Physiological and Biochemical Defects in Carboxyl-terminal Mutants of Mitochondrial DNA Helicase*

Received for publication, May 13, 2008, and in revised form, June 11, 2008. Published, JBC Papers in Press, June 30, 2008, DOI 10.1074/jbc.M803674200

Yuichi Matsushima‡, Carol L. Farr†, Li Fan§, and Laurie S. Kaguni∥

From the ‡Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824-1319 and the †Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Mitochondrial DNA helicase, also called Twinkle, is essential for mtDNA maintenance. Its helicase domain shares high homology with helicases from superfamily 4. Structural analyses of helicases from this family indicate that carboxyl-terminal residues contribute to NTP hydrolysis required for translocation and DNA unwinding, yet genetic and biochemical information is very limited. Here, we evaluate the effects of overexpression in Drosophila cell culture of variants carrying a series of deletion and alanine substitution mutations in the carboxyl terminal and identify critical residues between amino acids 572 and 596 of the 613 amino acid polypeptide that are essential for mitochondrial DNA helicase function in vivo. Likewise, amino acid substitution mutants K574A, R576A, Y577A, F588A, and F595A show dose-dependent dominant-negative phenotypes. Arg-576 and Phe-588 are analogous to the arginine finger and base stack of other helicases, including the bacteriophage T7 gene 4 protein and bacterial DnaB helicase, respectively. We show here that representative human recombinant proteins that are analogous to the alanine substitution mutants exhibit defects in nucleotide hydrolysis. Our findings may be applicable to understand the role of the carboxyl-terminal region in superfamily 4 DNA helicases in general.

The mitochondrial DNA (mtDNA) helicase, also known as Twinkle, was identified as one of the causative genes for autosomal dominant progressive external ophthalmoplegia (1). Mutations of the mtDNA helicase have been reported in patients with multiple mtDNA deletions or depletions (1–9). Patients with mutations of the mtDNA helicase have been reported in mitochondrial DNA helicase function in vivo. Likewise, amino acid substitution mutants K574A, R576A, Y577A, F588A, and F595A show dose-dependent dominant-negative phenotypes. Arg-576 and Phe-588 are analogous to the arginine finger and base stack of other helicases, including the bacteriophage T7 gene 4 protein and bacterial DnaB helicase, respectively. We show here that representative human recombinant proteins that are analogous to the alanine substitution mutants exhibit defects in nucleotide hydrolysis. Our findings may be applicable to understand the role of the carboxyl-terminal region in superfamily 4 DNA helicases in general.

The mitochondrial DNA (mtDNA) helicase, also known as Twinkle, was identified as one of the causative genes for autosomal dominant progressive external ophthalmoplegia (1). Mutations of the mtDNA helicase have been reported in patients with multiple mtDNA deletions or depletions (1–9). The helicase domain of the enzyme, located in the carboxyl-terminal region, shares high homology with helicases from superfamily 4 (SF4), which includes bacteriophage T7 gene 4 protein (T7 gp4) and Escherichia coli DnaB protein. These enzymes catalyze DNA helix unwinding, translocating 5’-3’ on single-stranded DNA by utilizing the energy of nucleotide hydrolysis (10, 11). Consistent with other ring-shaped SF4 enzymes, the mtDNA helicase forms a hexamer (1, 12–14).

The SF4 helicases share five conserved sequence motifs, H1, H1A, H2, H3, and H4. H1 and H2 are equivalent to the Walker A and Walker B motifs found in all AAA+ ATPases (10, 11). The H4 motif contributes to DNA binding, whereas the remaining four play a role in NTP binding and hydrolysis. Additionally, individual amino acids, termed the arginine finger and base stack, have been shown to serve specific roles (15–22). The arginine finger of one subunit interacts with the phosphate of the nucleotide bound to a neighboring subunit, stabilizing the transition state of the reaction (10, 11), whereas the amino acid functioning as the base stack contacts the base of the nucleotide bound on the same subunit. Furthermore, the extreme carboxyl-terminal region of many SF4 helicases has been shown to be required for interaction with other replication factors. Carboxyl-terminal residues of T7 gp4 interact directly with T7 DNA polymerase and thioredoxin, and the last three carboxyl-terminal residues in the RSF1010 plasmid-encoded RepA helicase are required for replication in vivo (23–28).

