rabbits for the antibody production. Polyclonal IgG specific to the mitochondrial protein and to enhance solubility, the portion

RESULTS

A BLAST search of GenBank for homologs to the Drosophila DNA polymerase γ accessory subunit identified a human homolog encoding a putative 43-kDa polypeptide (14). We obtained the clone (IMAGE Consortium clone number 44673) and amplified the published sequence for expression both in E. coli and in baculovirus-infected insect cells, but full-length p43 was not observed in either system. To mimic in vivo processing of the mitochondrial protein and to enhance solubility, the portion of the cDNA encoding 15 amino acid residues at the N terminus was deleted to remove a presumed mitochondrial targeting sequence. Expression of histidine-tagged, “mature” p43 in E. coli resulted in mostly insoluble protein, and the soluble fraction (~1%) did not cause the expected stimulation of Hp140 activity on poly(dA)poly(dT)12–15 (data not shown). Insoluble Hp43 was purified from inclusion bodies, and the denatured protein was used as antigen for the production of polyclonal antibodies (see “Experimental Procedures”). Previously we described the purification of monoclonal Hp43 from HeLa cell mitochondria (11), and possession of Hp43 antisera has allowed us to monitor the fate of the native accessory subunit during purification of pol γ from HeLa mitochondrial lysates. Although no detectable immunoreactive species could be identified in mitochondrial lysates (Fraction I) (11), a single immunoreactive 55-kDa band was readily detectable following phos-

\[ \text{Hp140} \rightarrow \text{Hp140}_{\text{N}} \]
phocellulose (Fraction II) and phenyl-Sepharose (Fraction III) chromatography (data not shown). Although the 55-kDa band co-chromatographed with the Hp140 catalytic subunit over these first two columns, the immunoreactive protein eluted from single-stranded DNA-cellulose at a significantly higher salt concentration than monomeric Hp140 (Fraction IV). Re-examination of DNA polymerase activity across the ssDNA-cellulose profile revealed an additional, cleanly resolved peak of polymerase activity that was coincident with the immunoreactive species. Silver staining and immunoblot analyses clearly identified the second peak as DNA polymerase γ (see below). The inability to express full-length Hp43, the insolubility and inactivity of mature Hp43 both in E. coli and baculovirus, and the identification from a natural source of a cross-reactive species larger than that predicted by the gene sequence led us to reinvestigate the cDNA clone for the human DNA polymerase γ accessory subunit.

Based on the existing sequence, polymerase chain reaction primers were designed to amplify a cDNA for the accessory subunit from both human cerebellum and HeLa cell cDNA libraries. Sequencing of these products in both directions revealed a continuous open reading frame of 474 amino acids that predicted a 54.9-kDa product with an N-terminal 12-kDa segment not reported in the original p43 sequence. Resequencing of the entire IMAGE Consortium clone number 44673 confirmed the 5′-extension, and the corrected sequence was submitted to GenBank™ (accession number AF142992). Two additional nucleotides in the new sequence shifted the reading frame for the 5′ portion of the gene and replaced the N-terminal 11 amino acids of the p43 sequence with 124 amino acids, adding 113 amino acid residues to the previously published sequence (14). Other minor changes included replacement of amino acids TNFTTI with NKLYYN at positions 287–292, Ser to Gly at position 136, and three other missense changes at positions 141, 295, and 306 (using the new amino acid numbers). Thus, the previously identified IMAGE Consortium clone is correct, but sequencing errors predicted an open reading frame that was a 12-kDa N-terminal deletion of the correct sequence (14).

The Hp55 appears to be a basic protein with a calculated isoelectric point of 8.3. Alignment of the Hp55 cDNA sequence with the Xenopus and Drosophila sequences reveals extensive conservation in the C-terminal region of the protein (data not shown). Kaguni and co-workers (14) previously described two leucine zipper motifs and a potential Zn\(^{2+}\) binding region in the human and Drosophila sequences that may act as anchors for binding the larger catalytic subunit. Additionally, these accessory subunits have high homology to Glycyl-tRNA synthetases (15). Computer analysis with MitoPlot predicts a mitochondrial targeting sequence with one cleavage site between amino acids 25 and 26.

