Mutations in human mitochondrial DNA influence aging, induce severe neuromuscular pathologies, cause maternally inherited metabolic diseases, and suppress apoptosis. Since the genetic stability of mitochondrial DNA depends on the accuracy of DNA polymerase γ (pol γ), we investigated the fidelity of DNA synthesis by human pol γ. Comparison of the wild-type 140-kDa catalytic subunit to its exonuclease-deficient derivative indicates pol γ has high base substitution fidelity that results from high nucleotide selectivity and exonucleolytic proofreading. pol γ is also relatively accurate for single-base additions and deletions in non-iterated and short repetitive sequences. However, when copying homopolymeric sequences longer than four nucleotides, pol γ has low frameshift fidelity and also generates base substitutions inferred to result from a primer dislocation mechanism. The ability of pol γ both to make and to proofread dislocation intermediates is the first such evidence for a family A polymerase. Including the p55 accessory subunit, which confers processivity to the pol γ catalytic subunit, decreases frameshifting and base substitution fidelity. Kinetic analyses indicate that p55 promotes extension of mismatched termini to lower the fidelity. These data suggest that homopolymeric runs in mitochondrial DNA may be particularly prone to frameshift mutation in vivo due to replication errors by pol γ.

The human mitochondrial genome (mtDNA) encodes 37 genes required for oxidative phosphorylation or mitochondrial protein synthesis (1). Loss of these essential gene functions clearly induces a multitude of severe metabolic disorders, and mutation of mtDNA is the cause of inheritable mitochondrial diseases (2–4). Early reports comparing nucleotide substitutions in mtDNA from somatic tissues of different primates revealed a 10-fold higher rate of evolution for mtDNA relative to the nuclear genome (5, 6), implying a relatively high mutation rate for mtDNA. More recently, the accumulation of deletions in mtDNA has been shown to correlate with increasing age (7), and a current longitudinal study strongly supports the age-dependent accumulation of non-inherited point mutations in human mtDNA (8). Recent data suggest that mutations in mtDNA can suppress apoptosis, a situation that would favor the growth of tumor cells (9). Additionally, human somatic cancer cells can acquire a homoplasmic mutant mtDNA genotype, presumably by mitotic segregation of mutant mitochondria during proliferation of tumors (10). The prevalence of mtDNA mutations in a variety of human cancers may be more than a passive association (11). Thilly and co-workers (12, 13) have developed sensitive methods to examine the spectrum of mtDNA mutations that form in human cells in vivo. Molecular genetic analyses such as these are the starting point for studying the biochemical mechanisms of mutagenesis of the mitochondrial genome.

Mutations in mtDNA arise from several sources, all of which involve DNA synthesis by the mitochondrial DNA polymerase, pol γ. Spontaneous replication errors produce mismatches, and replication through unrepaired mismatches can mutate mtDNA. Although Saccharomyces cerevisiae possesses the mismatch repair homolog Msh1 that can stabilize yeast mtDNA (14, 15), evidence for mitochondrial mismatch repair in higher eukaryotes is currently lacking (16). mtDNA chemically damaged by hydrolysis, reactive oxygen species, or environmental mutagens contains non-coding or mis-coding lesions (17, 18). Evidence for base excision repair of damaged mtDNA is abundant (17, 19–25), and pol γ has a well established role in mitochondrial base excision repair in vitro (26–28). Replication of DNA templates damaged by platinum adducts may also lead to mutations in mtDNA (29). Because pol γ is a component common to each mode of mutagenesis, knowledge of its biosynthetic fidelity is critical to understanding mitochondrial mutagenesis.

pol γ purified from chicken embryos or from pig liver mitochondria is accurate in vitro, with these enzymes exhibiting error frequencies at a 3-nucleotide mutational target of <3.8 × 10⁻⁶ per nucleotide and <2.0 × 10⁻⁶ per nucleotide, respectively (30, 31). Both enzymes contain intrinsic 3′- and 5′-exonuclease activities that prefer mispaired 3′ termini. Partial inhibition of these exonuclease activities with 20 mM dGMP increases the frequency of errors, suggesting the exonucleases proofread replication errors (30, 31). pol γ derived from chicken, pig, Drosophila melanogaster, Xenopus laevis, Saccharomyces cerevisiae, and human sources copurifies with 3′- to 5′-exonuclease activity (30–37), and the genes for all known mitochondrial DNA polymerases possess three highly conserved exonuclease motifs common to family A DNA polymerases (38–41). Several lines of evidence show the exonuclease contributes to replication fidelity in vivo. Disruption of the exonuclease motifs in the yeast MIP1 gene generates a mutator phenotype, as exhibited by a several hundred-fold increase in the spontaneous frequency of forming mitochondrial erythromycin-resistant mutants (42). Expression of exonuclease-deficient pol γ fusion proteins in cultured human cells also resulted in the accumulation of point mutations in mitochondrial DNA (43). Also, the loss of exonuclease function of pol γ in transgenic mice resulted in the rapid accumulation of point mutations and...
deletions in cardiac mtDNA, and the mutagenesis was accom-
panied by cardiomyopathy (44). We and others (39, 45, 46) have cloned and overexpressed the human gene encoding the catalytic subunit of DNA polymerase γ. In addition, we have constructed an exonuclease-deficient human DNA polymerase γ by replacing the conserved Glu and Asp residues in exonuclease region I of the catalytic subunit (45). The full-length human cDNA for the 55-kDa accessory subunit has been isolated and overexpressed (47–49), and the three-dimensional crystal structure of this protein has been determined (50). When associated with the catalytic subunit, the accessory subunit confers high processivity to pol γ through enhanced DNA binding (47, 48). However, the fidelity of DNA synthesis by structurally defined human pol γ with and without the accessory subunit or exonuclease proofreading is not known. In this report we present a comprehensive study of the fidelity of DNA synthesis by human DNA polymerase γ in vitro. We reconstituted the various forms of human pol γ and measured error rates during replication of natural DNA substrates. We also performed steady state kinetic analysis of base selection and mispair extension to determine the relative contributions of exonucleolytic proofreading and the accessory subunit to overall fidelity of replication.

