Active Site Mutation in DNA Polymerase γ Associated with Progressive External Ophthalmoplegia Causes Error-prone DNA Synthesis*

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Progressive external ophthalmoplegia (PEO) is a heritable mitochondrial disorder characterized by the accumulation of multiple point mutations and large deletions in mtDNA. Autosomal dominant PEO was recently shown to co-segregate with a heterozygous Y955C mutation in the human gene encoding the sole mitochondrial DNA polymerase, DNA polymerase γ (pol γ). Since Tyr-955 is a highly conserved residue critical for nucleotide recognition among family A DNA polymerases, we analyzed the effects of the Y955C mutation on the kinetics and fidelity of DNA synthesis by the purified human mutant polymerase in complex with its accessory subunit. The Y955C enzyme retains a wild-type catalytic rate (kcat) but suffers a 45-fold decrease in apparent binding affinity for the incoming nucleoside triphosphate (Km). The Y955C derivative is 2-fold less accurate for base pair substitutions than wild-type pol γ despite the action of intrinsic exonuclease proofreading. The full mutator effect of the Y955C substitution was revealed by genetic inactivation of the exonuclease, and error rates for certain mismatches were elevated by 10–100-fold. The error-prone DNA synthesis observed for the Y955C pol γ is consistent with the accumulation of mtDNA mutations in patients with PEO.

Disruption of mitochondrial energy metabolism causes mitochondrial disorders that play a central role in many degenerative diseases, aging, and cancer. Hundreds of mitochondrial and nuclear gene products are required for the proper functioning of the mitochondria. Accordingly heritable mitochondrial diseases exhibit both maternal and Mendelian modes of inheritance with considerable genetic heterogeneity (1–3).

Progressive external ophthalmoplegia (PEO)1 and mitochondrial neurogastrointestinal encephalomyopathy belong to a subclass of autosomal mitochondrial disorders associated with depletion of the mitochondrial genome and/or the accumulation of mutations and deletions within mtDNA (1, 4–6). Within the last two years, several nuclear genes controlling maintenance of mtDNA have been identified at disease loci, including the genes for adenine nucleotide translocator 1 (ANT1) at locus 4q34–35 (7), thymidine phosphorylase at locus 22q13.3-pter (8), a putative mitochondrial helicase (Twinkle) at locus 10q24 (9), an unidentified gene at locus 3p14–21 (10), and the sole mitochondrial DNA polymerase (pol γ) at locus 15q22–26 (11). Sequence analysis through the pol γ gene (12) in a Belgian pedigree with dominant PEO identified a heterozygous A to G mutation at codon 955 (Y955C) (11).

Located in the active site of pol γ, Tyr-955 is a highly conserved residue among a wide variety of DNA polymerases. As a family A DNA polymerase, pol γ is related to Escherichia coli DNA polymerase I and bacteriophage T7 DNA polymerase, and amino acid sequence alignments reveal that Tyr-955 is equivalent to Tyr-766 in E. coli pol I and Tyr-530 in T7 DNA polymerase (see Fig. 1A). The three-dimensional structure of T7 DNA polymerase (13) in a ternary complex with DNA and a nucleoside triphosphate places this conserved tyrosine residue in close proximity to the incoming dNTP (see Fig. 1B). Functionally Tyr-530 in T7 DNA polymerase hydrogen bonds with Glu-480 to form part of the binding pocket for the incoming dNTP and to help discriminate against ribonucleotides (13). Substitution of Tyr-766 in E. coli pol I with serine has only a minor effect on Kα(dNTP), and a slight decrease in kcat is attributed to a 2.5-fold increase in Kmic(dNTP) (14). The fidelity of a Y766F substitution in the Klenow fragment does not show an appreciable increase in nucleotide misinsertion; however, substitution with alanine or serine generates an error-prone DNA polymerase attributable to decreased stringency for selection of dNTPs (15, 16). Interestingly the Y766A- and Y766S-substituted enzymes exhibited a 17-fold increase in deletions between direct repeat sequences (16). These results predict that the Y955C substitution may lower the catalytic efficiency and/or the fidelity of human pol γ.

We have previously cloned and expressed the cDNAs for the catalytic subunit and the accessory subunit of human pol γ (12, 17, 18). We have also produced a 3’ to 5’ exonuclease-deficient derivative of pol γ to study the fidelity of DNA replication and selection of antiviral nucleotide analogs by pol γ (17, 19, 20). In this report we investigate the effects of the Y955C mutation in human pol γ on the kinetics and fidelity of DNA replication.