Overexpression and Purification of Hp55—The Hp55 cDNA sequence was amplified without the mitochondrial leader sequence and expressed as a histidine-tagged protein in E. coli. The soluble recombinant Hp55 protein was purified to apparent homogeneity by chromatography over Ni-NTA agarose, Sephadex G-25, and MonoS columns as described under “Experimental Procedures” (Fig. 1). Elution of Hp55 from MonoS at neutral pH required salt concentration in excess of 0.5 M, suggesting strong positive charges on the surface of the protein. The purified protein lacked detectable 3′ → 5′ and 5′ → 3′ exonuclease activities (“Experimental Procedures”), and ATPase activity could not be detected in the presence or absence of single- or double-stranded DNA as assayed by the method of Hughes and Jiricny (18).

The Hp55 Accessory Subunit Binds DNA and Confers Tighter DNA Binding to Polymerase γ—Identification of a second, distinct peak of HeLa pol γ activity coincident with Hp55 on ssDNA-cellulose prompted us to test the effects of recombinant Hp55 on the DNA binding properties of Hp140 (Fig. 2). For reference, purified Hp140 was applied to a single-stranded DNA-cellulose column, and the enzyme was eluted as expected at the relatively low salt concentration of 0.22 M. Recombinant Hp55 also bound intrinsically to ssDNA-cellulose, eluting at approximately 0.25 M NaCl. However, when an equal molar mixture of the proteins was applied to the column, they were eluted as a single, symmetrical peak at 0.38 M NaCl, and the free forms of both proteins were virtually undetectable. Quantitative conversion indicated that Hp55 and Hp140 were sufficient to form the more tightly binding species of polymerase γ. Analysis of each peak by SDS-PAGE and silver staining confirmed the purity of the subunit proteins and the successful in vitro reconstitution of recombinant human pol γ (Fig. 3A). The more tightly binding native form of HeLa pol γ is also shown (Fig. 3A, lane 4), indicating a greater than 75% purity for this preparation after only three chromatographic steps. Immunoblot analysis with antibodies directed against Hp140 or Hp55 discount proteolytic degradation of the proteins and confirm the molecular weight of the native accessory protein (Fig. 3, B and C). The biochemical properties of this native preparation are compared with those of reconstituted pol γ below.

Human DNA Polymerase γ Is a Heterodimer—DNA-cellulose chromatography with a 2-fold excess of Hp55 did not increase the yield of the Hp140-Hp55 complex, and recovery of approximately 50% of the Hp55 at 0.25 M salt strongly suggested a 1:1 stoichiometry between the proteins (data not shown). Gel exclusion chromatography and sedimentation analysis were performed to determine more critically the native molecular weight and stoichiometry of the pol γ complex. As measured by gel exclusion chromatography on a Superose12 fast protein liquid chromatography column, the estimated Stoke’s radii for Hp55 and Hp140 were 31.1 and 44.2 Å, respectively. These values did not deviate substantially from the sizes expected for monomeric forms of the two proteins. For example, assuming Hp55 is a spherical, globular protein, gel exclusion chromatography predicts a native molecular weight of 51,000. The Stoke’s radius of Hp140-Hp55 was 49.9 Å. To refine our understanding of the native conformations of the proteins, band sedimentation was performed. Hp55, Hp140, recombinant Hp140-Hp55, and the native HeLa pol γ complex were then analyzed by sedimentation velocity at neutral pH. Gel filtration profiles of each sample were compared to the profiles of unbound protein following Ni-NTA agarose (Fraction IV) and MonoS pool (1 M NaCl, Fraction III); lane 5, MonoS pool (1 M NaCl, Fraction IV). The arrow indicates the position of the Hp55 polypeptide. The positions of molecular mass standards (kDa) are indicated.