EXPERIMENTAL PROCEDURES

Proteins—The recombinant catalytic subunit of human DNA polymerase γ (p140), the exonuclease-deficient form (p140 Exo-), and their His₆-tagged derivatives were purified to homogeneity as described (45). The His₆-tagged accessory subunit (p55) was purified to homogeneity, and the heterodimeric forms of the polymerase (p140+p55 and p140 Exo-p55) were reconstituted as described (47). Protein concentration was determined relative to a BSA standard by quantitative digital imaging of protein bands that had been resolved by SDS-polyacryl-

amide gel electrophoresis and stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Fidelity Assays—The accuracy of DNA synthesis by human pol γ was measured by copying a single-stranded region of bacteriophage M13 DNA encoding the α-peptide region of the 3 β-galactosidase gene. Replication errors were scored by transfection and plating on chromogenic indicator plates to score plaque colors. The necessary bacterial strains, bacteriophage M13mp2 derivatives, and all procedures related to fidelity assays have been described (51–53). Gap-filling reaction mixtures (30 μl) contained 25 mM HEPES-KOH (pH 7.6), 2 mM dithiothreitol, 1 mM each dATP, dCTP, dGTP, and TTP, 4 mM MgCl₂, 50 μg/ml acetyl-
ated BSA, ~150 ng of gapped M13mp2 DNA, and 16–220 ng p140 or p140 Exo-, as needed to fill gaps within 60 min at 37 °C. Reactions utilizing p140+p55 were supplemented with 0.1 mM NaCl to achieve optimal activity (47). Uncomplexed p140 was not active under these conditions (47). Reaction products were isolated by phenol extraction and ethanol precipitation, and closure of the gaps was confirmed by agarose gel electrophoresis prior to transfection. Mutation frequencies were calculated as described, and specific nucleotide changes in mutant DNAs were determined by DNA sequencing.

Nucleotide Insertion Kinetics—The fidelity of nucleotide selection by pol γ was determined with a polyclamylide gel-based, single nucleotide extension assay utilizing synthetic oligodeoxyribonucleotides (54–56). Oligonucleotides were obtained from Oligos, Etc. (Wilsonville, OR), purified by denaturing polyacrylamide gel electrophoresis and ethanol precipitation, and 5′-labeled with T4 polynucleotide kinase (New England Biolabs) and γ⁻³²P-ATP (Amersham Pharmacia Biotech). La-

“labeled primer (18-mer) and unlabeled template (36-mer) were hybridized (ratio = 1:1.1) to form the following primer-template with a recessed 3′-end: G primer, 5′-TGA CCA TGT ACA TCA GAC-3′; C template, 3′-ACT GGT ACA TGT AGT CTC AGC CTG CAT ATA GTC ACT-5′. Reaction mixtures (10 μl) contained 25 mM HEPES-KOH (pH 7.6), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 50 μg/ml acetylated BSA, 5 mM MgCl₂, 1 pmol of primer-template, 10–30 fmol of exonuclease-deficient p140 or p140 Exo-p55, and one of the four common deoxynucleoside triphos-
phates. As the next correct nucleotide, TTP was varied from 0 to 0.2 μM, whereas the three incorrect nucleotides were varied from 0 to 1 mM. Following incubation at 37 °C for 5 min, reactions were terminated by the addition (10 μl) of 95% deionized formamide and 10 μl EDTA. Samples (2 μl) were boiled for 5 min and resolved by electrophoresis on 15% polyacrylamide gels containing 7 M urea. Radioactive bands were detected with a Storm 860 PhosphorImager (Molecular Dynamics) and quantified with NIH Image software (version 1.62). To remain within the limitations of steady state analysis, only values from reactions in which less than 25% of the primer had been extended were used to calculate initial reaction velocities. Kinetic constants were estimated by linear least-squares curve fitting of double-reciprocal plots.

Mispair Extension Kinetics—Four additional DNA substrates were constructed for mispair extension assays. Oligonucleotides were purified, labeled, and hybridized as before to generate primer-templates with recessed 3′-ends and A, T, C, or G mispairs at their 3′ termini as follows: A primer, 5′-TGA CCA TGT ACA TCA GAA-3′, and C primer, 5′-TGA CCA TGT ACA TCA GAC-3′; and T primer, 5′-TGA CCA TGT ACA TCA GAG-3′, and C template, 3′-ACT GGT ACA TGT AGT CTC AGC CTG CAT ATA GTC ACT-5′; G primer, 5′-TGA CCA TGT ACA TCA GAC-3′, and G template 3′-ACT GGT ACA TGT AGT CTC AGC CTG CAT ATA GTC ACT-5′.

The composition of reactions was the same as for nucleotide insertion, except the next correct nucleotide (TPP) was varied from 0 to 1 mM. Products were analyzed, and kinetic constants were determined as before.