EXPERIMENTAL PROCEDURES

Enzyme Production—The His6 affinity-tagged recombinant catalytic subunit of human DNA polymerase γ (p140 Exo−) were purified to homogeneity as described previously (17). The His6-tagged accessory subunit (p55) was purified to homogeneity, and the two subunit forms of the polymerase (p140p55 and p140 Exo−p55) were reconstituted as described previously (18).

The Y955C p140 derivative was made with the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenic primers 5-CCTG CCC AGC ACC ACA GAT GCG GCC GTA and 5-TAC CGC GCG ATC TGT GGT CCT GGC GAG were used to generate Y955C in wild-type and exonuclease-deficient backgrounds in the baculovirus transfer vectors pHyrpVL (17) and Exo−pQVSL1.4 (20), respectively. The Y955C polymerase; pol DNA polymerase; ANT1, adenine nucleotide translocator 1; WT, wild type.
Error-prone Pol γ in Mitochondrial Disorders

RESULTS AND DISCUSSION

Amino acid sequence alignments (Fig. 1A) and the active site structure of T7 DNA polymerase (Fig. 1B) suggest that Tyr-955 in human pol γ is crucial for dNTP binding and fidelity of nucleotide selection. To study the biochemical properties of the Y955C variant responsible for PEO, we made the Y955C substitution by site-directed mutagenesis and overproduced the mutant protein in baculovirus-infected Sf9 cells. As compared with the wild-type enzyme in the presence of the accessory subunit, the Y955C pol γ exhibited a 4-fold reduction in overall DNA polymerase activity in our standard DNA polymerase assay (data not shown). Steady state kinetic analyses (Table I) indicated that the basis for the reduced activity was a 45-fold increase in the apparent $K_m$ for incoming dNTP from 2.1 μM (WT Exo - ) to 92 μM (Y955C Exo - ) with no reduction in $k_{cat}$ and only a slight alteration in $K_m$.

<table>
<thead>
<tr>
<th>Pol γ</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Exo -</td>
<td>2.1</td>
<td>4.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Y955C Exo</td>
<td>92</td>
<td>5.9</td>
<td>0.064</td>
</tr>
</tbody>
</table>

Table I: Effects of Y955C mutation on kinetics of DNA synthesis by pol γ

All values are the averages of at least two independent determinations.

We previously determined the fidelity of wild-type and exonuclease-deficient pol γ with and without the p55 accessory subunit (19). Pol γ is highly accurate due to high nucleotide selectivity and exonucleolytic proofreading. Indeed genetic inactivation of the exonuclease activity was needed to reveal the complete error spectrum of the polymerase function. Also the p55 accessory subunit mildly decreased fidelity by promoting extension of misinserted nucleotides, and this negative effect may be balanced by the functional benefit of enhanced processivity conferred by the p55 subunit (18, 19). The accuracy of DNA synthesis by the human Y955C pol γ was measured in vitro by copying a single-stranded region of bacteriophage M13mp2 DNA encoding the α-peptide of the β-galactosidase gene as described previously (21). The first experiment utilized a sensitive reversion assay that measures eight different single base substitutions at a TGA codon within lacZα. In the absence of proofreading, the Y955C mutation raised the mutation frequency 42-fold from $13 \times 10^{-3}$ (WT pol γ) to $550 \times 10^{-3}$ (Y955C pol γ). In the exonuclease-proficient background the mutator effect of the Y955C mutation was 2-fold ($0.33 \times 10^{-3}$ for WT pol γ and $0.72 \times 10^{-3}$ for Y955C pol γ), implying that the majority of misinsertions produced by the Y955C polymase are proofread (Table II). Nevertheless this 2-fold increase in base substitution errors should significantly increase the mutant fraction of the heteroplasmic mtDNA pool during the preclinical onset period leading to PEO.

To gauge the broader effects of the Y955C mutation on fidelity of replication, a substrate bearing a 407-nucleotide mutator insert in each polymerase (Fig. 1A) contained 25 mM HEPES-KOH (pH 7.6), 2 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 4 mM MgCl₂, 50 μg/ml acetylated bovine serum albumin, ~150 ng of flapped M13mp2 DNA, 0.1 mM NaCl, and either 20 ng of p140, 20 ng of p140 Eco RI, 200 ng of p140/Y955C, or 830 ng of p140/Y955C/Exo , each reconstituted with 1.2-fold molar excess of p55 accessory subunit. 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 (19).
pairs, the average base substitution error rate of the Y955C polymerase was elevated 10-fold in the exonuclease-deficient background (Fig. 2). Perhaps the most striking effect of introducing the Y955C mutation was that the rates of forming point mutations due to TdTMP and TdGMP mispairs increased by factors of 110 and 81, respectively (Fig. 2). In fact, transition mutations caused by misinsertion of G opposite a template T represent 60% of all of the base substitution errors observed for Y955C pol γ (142 of 235 mutants). Interestingly analysis of point mutations in mtDNA from PEO patients revealed A:T → G:C transitions as the most common mutation (8 of 14 cases listed in the MitoMap data base), although the autosomal defects responsible for these cases of PEO are not known.