![Fig. 1. Purification of recombinant Hp55 from E. coli.](image-url)
Role of the Human DNA Polymerase \( \gamma \) Accessory Subunit

**Fig. 2.** Single-stranded DNA-cellulose chromatography of human DNA polymerase \( \gamma \) polypeptides. Purified recombinant Hp140 and Hp55 were resolved on a 1-ml column of single-stranded DNA-cellulose as described (11). The absorbance of the column effluent was monitored at 280 nm. Samples included 15.2 \( \mu \)g of Hp55 (solid line), 34.9 \( \mu \)g of Hp140 (dashed line), and 28.8 \( \mu \)g of Hp140 mixed with 12.7 \( \mu \)g of Hp55 (dotted line).

**Fig. 3.** Comparison of native and recombinant complexes. Purified pol \( \gamma \) polypeptides were resolved on 4–20% SDS-polyacrylamide gels and stained with ammoniacal silver (A) or detected by immunoblot (“Experimental Procedures”) with antibodies directed against Hp140 (B) or Hp55 (C). Lane 1, 150 ng of Hp140; lane 2, 40 ng of Hp55; lane 3, ~120 ng of reconstituted Hp140-Hp55 complex (15 \( \mu \)l of DNA-cellulose fraction 20, Fig. 2); lane 4, ~200 ng of native HeLa pol \( \gamma \) complex (see text). The positions of molecular mass standards (kDa) are indicated.

6.8, 8.1, and 8.1 S, respectively. These values, together with an assumed typical partial specific volume of 0.725 cm\(^3\)/g indicate a native molecular weight for the pol \( \gamma \) complex of 192,000 and a frictional ratio of 1.31. A calculated partial specific volume of 0.722 cm\(^3\)/g based on the predicted amino acid sequences of Hp140 and Hp55 in an equal molar ratio indicates a native molecular weight for the complex of 190,000 with a frictional ratio of 1.32 (19, 20). These values are in excellent agreement with the molecular weights deduced from the gene sequences of the proteins lacking mitochondrial targeting sequences. We conclude that our reconstituted, recombinant form of human DNA polymerase \( \gamma \) is chromatographically and hydrodynamically indistinguishable from the native, heterodimeric form of HeLa pol \( \gamma \).

The Accessory Subunit Binds the Catalytic Subunit with High Affinity in the Absence of DNA—As analyzed by DNA-cellulose chromatography, gel exclusion chromatography, and sedimentation analysis, Hp140 and Hp55 were quantitatively converted into heterodimeric Hp140-Hp55 complexes by simple stoichiometric mixing in vitro. We sought to determine the strength of this interaction by protein affinity chromatography. Purified proteins were mixed under physiological conditions, and each of the two proteins was immobilized by virtue of specific affinity to Ni-NTA agarose or DPG-Sepharose, a rabbit polyclonal antibody column specific for the catalytic subunit

**Fig. 4.** Co-precipitation assay of Hp55 and Hp140 in absence of DNA and in high salt. Purified Hp140 and Hp55 were mixed individually and together with either Ni-NTA agarose or DPG-Sepharose to precipitate the proteins. Immunoprecipitates and Ni-NTA agarose precipitates were washed in buffer containing 0.15 M NaCl for 120 column volumes before separating the proteins by 4–20% SDS-PAGE. After electrophoresis the proteins were transferred to Immobilon and probed with antibody. A, Western blot of Ni-NTA agarose precipitate probed with anti-Hp140 (anti-DPG). Lane 1, His-Hp55 alone; lane 2, His-Hp55 and Hp140; lane 3, Hp140 alone; lane 4, control Hp140. B, Western blot of DPG immunoprecipitate probed with mouse monoclonal antibodies directed against the histidine tag (anti-RGS-HIS, Qiagen). Lane 1, His-Hp55 alone; lane 2, His-Hp55 and Hp140; lane 3, Hp140 alone; lane 4, control His-Hp55.