RESULTS

Average Fidelity of Human pol γ—The accuracy of DNA synthesis by human pol γ was measured in vitro by copying a single-stranded region of bacteriophage M13mp2 DNA encoding a 407-nucleotide section of the β-galactosidase gene. Cor-

rect polymerization produces DNA that yields blue M13 plaques, whereas errors are scored as light blue or colorless plaques. A broad view of replication fidelity is provided by this forward mutation assay, which scores all 12 possible single-base substitutions, each in a variety of sequence contexts, as well as additions and deletions of 199 different template nucleo-

tides present as non-iterated or repeated sequences. We mea-

ured the fidelity of polymerization reactions catalyzed by the wild-type catalytic p140 subunit of human pol γ either alone or in the presence of its p55 accessory subunit. To determine the contribution of exonuclease-deficient proofreading to replication fidelity, we also measured the fidelity of exonuclease-deficient pol γ either alone or with p55. The frequencies of lacZ mutant plaques generated in the four different polymerization reac-

tions are shown in Table 1 (top section). The wild-type poly-

merase reactions generated products whose lacZ mutant fre-

quencies were only slightly above the background mutant frequency of the uncopied control DNA (7 × 10⁻⁶). Reactions catalyzed by the exonuclease-deficient polymerase had 1.8-fold higher lacZ mutant frequencies, indicating that this polymer-

ase was less accurate and that the majority of the lacZ mutants contained errors made during DNA synthesis in vitro. Inclu-

sion of the p55 accessory subunit further increased the mutant frequency for p140 Exo-, but proofreading was able to balance this apparent mild reduction in fidelity caused by p55. DNA extracted from collections of independent lacZ mutants were sequenced to classify the types of errors made by each form of the enzyme and to permit calculation of specific error rates (51).

Single-base substitutions were found in the majority of lacZ mutants recovered from all four copying reactions (Table 1). In the case of wild-type pol γ without p55, the average substitu-

tion error rate when considering all mispairs (≥8 × 10⁻⁶) and the rates for individual mismatches (TdGMP and AdAmp listed as an example, the other 10 mismatches not shown) are all “less than or equal to” values, since these values are similar to those for uncopied DNA. This indicates that the exonuclease-deficient catalytic subunit has relatively high base substitution fidelity. As expected, the exonuclease-deficient enzyme is less accurate, e.g. by ≥30-fold for the TdGMP mismatch and by ≥14-fold for the AdAmp mismatch (Table 1). This suggests that the majority of misinsertions made by the catalytic subunit are proofread by its intrinsic exonuclease. Proofreading is
Frequency and classes of errors made by pol γ in the presence and absence of exonucleolytic proofreading and the accessory subunit

Fidelity determinations were performed as described under “Experimental Procedures” and “Results.” The mutant frequency values shown here are for single determinations, but previous studies have shown that standard deviations are about 20% of mean values when multiple determinations are performed. The numbers in parentheses are the number of template nucleotides (sites) in the lacZ template at which each type of error can be scored. Error rates were calculated as described (51). Error rates listed as ≤ are half within 2-fold of previously published values for uncopied DNA and therefore are unlikely to reflect errors made during gap-filling DNA synthesis. The background mutant frequency for uncopied control DNA was 7 × 10⁻⁶.

| Type of error (Sites) | Mutants | Error rate | Mutants | Error rate | Mutants | Error rate | Mutants | Error rate |
|----------------------|---------|------------|---------|------------|---------|------------|---------|------------|
| All substitutions (125) | 52 | 10⁻⁶ | 59 | 6 | 10⁻⁶ | 91 | 10 | 6 | 10⁻⁶ |
| Wild type | 103 | 114 | 127 | 140 | 119,079 | 10⁻⁶ | |
| Exo-deficient | 52,224 | 52,224 | 22,699 | 22,699 | 62 | 10⁻⁶ | |
| Mutant plaques | 12 × 10⁻⁴ | 12 × 10⁻⁴ | 11 × 10⁻⁴ | 11 × 10⁻⁴ | |
| Total plaques | 86,262 | 52,224 | 119,079 | 22,699 | |
| Mutant frequency | | | 119,079 | 22,699 | |

The p55 subunit has no apparent effect on the frameshift fidelity of wild-type p140. In contrast, p55 increases the frameshift error rates of exonuclease-deficient p140 but in a sequence-dependent manner. Thus, p55 increases the deletion rate by 9-fold for non-iterated nucleotides and by 3-fold for nucleotides present in homopolymeric runs of two bases. However, it does not affect the rate of deletions in runs of 3–5 bases. These data suggest that p55 may promote extension of misaligned primer-templates in which the unpaired base is at or 1 base pair upstream of the 3’ terminus but has little effect when two correct terminal base pairs are possible in the misaligned intermediate. A comparison of wild-type p140 and exonuclease-deficient p140 also suggests that even when p55 is present proofreading still corrects most of addition misalignments that have an extra base in the primer strand. Proofreading is also apparent for intermediates that have the extra base in template strand and lead to deletions of non-iterated nucleotides or nucleotides present in 2- and 3-base homopolymeric runs.