We have shown that the Drosophila (d-) mtDNA helicase is essential for mtDNA maintenance in vivo, and overexpression of protein variants carrying mutations in the H1 or H2 motifs or those with amino acid substitutions equivalent to human autosomal dominant progressive external ophthalmoplegia mutations results in the depletion of mtDNA in cultured cells (13). Recently, similar results were obtained with human cultured cells (29). Here, we extend our study by investigating the importance of the carboxyl-terminal region. We evaluate the effects of overexpression in Drosophila cell culture of variants carrying mutations in the carboxyl-terminal region and characterize biochemically several recombinant human helicases carrying amino acid substitutions that are shown to cause dominant negative phenotypes in vivo.

EXPERIMENTAL PROCEDURES

Generation and Induction of Stable Cell Lines—Drosophila Schneider S2 cells were cultured at 25 °C in Drosophila Schneider Medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were subcultured to 5 × 10⁶ cells/ml every third day. Cells were transfected using Effectene (Qiagen). Hygromycin-resistant cells were selected with 200 μg/ml hygromycin. Cells were passed at least five times in hygromycin-containing medium and then cultured in standard medium. The cell lines were grown to a density of 3 × 10⁶/ml and then treated with 0.2 mM CuSO₄ to induce high level expression from the metallothionein promoter.
Immunoblotting—Total cellular protein (20 μg/lane) was fractionated by SDS-PAGE in 9% gels and transferred to nitrocellulose filters. Filters were preincubated for 1 h with 5% skim milk in phosphate-buffered saline (PBS) followed by incubation for 1 h with d-mtDNA helicase antibody (1:20 ml in PBS containing 0.1% Tween 20). Filters were washed 4 times with PBS containing 0.1% Tween 20, incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad), and washed with PBS containing 0.1% Tween 20. Protein bands were visualized using ECL Western blotting reagents (Amersham Biosciences). Rabbit polyclonal antibody against the human mtDNA helicase domain was provided by Rafael Garesse (Universidad Autonoma de Madrid-Consejo Superior de Investigaciones Cientificas, Madrid).

Protein Cross-linking Analysis—1 × 10^8 cells that were induced with 0.2 mM CuSO4 for 7 days were washed twice with PBS and resuspended in 200 μl cross-linking buffer (PBS containing 1% formaldehyde). After incubation for 5 min at room temperature, 200 μl of quenching solution (PBS containing 250 mM glycine, 10 mM EDTA, and 4% SDS) was added, and the samples were mixed thoroughly. 20 μl of the protein solution was fractionated by 6% SDS-PAGE and transferred to nitrocellulose filters. Immunoblot analysis was performed as described above.

Southern Blotting—Genomic DNA was purified from Drosophila Schneider S2 cells by standard methods. DNA (10 μg/lane) was cleaved with XhoI, fractionated on 0.7% agarose/Tris-buffered EDTA gels, and transferred to Hybond-N+ nylon membrane (Amersham Biosciences). Hybridization was performed as described previously (31, 32). Filters were washed 3 times for 10 min at room temperature with 2× SSC containing 0.1% SDS, once for 30 min at 65 °C with 0.2× SSC containing 0.1% SDS, and then analyzed with a PhosphorImager. Blots were probed with radiolabeled DNAs for the mitochondrial gene Cytb and the nuclear histone gene cluster. The ratio of the signals for these two genes was used to determine the relative copy number of mtDNA. The Southern blot experiments shown in Figs. 2 and 4 were performed twice with each of the two independent cell lines carrying each plasmid construct, including the control (no plasmid), vector only, wild type, and each of the mutant d-mtDNA helicas. The data presented represent one such experiment, and quantitation is provided for the duplicate experiments from one of the two cell lines. All of the comparable data for each construct vary by less than 15%.