(11). The ability of an immobilized protein to retain the other subunit after extensive washing was determined by immunoblot analysis. Purified histidine-tagged Hp55 was mixed with native Hp140, and the complex was separately immobilized with Ni-NTA agarose (Fig. 4A) or DPG-Sepharose (Fig. 4B). Exhaustive washing with a solution containing of 0.05 M KP0\(_4\) (pH 7.5), 0.1% Nonidet P-40, and 0.15 M NaCl was followed by immunoblot analysis of captured proteins with anti-Hp140 antibodies (Fig. 4A) or anti-histidine tagged monoclonal antibodies (Fig. 4B). Lane 2 in both panels A and B of Fig. 4 clearly demonstrates that immobilized His-Hp55 captured Hp140 and immobilized Hp140 captured His-Hp55, respectively. The immobilized component was required in both orientations (lanes 3), indicating the absence of nonspecific binding of the proteins to each resin. Identical results were obtained when the experiment was repeated with wash buffers containing no salt or 0.5 M NaCl (data not shown), indicating that complexes formed under physiological conditions are not disrupted by extremes of ionic strength.

Hp55 Confers Salt Tolerance to Hp140—Previously we documented the unexpected salt sensitivity of the isolated Hp140 catalytic subunit on natural DNA substrates (11). Because Hp55 forms a salt-stable complex with Hp140 and enhances binding to DNA, we tested the ability of Hp55 to restore salt-tolerant activity to Hp140. In reactions that utilized activated salmon sperm DNA, addition of excess Hp55 completely abolished the salt sensitivity of isolated Hp140 and enabled the polymerase to act over a broad range of ionic strength, with optimal activity between ~75 and ~175 mM NaCl (Fig. 5A). Hp55 also expanded the window of optimal activity on poly(dA)/oligo(dT), presumably through enhanced binding of the pol \( \gamma \) complex to this substrate. Isolated Hp140 exhibited salt sensitivity on poly(dA)/oligo(dT), and Hp55 raised the salt optimum to ~100 mM NaCl (data not shown), similar to what we observed previously with the X. laevis accessory subunit (15). The observed stimulatory effects of Hp55 on Hp140 activity were more closely examined in Hp55 titration experiments at the fixed, optimal salt concentrations identified in Fig. 5A.
H55 was unable to stimulate H140 activity on activated salmon sperm DNA in the absence of added salt, but activity at 150 mM NaCl was stimulated linearly with increasing amounts of H55 until a 1:1 stoichiometry was reached (Fig. 5B). Excess H55 continued to stimulate activity but at a reduced level. Together these observations suggest that salt is necessary for enhanced binding of the pol γ complex during replication of natural DNA. Similarly, stimulation of activity on poly(rA) oligo(dT) became less pronounced when H55 was no longer limiting. Interestingly, substoichiometric addition of H55 caused marked stimulation at 50 mM NaCl on this substrate, but further addition of H55 inhibited polymerase activity to the point of negating the stimulatory effect. It is possible that excess H55 occupies primer termini or otherwise blocks extension by H140 on this substrate.

The 3′ → 5′ exonuclease activity of DNA polymerase γ has been previously shown to display a high tolerance for salt (21, 22), and we have documented the salt sensitivity of the exonuclease activity of the isolated human catalytic subunit (11). We therefore tested the effect of H55 on 3′ → 5′ exonuclease activity and observed modest stimulation with an elevated salt optimum, similar to the results for DNA polymerase activity (data not shown).

**Fig. 5. Salt-dependent stimulation of the pol γ catalytic subunit by H55.** DNA polymerase γ activity was measured on activated salmon sperm DNA (squares) or poly(rA)oligo(dT)12–18 (circles) as described under “Experimental Procedures.” A, reactions contained the indicated concentrations of NaCl and 6 ng of H140 alone (open symbols) or in combination with a ~4-fold excess of H55 (closed symbols). Reaction times were 15 min. B, reactions contained 6 ng of H140, the indicated stoichiometric equivalents of H55, and either 0 mM (open square), 0.05 mM (open circle), or 0.15 mM (closed symbols) additional NaCl.