Dislocation Mutagenesis—Hypothetically, strand slippage in repetitive sequences may also result in substitution errors by a dislocation mechanism. This model suggests that strand slippage in a template run is followed by incorporation of the next correct nucleotide. Continued DNA synthesis results in a −1 frameshift mutation, and realignment followed by DNA synthesis results in a base substitution error. In previous studies of mammalian DNA polymerase β (59–61), this transient misalignment mechanism explained a hot spot for T→G substitutions at position 70 (underlined) in the sequence 5’-GTTTT in the lacZ template. For three of the four reactions catalyzed by pol γ in the present study, the spectra of errors (not shown) revealed that position 70 is again a hot spot for T→G substitutions. The error rate for this particular substitution is much higher than is the average rate for T→G substitutions at the other 22 sites in the template where this substitution can be scored (Table II). Consistent with the dislocation model and previous studies, pol γ also generated single T deletions at a high rate in the TTTT run (Table II). A comparison of rates for the wild-type and exonuclease-deficient p140 reactions (≤ 19 × 10⁻⁶ versus 96 × 10⁻⁶) suggests that T→G substitutions at position 70 are proofread. A parallel comparison in the pres-
ence of p55 (84 × 10^{-6} versus 150 × 10^{-6}) suggests that p55 suppresses the efficiency of proofreading, perhaps by promoting extension of a mismatch. For the exonuclease-deficient pol γ reactions, the presence of p55 reduces the rate of deletions in the TTTT run by 4-fold while enhancing the rate of T/G substitutions at position 70 by about 2-fold (Table II). This implies that p55 shifts the balance from extension of the initial misalignment to extension of a terminal mismatch after realignment.

**Fidelity Measurements with Reversion Assays**—To reinforce and extend the results of the forward mutation assay, we measured the rates of base substitution and frameshift errors at specific template positions using more sensitive (i.e., lower background) reversion assays. In this approach, replication errors are scored as blue plaque revertants of preexisting substitutions or frameshift mutations with colorless plaque phenotypes. Substrates previously developed to score base substitutions, additions, and deletions in repetitive sequences were employed to monitor the fidelity of the four different pol γ DNA synthesis reactions studied above. The first substrate monitors eight different single-base mismatches that revert a TTA termination codon at positions 87–89 of the lacZ gene. Wild-type p140 did not generate errors above the background reversion frequency of uncopied DNA (0.5 × 10^{-6}), yielding an average substitution rate at the TGA codon of ≤1.7 × 10^{-6}. The exonuclease-deficient mutant was at least 20-fold less accurate (Table III, top line), implying that >95% of polymerase misinsertions within this opal codon are proofread. As observed in the forward mutation assay, p55 raised substitution error rates for both forms of p140, and proofreading was still apparent. By comparison, heterodimeric D. melanogaster pol γ displayed a reversion frequency of 0.18 × 10^{-5} in the δX174am3-based single nucleotide reversion assay (62).

Three sets of frameshift reversion substrates were used. One set contains runs of either four, six, or seven consecutive template T residues in the 1 reading frame. Theoretically, blue plaque revertants can result from restoration of the correct reading frame by additions (e.g., +1, +4, etc.) or by deletions (e.g., −2, −5). However, the results of the forward mutation assay demonstrate that the rate of single-base additions far exceeds the rate of the other events that might yield blue plaques, implying that results obtained with this set of reversion substrates can be interpreted in terms of single-base addition error rates. Synthesis by wild-type p140 failed to generate revertants above the background reversion frequency of the control DNA with any of the three substrates tested (2nd to 4th lines in Table III). This is consistent with results from the forward mutation assay and extends the observation of high base addition fidelity by wild-type p140 to include a homopolymeric run of seven Ts. The exonuclease-deficient p140 was substantially less accurate in all three assays, suggesting effective proofreading of misaligned addition intermediates by the catalytic subunit in runs of four, six, or seven Ts. In the presence of p55, addition fidelity due to efficient proofreading is again observed for runs of four and six Ts (2nd and 3rd lines in Table III). However, p55 increases the addition error rate of wild-type p140 in the run of seven Ts (compare 6 × 10^{-6} to 55 × 10^{-6} in 4th line of Table III), while having little effect on the fidelity of exonuclease-deficient p140. This suggests that p55 reduces the ability of wild-type p140 to proofread misalignments when the extra primer-strand nucleotide can reside as many as 7 base pairs upstream of the polymerase active site. These results are reminiscent of the previous demonstration that porcine pol γ could not efficiently recognize and excise pre-formed mispairs positioned more than four nucleotides from the 3' terminus (34). Interestingly, Fan and Kaguni (63) have recently described a possible physical interaction between the accessory subunit and the exonuclease domain of pol γ.

The second set of frameshift substrates contains runs of template Ts in the +1 reading frame. Based on the results of the forward assay, revertants primarily result from single nucleotide deletions. Synthesis by wild-type p140 ± p55 was accurate with the T₃ run, and the exonuclease-deficient p140 was substantially less accurate (5th line in Table III). This pattern changes as the run length increases. For example, with the T₄ run (6th line), p140 is at least 20-fold more accurate than the exonuclease-deficient p140, but only 8-fold more accurate when p55 is present. In the T₅ run even wild-type p140 is inaccurate, such that inactivation of the exonuclease and the presence of p55 only enhance the error rate for single-base deletions by a factor of 2 or 3. This trend of decreasing fidelity together with decreasing effects for the exonuclease or p55 continues as the length of the run increases, such that fidelity within the T₈ run is similar for all four polymerization reactions (10th line in Table III). The lacZ reversion frequencies in Table III were corrected for differences in run length to calculate error rates per detectable nucleotide polymerized. The results (Fig. 1) illustrate a quantitative relationship between increasing run length and increasing error rate that is predicted by the strand slippage model for formation of frameshift errors. The results in Table III also reveal that the wild-type p140+p55 complex deletes AT base pairs in the T₈ run at a rate of 3800 × 10^{-6}, a value 380–3200-fold higher than the base substitution error rates of this complex (range of 1.2 to 10 × 10^{-6}).