Pathogenic base substitutions in mtDNA are well documented for PEO. Our in vitro data indicate that pol γ makes both +1 and −1 frameshift errors at almost 20% of the base substitution error rate, although proofreading substantially reduces the frameshift error rate. The Y955C mutation increases the frameshift error rate by 4-fold, but base substitution errors remain the primary replication error catalyzed by Y955C pol γ. Clinical tests based on Southern blots or PCR are designed to detect deletions in mtDNA, and multiple deletions in mtDNA have become a clinical hallmark of PEO. Large deletions in mtDNA between direct repeats, such as the 4977-bp deletion between nucleotides 8470 and 13447, are found in both dominant and recessive forms of PEO (24, 25). We present a model in which the enhanced base substitution error rate of Y955C pol γ promotes deletions between direct repeat sequences in mtDNA (Fig. 3). This mechanism invokes a misinsertion event following correct synthesis through a direct repeat sequence. Failure of the polymerase to proofread the error or to extend the mismatch favors a slippage event between the direct repeats that creates a matched DNA terminus at a downstream template sequence (Fig. 3). Large deletions between direct repeats were observed for the analogous Y766S mutation in the Klenow fragment (16), and we propose that a common TdTMP misinsertion by Y955C pol γ (Fig. 2) initiates the 4977-bp deletion observed in some PEO patients.

Mitochondrial disorders marked by deletion, deletion, or base substitution of mtDNA are most likely caused by defects in nuclear genes that function to maintain mtDNA. Genetic predisposition to the accumulation of mtDNA mutations, as in PEO patients with the Y955C allele, is consistent with the delayed-onset and progressive course of such degenerative mitochondrial diseases. In addition to our identification of exonuclease-deficient pol γ (19) and Y955C pol γ as mutator DNA polymerases, Zassenhaus (26) has demonstrated that transcriptional overexpression of exonuclease-deficient pol γ in mice is associated with cardiomyopathy and results in accumulation of point mutations, deletions, and large deletions flanked by direct repeats. Thus, mutations that reduce the fidelity of pol γ cause mitochondrial disease through mutagenic replication of mtDNA. The ability of other pol γ mutations linked with disease (11) to affect the fidelity of the polymerase has not been tested. Multiple mutations within the ‘Twinkle’ gene encoding a putative mitochondrial helicase are also causally linked to dominant PEO with mtDNA deletions (9), and a dysfunctional replicative helicase (dnaB) has been shown to enhance deletions in E. coli, possibly by stalling the replication fork (27).

Mutations in the nuclear genes for ANT1 or thymidine phosphorylase also induce pathogenic mutation of mtDNA (7, 8) perhaps by unbalancing or reducing the available intramitochondrial pool of deoxynucleoside triphosphates. Nucleotide pool imbalance is known to enhance base substitution errors by pol γ (28, 29). Additionally Wallace (23) observed mtDNA rearrangement and increased production of reactive oxygen species in the mitochondria of ANT1−/− knockout mice, suggesting pathogenesis results from enhanced oxidative damage to mtDNA. In summary, we believe that autosomal mitochondrial disorders exhibiting point mutations, deletions, and rearrangements have a higher mtDNA mutation rate due to enhanced damage or compromised mechanisms of mtDNA maintenance, and we predict a general mechanism in which point mutations

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**TABLE II**

<table>
<thead>
<tr>
<th>Mutation assay</th>
<th>Wild type</th>
<th>Y955C mutant</th>
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<tbody>
<tr>
<td></td>
<td>Exo-proficient</td>
<td>Exo-deficient</td>
</tr>
<tr>
<td>TGA reversion</td>
<td>0.33 (1)</td>
<td>0.72 (2)</td>
</tr>
<tr>
<td>Forward mutation</td>
<td>130 (500)</td>
<td>550 (42)</td>
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</tbody>
</table>

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2 MitoMap data base at www.gen.emory.edu/mitomap.html.
are an early event in PEO and other mitochondrial diseases affecting the integrity of mtDNA.

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REFERENCES