**Fig. 6. Influence of H55 accessory protein on the processivity of DNA polymerase γ on singly primed M13 DNA.** Processivity reactions (“Experimental Procedures”) utilized 12 ng of H140 (lanes 1–4), 8 ng of H55 (lanes 3 and 4), or ~28 ng of HeLa pol γ complex (lanes 5 and 6). Reactions in lanes 2, 4, and 6 were supplemented with 0.15 mM NaCl. Lane 0 contained no enzyme. A, samples were analyzed by denaturing 7% polyacrylamide gel electrophoresis. B, identical samples were analyzed by alkaline agarose gel electrophoresis in 0.03 M NaOH, 1 mM EDTA. The relative mobilities (kilobases, kb) of radiolabeled, HindIII-digested, bacteriophage λ DNA fragments are shown in lane M. The position of the unextended primers (arrow) is indicated. nt, nucleotides.
apparent (lane 3). However, addition of 150 mM NaCl eliminated most of the stalling within 100 nucleotides of the primer (lane 4), and the bulk of the primer was converted to a form that could not enter the polyacrylamide gel. In addition, substantial reduction of exonuclease activity under these conditions may indicate a more stringent selection for the polymerase active site of Hp140 in the presence of Hp55. Almost identical results were obtained for the native HeLa pol γ complex (lanes 5 and 6). To resolve fully the high molecular weight products we separated the same reactions on a denaturing agarose gel (Fig. 6B). Products synthesized by isolated Hp140 at both salt concentrations were too short to be visualized (lanes 1 and 2), but the Hp140-Hp55 complex synthesized products as long as 7 kilobases or the entire length of the M13 substrate (lanes 3 and 4). 3–5-fold more product was apparent for the 150 mM NaCl reaction, consistent with the salt-dependent stimulation of Hp140-Hp55 activities (compare lanes 3 and 4).

The Hp55 Subunit Specifically Binds Double-stranded DNA—Retention of Hp55 by the single-stranded DNA-cellulose column was unexpected (Fig. 2). To better understand the intrinsic affinity of isolated Hp55 to single-stranded or double-stranded DNA, we performed gel mobility shift assays with a single-stranded, 32P-labeled oligonucleotide annealed, as indicated, to its complementary sequence (Fig. 7). Less than 15% of the single-stranded oligonucleotide was shifted by increasing amounts of Hp55 (Fig. 7, lanes 2–4 compared with lane 1). In contrast, equivalent amounts of human mitochondrial single-stranded DNA-binding protein shifted all of the single-stranded primer (lanes 6 and 7). When this same single-stranded primer was annealed to its complementary sequence, as little as 2 pmols of Hp55 shifted 55% of this primer-template substrate. Increasing the Hp55 to 8 pmols shifted an additional 10% of the original primer-template (lane 11) and caused an aggregation of the DNA-protein complex at the origin. Addition of salt to the mixture (lane 12) resulted in no additional binding of the original primer-template (compare lanes 10 and 12). Our negative control, HmtSSB, bound very little of the double-stranded primer-template. These results demonstrate that Hp55 binds DNA intrinsically and has a strong preference for double-stranded over single-stranded DNA.

**FIG. 7.** Hp55 binds preferentially to double-stranded DNA. Autoradiogram of a gel mobility shift experiment with a single-stranded primer (lanes 1–7) or double-stranded primer-template (lanes 8–14) as described under “Experimental Procedures.” Lanes 1 and 8 contain the oligonucleotide substrates with no added protein. Single-stranded 38mer or primer-template was mixed with either 2 pmols of Hp55 (lanes 2 and 9), 4 pmols of Hp55 (lanes 3, 5, 10, and 12), 8 pmols of Hp55 (lanes 4 and 11), 25 pmols of HmtSSB (lanes 6 and 13), or 5 pmols of HmtSSB (lanes 7 and 14). An additional 100 mM NaCl was added to the reactions in lanes 5 and 12. Arrows indicate the unshifted primer or primer-template substrates.