A similar pattern of error rates as a function of protein composition and run length emerged when we used a third set of frameshift substrates involving runs of template Cs in the +1 reading frame. However, in these runs, frameshift fidelity is substantially higher than with the T run substrates, illustrating the well known but poorly understood effects of run composition and location on frameshift fidelity that have been seen before with other DNA polymerases (reviewed in Ref. 64). Misincorporation by pol γ—Base substitutions generated by pol γ require selection and insertion of an incorrect nucleotide, failure to proofread the misinserted nucleotide, and extension of the resulting mispair. Possession of the exonuclease-proficient and -deficient forms of pol γ allows an examination of these steps with proofreading uncoupled from extension. To evaluate the selectivity of nucleotide insertion by pol γ, we...
Fidelity of Human DNA Polymerase γ

Table III
Reversion frequencies for pol γ at a point mutation and in homopolymeric runs

| Target sequence | p140 | p140 + p55 |
|-----------------|------|-----------|
|                 | Wild-type pol γ | Exo-deficient pol γ | Mutant frequency (× 10⁻⁵) | Wild-type pol γ | Exo-deficient pol γ |
| TGA Codon       | 0.3  | 6         | 1 | 18 |
| +1/-2 frameshift reversion assays |
| TTT             | <1  | 17        | <1 | 44 |
| TTTT            | <1  | 34        | <1 | 22 |
| TTTTTT          | 6   | 120       | 55 | 130 |
| −1 frameshift reversion assays |
| TTT             | 2   | 24        | <1 | 23 |
| TTTT            | <1  | 21        | 12 | 90 |
| TTTTT           | 96  | 220       | 310| 860|
| TTTTTT          | 100 | 290       | 380| 570|
| TTTTTTTT        | 800 | 1700      | 770| 1100|
| CCC             | 2000| 1900      | 1500| 1800|
| CCCCCC          | <1  | 8         | 2 | 15 |
| CCCCC           | 2   | 7         | <1 | 6 |
| CCCCCCC         | 5   | 22        | 7 | 18 |
| CCCCCCCCC       | 16  | 53        | 24 | 33 |

FIG. 1. Deletion error rates of wild-type and exonuclease-deficient p140 within homonucleotide runs of increasing length. A series of gapped M13mp2 DNA substrates containing oligo(dT) tracts three to eight nucleotides in length were utilized in in vitro mutational assays as described under “Experimental Procedures.” Gap-filling reactions utilized exonuclease-proficient (open bars) or exonuclease-deficient (closed bars) forms of human p140. Error rates were calculated as described (51), using the data shown in Table III.

determined steady state kinetic parameters for incorporating a single correct or incorrect nucleotide onto a 3′-terminally matched primer-template. As expected for a polymerase with a proofreading exonuclease, initial experiments with exonuclease-proficient pol γ did not result in the accumulation of improperly extended primers (data not shown). Subsequent experiments utilized the exonuclease-deficient form of the enzyme. The p140 subunit alone efficiently inserted dTMP opposite template A (Table IV). The catalytic efficiencies of inserting the three incorrect nucleotides were 25–60% as efficient as insertion of dTMP, but significantly higher concentrations of the incorrect dNTPs were required to achieve these efficiencies. The ratio of the catalytic constants (kcat/Km) is an estimation of the overall propensity of inserting a given nucleotide, and values for incorrect nucleotides can be compared relative to the correct nucleotide. As typified for a faithful DNA polymerase, p140 discriminated against insertion of the three incorrect nucleotides by factors of 10²–10⁵ (Tables IV), and these data are generally consistent with the error rates determined in the gap-filling assays. Interestingly, inclusion of the p55 accessory subunit had little effect on the kinetics of nucleotide insertion, suggesting the effects of p55 on fidelity that were observed in the gap-filling assays may stem from altered efficiencies of extending mismatched or misaligned primer termini.

**Mispair Extension**—The propensity of pol γ to insert a single dTMP residue onto a variety of 3′-terminally mismatched primer-templates was examined by steady state kinetic analysis. As expected, mispaired 3′ termini were extremely short lived when incubated with wild-type p140 (mean kcat for excision was >100/min, data not shown), so subsequent mispair extension reactions were performed with exonuclease-deficient p140. Whereas only nanomolar concentrations of dTTP were needed to permit efficient extension of the matched G-C terminus by p140, Km(dTTP) values of 5–55 μM were required for extension of A-C, T-C, G-G, and C-C mispairs (Table V). The catalytic efficiencies (kcat) of extending mispairs were consistently less than 20% as efficient as extension of a paired terminus at all dTTP concentrations. With values normalized to those for a matched primer terminus, p55 enhanced the overall efficiency of mismatch extension by factors ranging from about 2-fold for the A-C mismatch to 70-fold for the C-C mismatch. The high concentration of dTTP needed to extend a mismatched (C-C) primer-template and the strong influence of p55 on efficiency of extension are illustrated in Fig. 2. Nanomolar dTTP concentrations were needed for efficient extension of a matched G-C terminus by p140-p55. In contrast, micromolar dTTP concentrations were required for extension of a 3′-terminally C-C mispair. Inclusion of p55 reduced the concentration of dTTP needed for extension of the C-C mismatch while it increased the kcat by 10-fold (Fig. 2 and Table V). Higher concentrations of dTTP also stimulated additional misincorporation events.