Preparation of an Inducible Plasmid Expressing d-mtDNA Helicase Variants—The construction of the plasmid pMt/WT/Hy was performed as described previously (13). The expression vectors carrying mutant d-mtDNA helicases were prepared by QuikChange mutagenesis or PCR with Pfu DNA polymerase. A typical PCR was carried out in a 50-μl reaction mixture with 50 ng of pMt/WT/Hy and 2 units of Pfu DNA polymerase. A typical PCR was carried out in a 50-μl reaction mixture with 50 ng of pMt/WT/Hy and 2 units of Pfu DNA polymerase. Aspecific primer pair was used for each mutant—1 of the protein solution was fractionated by 6% SDS-PAGE and transferred to nitrocellulose filters. Immunoblot analysis was performed as described above.

RESULTS
Overexpression of Carboxyl-terminal Deletion Mutants of Drosophila mtDNA Helicase in Schneider Cells—To examine the role of the carboxyl-terminal region of the d-mtDNA helicase, we created four variants of the enzyme lacking 7, 17, 28, or 42 amino acids at the carboxyl terminus (Fig. 1). The constructs were transfected into Schneider cells, and after a 14-day incubation in the presence of 0.2 mM CuSO4, an immunoblot anal-
pressed proteins. To do so, we induced stable cell lines carrying
Schneider cells overexpressing the helicase function.

indicate that the region between 572 and 596 is essential for
pressing the control cells, values that are comparable with the
mtDNA copy number was determined from the ratio of
hybridization to histone gene cluster hybridization. After 14
days of incubation in the absence or presence of 0.2 mM CuSO4, immunoblot analysis indicated increases in the
levels of all of the variants in the range of 2.5–3.5- and
dult showed no significant change in expression level. Cell lines expressing the Δ(586–613) and Δ(572–613) mutants showed increases of only 1.5-
and 1.3-fold, respectively, relative to the endogenous d-mtDNA helicase level. As a result, we could not isolate high level expres-
cell lines for these two variants.

The effects of overexpression of the d-mtDNA helicase dele-
tion mutants on mtDNA maintenance were examined in cells expressing the mutants. To determine the mtDNA copy num-
ber, total cellular DNA was isolated, cleaved with XhoI, and
analyzed by Southern blot (see "Experimental Procedures.")

FIGURE 1. Sequence alignment and location of the deletion and amino acid substitution mutations in the
carboxyl-terminal region of Drosophila mtDNA helicase. Schematic diagram of the sequence organization of the d-mtDNA helicase; five amino acid sequence motifs common to ring helicases are indicated in gray, and the bacteriophage T7 gp4 linker region is indicated in black. The deletion mutations in d-mtDNA helicase used in this study are designated below the schematic diagram along with the sequence alignments of the regions containing altered amino acids. Dm, mitochondrial DNA helicase of the fly; Ag, mosquito; Xi, frog; Hs, humans; DnaB, Thermus aquaticus DnaB helicase; T7, bacteriophage T7 gp4. Altered and conserved amino acids are
indicated in gray. The positions of the deletion mutants and alanine substitutions are shown above the alignment as asterisks and A, respectively. The positions of the puta-
tive arginine finger and base stack are indicated below the alignment.

ysis revealed a 10–20-fold increase in the levels of the wild type, Δ(607–613) mutant, and Δ(597–613) mutant relative to the endogenous d-mtDNA helicase level (Fig. 2A). The ATP synthase β subunit probed as a control showed no significant change in expression level. The relative mtDNA copy number was determined from the ratio of CytB hybridization to histone gene cluster hybridization. After 14
days of induction, the relative mtDNA copy number was increased 1.2-fold in cells overexpressing the wild type enzyme as compared with the control cells (Fig. 2B). In the case of the overexpression of the Δ(607–613) and Δ(597–613) mutants, the relative mtDNA copy number was increased to 105–120%
that of the control cells, values that are comparable with the wild type-overexpressing cells. In contrast, in the cells over-
pressing the Δ(586–613) and Δ(572–613) mutants, the relative mtDNA copy number was decreased to 57–65% that of the control despite their very low levels of expression. These results indicate that the region between 572 and 596 is essential for helicase function.