| Enzyme          | $K_m$ (nM 3’-OH ends) | $k_{cat}$ (dTTP) | $k_{cat}$/[$S$] | $k_{cat}$ (dTTP) | $k_{cat}$/[$S$] |
|-----------------|------------------------|------------------|----------------|-----------------|----------------|
| Hela pol γ complex |                         |                  |                |                 |                |
| No NaCl         | 74                     | 3.2              | 0.08           | 0.03            |                |
| 0.1 mM NaCl     | 41                     | 4.0              | 0.28           | 0.07            |                |
| Hp140 · Hp55    | 46                     | 3.1              | 0.27           | 0.09            |                |
| No NaCl         | 43                     | 3.1              | 0.73           | 0.23            |                |
| Hp140 alone     | 5800                   | 6.7              | 0.60           | 0.09            |                |
| 0.1 mM NaCl     | ND                     | ND               | ND             | ND              |                |

*ND, no detectable activity at this salt concentration.

The Accessory Subunit Increases the Affinity of the DNA Polymerase γ for DNA—To investigate the cause of the high processivity conferred by the accessory subunit we performed steady state kinetic analysis of the native HeLa pol γ complex, reconstituted recombinant Hp140-Hp55, and the isolated catalytic subunit (Table I). Reactions utilized poly(dA)·oligo(dT)$_{12-18}$ as the substrate and were performed with physiological buffer containing no salt or 0.1 mM salt. The $K_m$ for dTTP insertion was independent of salt and similar for all three forms of the enzyme. Values for $k_{cat}$ of dTTP insertion were also similar, and the −3-fold salt effect on $k_{cat}$ more precisely explains the general salt stimulation observed for complexes on activated DNA and poly(rA)·oligo(dT) (Fig. 5A). The most striking difference in the kinetic constants for the different forms of the enzyme was the $K_m$ for DNA 3’-OH ends. The two subunit pol γ, either reconstituted or native, had an apparent $K_m$ for DNA 100-fold lower than that of free Hp140. For an ordered bisubstrate reaction, binding of the first substrate (primer-template) is given by the ratio of $k_{cat}$/[$S$]. The effect of Hp55 was likely to increase $k_{cat}$ for DNA. A lower $K_m$ for DNA allows the polymerase to remain bound to the substrate at higher salt concentrations, whereas isolated Hp140 has little measurable activity at 100 mM NaCl (Table I).

The Hp55 Accessory Subunit Protects the DNA Polymerase γ Catalytic Subunit from N-Ethylmaleimide Inhibition—NEM irreversibly and covalently modifies sulfhydryl groups in proteins, and inhibition of an enzyme by NEM indicates solvent accessible cysteine residue(s) that are critical for activity. Traditionally, DNA polymerase γ has been viewed as a NEM sensitive DNA polymerase, and this sensitivity is one of the few inhibitory characteristics that set it apart from the NEM-resistant DNA polymerase β (5, 23). We previously reported the extreme sensitivity of the isolated Hp140 enzyme (11). Our initial analysis of the native heterodimeric HeLa pol γ complex indicated it had the classical inhibitory properties of DNA polymerase γ (sensitive to dideoxynucleotides but resistant to aphidicolin) but appeared to be resistant to N-ethylmaleimide. We sought to exploit the ability to reconstitute the pol γ complex from individual components to investigate the cause of this NEM resistance. Both the native and recombinant Hp140-Hp55 complexes displayed nearly complete resistance to NEM up to 1 mM and still retained >50% activity with 10 mM NEM (data not shown). In marked contrast, the single subunit was inhibited to 50% with less than 0.1 mM NEM and >90% inhibited at 0.5 mM NEM. Thus, the Hp55 accessory subunit protects the catalytic subunit from NEM inhibition by over
100-fold. This inhibition was independent of salt, and order of addition experiments demonstrated that DNA binding was not required for this protection.