**DISCUSSION**

This study has several implications related to the accuracy of human mitochondrial DNA transactions mediated by DNA polymerase γ. Our results indicate that the base substitution fidelity of wild-type human DNA polymerase γ is high, consistent with its central role in mitochondrial DNA replication and repair. This high fidelity results from high nucleotide selectivity, slow mismatch extension, and efficient proofreading by the intrinsic 3′- to 5′-exonuclease. The data with the recombinant proteins studied here are consistent with a number of previous observations (30, 31, 62, 65) on the base substitution fidelity of pol γ purified from natural sources. The data also reveal that
Fidelity of Human DNA Polymerase γ

Table IV
Single nucleotide insertion kinetics

Steady state kinetic values were measured for incorporation of a single correct or incorrect dNMP residue onto a 3’-terminally matched primer-template as described under “Experimental Procedures.” Reactions utilized 10–30 fmol of p140 Exo− or p140 Exo−’–p55. All values are the averages of at least two independent determinations.

| Mismatch   | p55 | $K_M$  | $k_{cat}$ | $k_{cat}/K_M$ | $k_{cat}/K_M$(mispair)/$k_{cat}/K_M$(right) |
|------------|-----|--------|-----------|--------------|---------------------------------------------|
| AdTMP      | –   | 0.011  | 4.3       | 380          | 1                                           |
|            | +   | 0.015  | 5.0       | 250          | 1                                           |
| AdAMP      | –   | 3.8    | 2.8       | 0.73         | 1.9 $\times 10^{-3}$                        |
|            | +   | 5.4    | 2.3       | 0.42         | 2.2 $\times 10^{-3}$                        |
| AdCMP      | –   | 87     | 2.0       | 0.023        | 6.2 $\times 10^{-5}$                        |
|            | +   | 104    | 1.0       | 0.010        | 5.2 $\times 10^{-5}$                        |
| AdGMP      | –   | 69     | 1.1       | 0.015        | 4.0 $\times 10^{-5}$                        |
|            | +   | 58     | 0.76      | 0.013        | 6.9 $\times 10^{-5}$                        |

Table V
Kinetics of mismatch extension

Steady state kinetic values were measured for incorporation of a single dTMP residue onto the indicated 3’-terminally matched or mismatched primer-template, as described under “Experimental Procedures.” Reactions utilized 10–30 fmol of p140 Exo− or p140 Exo−’–p55. All values are the averages of at least two independent determinations.

| Mismatch   | p55 | $K_M$  | $k_{cat}$ | $k_{cat}/K_M$ | $k_{cat}/K_M$(wrong)/$k_{cat}/K_M$(right) |
|------------|-----|--------|-----------|--------------|---------------------------------------------|
| G-C        | –   | 0.011  | 4.3       | 380          | 1                                           |
|            | +   | 0.015  | 3.7       | 250          | 1                                           |
| A-C        | –   | 5.5    | 0.82      | 0.15         | 3.9 $\times 10^{-4}$                        |
|            | +   | 7.6    | 1.2       | 0.16         | 6.5 $\times 10^{-4}$                        |
| T-C        | –   | 10     | 0.56      | 0.055        | 1.4 $\times 10^{-4}$                        |
|            | +   | 5.5    | 1.0       | 0.19         | 7.5 $\times 10^{-4}$                        |
| G-G        | –   | 13     | 0.19      | 0.015        | 3.9 $\times 10^{-5}$                        |
|            | +   | 11     | 0.44      | 0.041        | 17 $\times 10^{-5}$                         |
| C-C        | –   | 55     | 0.013     | 0.00023      | 6.1 $\times 10^{-7}$                        |
|            | +   | 11     | 0.11      | 0.011        | 440 $\times 10^{-7}$                        |

*Reactions with C-C DNA required 80–500 fmol of enzyme to detect primer extension.

Fig. 2. Addition of p55 enhances extension of C-C mismatches by exonuclease-deficient p140. Mispair extension reactions (“Experimental Procedures”) utilized oligonucleotide substrates with a G-C pair or a C-C mispair at the 3’ terminus of the primer, as indicated. Reactions with G-C DNA included 10 fmol of p140–p55 and dTTP at the indicated concentrations. Reactions with C-C DNA included 0.5 pmol of p140 or p140–p55 and the indicated concentrations of dTTP. The leftmost lane contained no enzyme. Products were resolved by denaturing PAGE and visualized with a PhosphorImager as described under “Experimental Procedures.” The position of the unextended oligonucleotide primer is indicated by the arrow. The nucleotide sequence of the template strand is indicated on the right side of the figure.

pol γ has high single-base addition and deletion fidelity for errors at non-iterated and short repetitive sequences (Tables I and III). Such events likely involve misalignments where the unpaired base is at or near the primer terminus, at situations relevant to most of the sequences in the 16,569-base pair human mitochondrial genome. The data in Tables I and III and Fig. 1 further suggest that pol γ frameshift fidelity in most sequence contexts is enhanced by proofreading, as also indicated by previous studies of other DNA polymerases (66).

As predicted by the classical strand slippage hypothesis for synthesis of repetitive sequences (57, 58), pol γ error rates increase with increasing homopolymeric run length (Table III and Fig. 1). The wild-type catalytic subunit and the p140–p55 complex both have low frameshift fidelity in long runs despite the presence of an intrinsic proofreading exonuclease that effectively proofreads most base substitution intermediates. This suggests that proofreading efficiency diminishes with increasing distance between the terminus and the location of the extra nucleotide in the misaligned intermediate. This effect of location can actually negate the contribution of proofreading to the frameshift fidelity of pol γ in tracts of 6–8 nucleotides (Table III). When combined with the increased error rate of pol γ as a function of increasing run length (Fig. 1, results with exonuclease-deficient p140), the minimal contribution of proofread-
ing implies that repetitive sequences in mtDNA are at relatively high risk for insertion and deletion mutagenesis due to spontaneous strand slippage during mitochondrial DNA replication. Consistent with this, frameshift mutations within homopolymeric runs have been documented to occur in vivo (67, 68). Interestingly, no homopolymeric runs longer than eight nucleotides exist in human mtDNA, and only a single homopolymeric run of eight nucleotides exists (1).