d-mtDNA Helicase Deletion Mutants Form Hexamers—To explain the dominant negative effects we observed in the Schneider cells overexpressing the d-mtDNA helicase deletion mutants, we examined the oligomeric state of the over-
expressed proteins. To do so, we induced stable cell lines carrying
either no construct or the wild type
or mutant constructs for 7 days in the presence of 0.2 mM CuSO4. Cells were harvested and treated with formaldehyde to cross-link the proteins as described under "Experimental Procedures." Immunoblot analysis after native gel electro-
phoresis of the wild type d-mtDNA helicase revealed monomers, dimers, and hexamers (Fig. 3A, lane 2), whereas the endogenous protein in cells carrying no construct was undetectable under these condi-
tions (lane 1). Analysis of the deletion mutants (lanes 3 and 4) showed that the efficiency of hexamer for-
mation with the Δ(607–613) and Δ(597–613) mutants is nearly
equivalent to that of the overex-
pressed wild type protein. Because of the low level of expression of the Δ(586–613) and Δ(572–613)
mutants, we were unable to detect multimeric helicase forms using the formaldehyde cross-linking analysis. However, upon velocity sedimentation of these two mutants from mitochondrial extracts of Schneider cells, we found them to have sedimentation co-
efficients similar to endogenous mtDNA helicase (data not shown).

Thus, we conclude that the dominant-negative phenotype and mtDNA depletion observed in Drosophila Schneider cells expressing these deletion mutants also results from the formation of functionally defective heterohexamers.

Overexpression of Carboxyl-terminal Point Mutants of the d-mtDNA Helicase in Schneider Cells—In the carboxyl-termi-
nal region between amino acids 572 and 596 of the d-mtDNA helicase, eight charged or aromatic amino acids, Lys-574, Arg-576, Tyr-577, Asp-580, Glu-587, Lys-590, and Tyr-
595, are well conserved between the various mtDNA helicases
aligned in Fig. 1. Arg-576 and Phe-588 of the
helicase are analogous to the arginine finger, Arg-522, and base stack,
Tyr-535, of T7 gp4, respectively. To examine their physiologi-
cal significance in the mitochondrial enzyme, we constructed metallothionein-inducible plasmids expressing eight d-
mtDNA helicase variants carrying the alanine substitutions K574A, R576A, Y577A, D580A, E587A, F588A, K590A, and Y595A. The constructs were transfected into Schneider cells, and two independent stable cell lines were established for each of them. After 14 days of incubation in the absence or presence of 0.2 mM CuSO4, immunoblot analysis indicated increases in the
levels of all of the variants in the range of 2.5–3.5- and
12–20-fold, respectively, relative to the control cell lines (Fig.
4A). At the same time, the ATP synthase β subunit showed no significant change in expression levels. Cross-linking analysis of the mutants as described above showed that all were capable of forming hexamers (Fig. 3B). Next, we evaluated their effects on mtDNA maintenance. In cells overexpressing the putative argi-
nine finger mutant R576A, the relative mtDNA copy number was reduced to 70 and 9% of the control cells (Fig. 4B), corre-
that of the control upon induction, values that are comparable with those we obtained upon overexpression of the wild type d-mtDNA helicase. Correspondingly, whereas copper induction had no adverse effects on the growth of cells expressing the Y577A, D580A, E587A, and K590A mutants, cells expressing the K574A, R576A, F588A, and Y595A mutants grew very poorly. After 4 weeks of induction, the mtDNA levels in the latter mutants decreased to an undetectable level and produced a lethal phenotype between 4 to 6 weeks (data not shown).