DISCUSSION

The correct sequence of the cDNA encoding the human mitochondrial DNA polymerase γ accessory subunit encodes 113 more amino acids at the N terminus than the previously published sequence, which encoded a 43-kDa polypeptide (14). The full-length cDNA without the mitochondrial leader sequence was expressed in E. coli, purified to homogeneity, and shown to produce a functional polypeptide of 55 kDa. The Hp55 subunit forms a tight 1:1 stoichiometric complex with the catalytic subunit in the presence or absence of DNA, and the 190-kDa complex can survive extremes of ionic strength. Kinetic values indicate that the presence of the second subunit decreases the $K_m$ of the catalytic subunit for primer termini by 2 orders of magnitude with only a modest stimulation of $V_{max}$. We have also demonstrated that Hp55 increases the average number of nucleotides incorporated per productive binding event to several thousand, 50–100 times greater than the processivity of the isolated catalytic subunit. Thus, the greatly enhanced DNA binding caused by Hp55 produces a highly processive DNA polymerase, capable of replicating the entire human mitochondrial genome of 16,569 base pairs within ~45 min. This prediction agrees with the work of Clayton (4), who showed that mouse mitochondrial DNA doubles every 1–2 h.

Purification of DNA polymerase γ from HeLa mitochondrial lysates resolved the enzyme into two native forms: a heterodimeric complex and the isolated catalytic subunit. Our ability to detect and to resolve the two forms was entirely dependent upon the selection of chromatographic resins, DNA substrates, and in vitro assay conditions. For example, Hp55 raised the salt optima and stimulated activity on all substrates tested. In contrast, salt was inhibitory to isolated Hp140 on activated DNA. Because of the marked differences in activity of the two forms under any single set of reaction conditions, previous purification procedures of DNA polymerase γ from a variety of species may have been unable to discriminate, possibly resulting in the uncomplexed catalytic subunit being discarded. More importantly, established preparations of pol γ may be a mixture of the two forms, and biochemical analysis of such mixtures would yield composite values dependent on the relative abundance and inhibition of the two forms. For example, the two recombinant forms had independently determined sedimentation constants ($s_{20,w}$) of 6.8 and 8.1 S, whereas a previously reported value for highly purified HeLa cell DNA polymerase γ was 7.8 S (8). Such a scenario could also help to explain the apparently poor recovery of second subunit with respect to the catalytic subunit (8). Additionally, because only the catalytic subunit is sensitive to inhibition by NEM, we believe that previous reports of NEM sensitivity for DNA polymerase γ are the natural conclusions for preparations containing excess catalytic subunit (6, 8, 24).

Highly purified preparations of DNA polymerase γ from Droso phila embryos and X. laevis oocytes resulted in apparently equal abundance of the large and small subunits (6, 15). Nevertheless, we were able to resolve the two forms of HeLa cell DNA polymerase γ in nearly equal quantities. Because purified Hp55 and purified Hp140 form a very stable complex in vitro, dissociation of the subunits during purification seems unlikely. Therefore, we propose that DNA polymerase γ in HeLa cells exists in two forms, possibly reflecting its dual roles in mitochondrial DNA replication and repair (11, 12). Because the Xenopus and Dro sophila enzymes were derived from developmenta lmental tissues, the accessory subunit may be required for proliferation and replication synthesis, whereas the individual catalytic subunit is sufficient for maintenance and/or post replication repair processes.

Gel mobility shift assays indicate that Hp55 specifically binds double-stranded primer-template DNA compared with single-stranded DNA, and ATP is not required for this interaction. Also, Hp55 behaves as a monomer when free from the Hp140 subunit. In this sense, Hp55 differs from the homodimeric E. coli β-subunit and the homotrimeric nuclear processivity factor proliferating cell nuclear antigen by not requiring a clamp loader to bind DNA (25–27). Because of its simplicity and close sequence similarity, T7 DNA polymerase is thought to be a structural and mechanistic model for DNA polymerase γ, and Hp55 may be more reminiscent of thiore doxin. Thiore doxin binds to T7 DNA polymerase and potentially causes a closure of the complex around DNA (28). Similarly, association of Hp55 with Hp140 enhances binding of the complex to primer-template DNA. However, thiore doxin and Hp55 differ in that thiore doxin does not bind DNA in the absence of T7 DNA polymerase (29, 30). The intrinsic affinity of Hp55 to double-stranded DNA may help to tether or recruit Hp140 to primer termini.