Strand slippage may also produce base substitution errors via a transient misalignment (Table II). Such a dislocation mechanism has been invoked previously to explain synthesis errors by two naturally exonuclease-deficient DNA polymerases, pol β and human immunodeficiency virus, type I reverse transcriptase. This study of pol γ provides the first evidence for substitutions by dislocation for any family A DNA polymerase, and it also provides the first evidence that dislocation errors can be suppressed by proofreading. Theoretically this could involve editing either the misaligned primer-template or the terminally mismatched intermediate. In the absence of editing, the data in Table II indicate that p55 diminishes the probability of extending mismatched termini (compare $64 \times 10^{-6}$ to $18 \times 10^{-6}$) while increasing the probability of extending terminally mismatched primer termini ($96 \times 10^{-6}$ to $150 \times 10^{-6}$). This shift in mutational specificity is the first indication that a replication accessory protein can modulate the rate of base substitutions arising by dislocation. Although it suggests a model in which p55 promotes realignment prior to continued DNA synthesis, addition of the accessory subunit also seems to interfere with the ability of p140 to proofread realigned but mispaired primer termini at dislocation loci. The two competing effects, promoting realignment of primer-templates and enhancing extension of mismatches, result in a clear base substitution hot spot even with the wild-type p140:p55 enzyme complex (error rate of $84 \times 10^{-6}$).

Indeed, the results in Tables I, III, and V and Fig. 2 indicate that p55 enhances the ability of the p140 pol γ catalytic subunit to extend a variety of mismatched primer-templates but had no detectable influence on the misinsertion rates of p140. This enhancement is generally strongest for primer-templates wherein the mismatched or unpaired bases can reside at or within a few base pairs of the primer terminus, such as for base substitutions and frameshifts at non-iterated or short repetitive sequences. In contrast, p55 has little or no effect on frameshifts involving extra bases in runs of 5–8 base pairs (Table III). Although the mechanistic explanation for this p55 enhancement must await further kinetic and structural analysis, the error rate data suggest that p55 is somehow modulating the interactions between p140 and the duplex primer-template that occur at and within about 4–5 base pairs of the active site. The negligible p55 effect on frameshift error rates in long homopolymeric tracts may be also eventually be instructive in high of its ability to enhance the processivity of the catalytic subunit (47). Processivity factors, such as PCNA, thioredoxin, and the 55-kDa accessory subunit of pol γ increase the number of nucleotides incorporated each time a polymerase binds to DNA by decreasing dissociation of the enzyme from DNA (47, 69, 70). Thus, the enhanced binding afforded by these processivity factors reduces the options available to the polymerase following a replication error. Fidelity studies in vitro have shown that thioredoxin increases base substitution errors for T7 polymerase copying non-iterated DNA sequences (71), and gel-based oligonucleotide extension assays have shown that PCNA enhances misincorporation by calf thymus DNA polymerase δ by a factor of 27 (72). The reduction in base substitution fidelity caused by the accessory subunit of pol γ is consistent with this model; however, the analysis is somewhat more complex for frameshift mutations. Thioredoxin increases the frequency of −1 and −2 frameshift errors made by exonuclease-deficient T7 DNA polymerase when copying both non-iterated sequences and short homopolymeric runs (71). Similarly, p55 enhances −1 frameshifts for random DNA sequences and shorter homonucleotide runs (Tables I and III). Since frameshift mutations are caused by misalignment of the primer and template strands during DNA synthesis, the fidelity effects observed for these accessory factors could be explained by a reduced opportunity for template realignment during disassociation/reassociation events. In general homopolymeric runs are particularly prone to frameshift mutations due to an increased probability of slippage between the primer and template strands during DNA synthesis. Misalignments in longer homonucleotide runs can be stabilized by correctly paired nucleotides proximal to the primer terminus, and such intermediates may not provoke transfer to the exonuclease site. The three-dimensional structure of the T7 polymerase-thioredoxin complex shows that thioredoxin acts as a flap or lid to the DNA-binding cleft of T7 DNA polymerase (73). Restricting the geometry of the DNA-binding cleft may serve to suppress stabilization of misaligned intermediates, resulting in an antimutator effect. Indeed, +1 frameshift errors catalyzed by exonuclease-deficient T7 DNA polymerase at a Tα run are reduced 46-fold by thioredoxin (71).

We have shown that proofreading-deficient pol γ synthesizes DNA with substantially reduced fidelity, thus predicting a mutator phenotype in vivo. Consistent with this expectation, Zassenhaus and co-workers(44) have clearly demonstrated that heart-specific overexpression of a transgene encoding exonuclease-deficient pol γ generated mutations in mtDNA and induced cardiomyopathy in their mice. Interestingly, among 11 mtDNA mutations specifically attributed to the transgene (as opposed to random errors or naturally occurring polymorphisms in the heart mtDNAs), 6-base substitution mutations occurred within homopoly nucleotide runs (44). Similarly, stable episomal overexpression of exonuclease-deficient p140 in cultured human cells resulted in a variety of point mutations, with C to T transitions and G to A transitions dominating the mutant collection (43). The subunit composition of pol γ in these two studies is uncertain. Overexpression of the mutant catalytic subunit relative to natural expression of the accessory subunit suggests pol γ may be functioning as a monomer; however, the more processive heterodimer would likely dominate synthesis of mtDNA in vivo. We and others (47, 49, 50, 63, 74, 75) have suggested the accessory subunit may help initiate mtDNA synthesis. We have further suggested that efficient replication of the mitochondrial chromosome requires the high processivity afforded by the accessory subunit but that filling of short gaps during mitochondrial base excision repair need not be processive and need not require the accessory subunit (27, 45, 47). In Drosophila, expression of the two pol γ genes is regulated by distinct mechanisms (75), leaving open the possibility of unequal gene expression in human cells. Our results predict a scenario in which differential expression of the two subunits could modulate the fidelity of replication. Thus age-related or tissue-specific changes in expression of the two subunits might contribute to the accumulation of mutations associated with mitochondrial disease and dysfunction. Additional articles addressing mitochondrial DNA polymerase fidelity have been published recently, indicating a calculated increase in fidelity upon the addition of the accessory subunit for their in vitro reaction conditions and specific sequence context (76, 77). We also observed enhanced fidelity at certain sequence contexts, but the overall effect of the accessory subunit was to lower the fidelity of DNA replication.
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REFERENCES

1. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and Young, I. G. (1981) Nature 290, 457–465
2. Wallace, D. C. (1992) EMBO J. 11, 2717–2726
3. Spellicy, J. N., Toivenen, J. M., Hakkarila, G. A., Kurkela, J. M., Cooper, H. M., Lehtinen, S. K., Lercren, N., Back, J. W., Speijer, D., Fory, F., and Jacobs, H. T. (2000) J. Biol. Chem. 275, 24818–24828
4. Yang, C., Mott, J. L., Chang, S. W., Denninger, G., Peng, Z., and Zassenhaus, H. P. (2000) Genomics 69, 151–161
5. Longley, M. J., Ropp, P. A., Lim, S. E., and Copeland, W. C. (1998) Biochemistry 37, 10529–10539
6. Graves, S. W., Johnson, A. A., and Johnson, K. A. (1998) Biochemistry 37, 6050–6058
7. Austin, E., Longley, M. J., and Copeland, W. C. (1999) J. Biol. Chem. 274, 31997–32003
8. Johnson, A. A., Tsai, Y., Graves, S. W., and Johnson, K. A. (2000) Biochemistry 39, 1702–1708
9. Carrodeguas, J. A., and Bogenhagen, D. F. (2000) Nucleic Acids Res. 28, 1237–1244
10. Carrodeguas, J. A., Theis, K., Bogenhagen, D. F., and Kisker, C. (2001) Mol. Cell 7, 43–54
11. Bebenek, K., and Kunkel, T. A. (1995) Methods Enzymol. 262, 217–232
12. Krituli, L. C. Register, K., Bebenek, K., and Kunkel, T. A. (1996) Biochemistry 35, 1046–1053
13. Krituli, L. C., and Kunkel, T. A. (1999) Nucleic Acids Res. 24, 2922–2929
14. Bogenhagen, D. F., and Bjerke, S. B. (1988–1989) Nucleic Acids Res. 16, 2369–2374
15. Minkernig, H., Okada, K., and Kuzin, A. (1990) Adv. Cancer Res. 53, 153–220
16. Krituli, L. C., and Kunkel, T. A. (1999) J. Biol. Chem. 274, 3481–3486
17. Bebenek, L. S., Petruska, J., and Goodman, M. F. (1997) J. Biol. Chem. 272, 14784–14789
18. Osherf, W. P., Jung, H. K., Beard, W. A., Wilson, S. H., and Kunkel, T. A. (1999) J. Biol. Chem. 274, 3642–3650
19. Kaguni, L. S., Wernette, C. M., Conway, M. C., and Yang-Cashman, P. (1988) Eukaryotic DNA Replication, Vol. 6, Ed. 425–432, Cold Spring Harbor Press, NY
20. Fan, L., and Kaguni, L. S. (2001) Biochemistry 40, 4780–4791
21. Krituli, T. A. (1990) Biochemistry 29, 8003–8011
22. Vanderstraeten, S., Van den Brule, S., Hu, J., and Fory, F. (1998) J. Biol. Chem. 273, 23690–23697
23. Krituli, T. A., and Bebenek, K. (2000) Annu. Rev. Biochem. 69, 497–529
24. Habano, W., Nakamura, S., and Sugai, T. (1998) Oncogene 17, 1831–1837
25. Kogelnik, A. M., Lott, M. T., Brown, M. D., Navathe, S. B., and Wallace, D. C. (1996) Nucleic Acids Res. 24, 177–179
26. Karyyan, J., and O'Donnell, M. (1995) J. Mol. Biol. 243, 915–925
27. Huber, H. P., Tabor, S., and Richardson, C. C. (1987) J. Biol. Chem. 262, 16224–16232
28. Krituli, T. A., Patel, S. S., and Johnson, K. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6830–6834
29. Mozherin, D. J., McConnell, M., Jasko, M. V., Krayevsky, A. A., and Kline, K., Downey, K. M., and Fisher, P. A. (1996) J. Biol. Chem. 271, 31711–31717
30. Doubley, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. H. (1998) Nature 391, 521–528
31. Fan, L., Sanschagrin, P. C., Kaguni, L. S., and Kuhn, L. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9527–9532
32. Lefay, E., Fernandez-Moreno, M. A., Alahari, A., Kaguni, L. S., and Garresse, R. (2000) J. Biol. Chem. 275, 33125–33133
33. Johnson, A. A., and Johnson, K. A. (2001) J. Biol. Chem. 276, 38990–38996
34. Johnson, A. A., and Johnson, K. A. (2001) J. Biol. Chem. 276, 38997–38107