The role of the accessory subunit as a processivity factor also implies it will have effects on the fidelity of DNA synthesis. In the case of T7 DNA polymerase, thiore doxin was shown to lower the fidelity for single nucleotide insertion while increasing fidelity through homopolymeric runs by preventing strand slippage (31). The effect of the accessory subunit on the fidelity of DNA synthesis by wild type and mutator DNA polymerase γ proteins is presently being investigated in our laboratory.

Acknowledgments—We thank Olivia Dominick for assistance with DNA sequencing and Dr. Leroy Worth for performing the ATPase assay. We thank Drs. K. Bebenek and L. Worth for critical reading of this manuscript.

REFERENCES

1. Shoffner, J. M., and Wallace, D. C. (1994) Annu. Rev. Nutr. 14, 535–568
2. Bourgeron, T., Rustin, P., Chretien, D., Birch-Machin, M., Bourgeois, M., Viegas-Pequignon, E., Munnich, A., and Rotig, A. (1995) Nat. Genet. 11, 144–149
3. Wallace, D. C. (1999) Science 283, 1482–1488
4. Clayton, D. A. (1992) Cell 72, 693–705
5. Kornberg, A., and Baker, T. A. (1992) DNA Replication, 2nd Ed., pp. 209–210, W. H. Freeman and Co., New York
6. Wernette, C. M., and Kaguni, L. S. (1986) J. Biol. Chem. 261, 14764–14770
7. Insdorf, N. F., and Bogenhagen, D. F. (1989) J. Biol. Chem. 264, 21491–21497
8. Gray, H., and Wong, T. W. (1990) J. Biol. Chem. 265, 5835–5841
9. Ropp, P. A., and Copeland, W. C. (1995) Gene (Amst.) 165, 103–107
10. Ropp, P. A., and Copeland, W. C. (1996) Genomics 36, 449–458
11. Longley, M. J., Ropp, P. A., Lim, S. E., and Copeland, W. C. (1998) Biochemistry 37, 10529–10539
12. Longley, M. J., Prasad, R., Srivastava, D. K., Wilson, S. H., and Copeland, W. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12244–12248
13. Williams, A. J., Wernette, C. M., and Kaguni, L. S. (1993) J. Biol. Chem. 268, 24855–24862
14. Wang, Y., Farr, C. L., and Kaguni, L. S. (1997) J. Biol. Chem. 272, 13640–13646
15. Carrodegues, J. A., Kohayashi, R., Lim, S. E., Copeland, W. C., and Bogenhagen, D. F. (1999) Mol. Cell. Biol. 19, 4039–4046
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 18.17–18.18, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
18. Hughes, J. M., and Jiricny, J. (1992) J. Biol. Chem. 267, 33876–33882
19. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
20. Siegel, L. M., and Monty, K. J. (1966) Biochem. Biophys. Acta 112, 346–362
21. Longley, M. J., and Mosbaugh, D. W. (1991) J. Biol. Chem. 266, 24703–24711
22. Olson, M. W., and Kaguni, L. S. (1995) J. Biol. Chem. 270, 23136–23142
23. Wang, T. S.-F. (1991) Annu. Rev. Biochem. 60, 513–552
24. Mosbaugh, D. W. (1988) Nature Struct. Mol. Biol. 26, 12244–12248
25. Wyman, C., and Botchan, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 36, 18.17–18.18, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Nano, J., and Jiricny, J. (1992) J. Biol. Chem. 267, 33876–33882
27. Karlson, Z., and O’Donnell, M. (1995) Nucleic Acids Res. 23, 3613–3620
28. Jonsson, Z. O., and Huber, U. (1997) Bioessays 19, 967–975
29. Dubois, S., Tabor, S., Leng, A. M., Richardson, C. C., and Ellenberger, T. (1998) Nature 391, 251–258
30. Huber, H. E., Tabor, S., and Richards, C. C. (1987) J. Biol. Chem. 262, 16224–16232
31. Tabor, S., Huber, H. E., and Richardson, C. C. (1987) J. Biol. Chem. 262, 16212–16223
32. Kunkel, T. A., Patel, S. S., and Johnson, K. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6830–6834