**ATPase Activities of the Human mtDNA Helicase and Its Variants**—To evaluate biochemically mutants producing dominant negative phenotypes in *Drosophila* cells, we produced recombinant human proteins carrying alanine substitutions at analogous positions: R609A, F621A, and F628A. Consistent with the dominant negative effects seen in vivo for the *Drosophila* Arg-576, Phe-588, and Tyr-595 helicase mutants, the human R609A variant lacks ATP hydrolysis activity, and the

---

**FIGURE 2. Overexpression of Drosophila mtDNA helicase and deletion mutants in Schneider cells.** A, Schneider cells containing no plasmid (control), carrying pMt/Hy (vector), pMt/WT/Hy (WT), pMt/Δ607–613/Hy (Δ607–613), pMt/Δ597–613/Hy (Δ597–613), pMt/Δ586–613/Hy (Δ586–613), or pMt/Δ572–613/Hy (Δ572–613), were cultured for 14 days in the presence of 0.2 mM CuSO₄. Protein extracts (20 μg) were fractionated by 9% SDS-PAGE, transferred to nitrocellulose filters, and probed with affinity-purified rabbit antiserum against d-mtDNA helicase or antiserum against d-ATPase β as indicated. B, effect of overexpression of the deletion mutants on mtDNA copy number. Upper panel, total mtDNA (10 μg) was extracted from Schneider cells (control) or Schneider cells carrying pMt/Hy (vector), pMt/WT/Hy (WT), pMt/Δ607–613/Hy (Δ607–613), pMt/Δ597–613/Hy (Δ597–613), pMt/Δ586–613/Hy (Δ586–613), or pMt/Δ572–613/Hy (Δ572–613) that were cultured for 14 days in the presence of 0.2 mM CuSO₄ DNA was digested with XhoI, fractionated in a 0.7% agarose/Tris-buffered EDTA gel, and then blotted to a nylon membrane. The membrane was hybridized with a radiolabeled probe for the histone gene cluster (his genes) and then stripped and re-hybridized with radiolabeled probe for CytB (designated as mtDNA). Lower panel, relative mtDNA copy number was quantitated as described under “Experimental Procedures.”

---

**FIGURE 3. Intramolecular cross-linking of Drosophila mtDNA helicase and its variants.** Schneider cells carrying overexpressing constructs were treated with 1% formaldehyde for 5 min, and the reaction was quenched with glycine. Cell extracts were fractionated by 6% SDS-PAGE, transferred to nitrocellulose filters, and probed with affinity-purified rabbit antisera against d-mtDNA helicase as above. A, lane 1, Schneider cells containing no plasmid (control); lane 2, wild type (WT); lane 3, Δ607–613; lane 4, Δ597–613. B, lane 1, Schneider cells containing no plasmid (control); lanes 2–10, wild type and mutant helicase as indicated above the lanes.
mtDNA helicase using the crystal structure of the T7 gp4 helicase as the template (PDB code 1EOJ (18); see “Experimental Procedures”) illustrates that the positions of these amino acids are consistent with their proposed roles in the human protein (Fig. 6).

**DISCUSSION**

The ring-shaped helicases require the energy nucleotide hydrolysis for helicase movement and double-stranded DNA unwinding. Nucleotide binding and hydrolysis requires amino acids within the Walker A and B motifs, with additional contributions from a number of other signature sequences (10, 11). Amino acids within the Walker A motif stabilize the nucleotide phosphate, those within the Walker B motif stabilize the Mg$^{2+}$ ion, an arginine finger coordinates the γ phosphate of the bound nucleotide, and a base stack residue is involved in positioning the nucleotide base. In our previous paper we demonstrated that the overexpression of d-mtDNA helicase variants carrying either mutations in the Walker A or Walker B motifs results in a dose-dependent depletion of mtDNA (13). Very recently, Falkenberg and co-workers (14, 35) reported on mutational studies of the amino-terminal domain and linker region of the human mtDNA helicase. Here, we show that specific amino acids in the carboxyl-terminal region are essential for helicase function both in vivo and in vitro.

In several enzymes belonging to the SF4 group of helicases, a deletion or substitution of carboxyl-terminal residues abolishes replication in vivo without affecting enzymatic activities, and these residues were found to be essential for interaction of the helicase with other replication factors (24, 26). For example, 7 of the 17 carboxyl-terminal amino acids in T7 gp4 are negatively charged, and the deletion or substitution mutations in these acidic residues results in loss of T7 phage replication (24). Furthermore, a biochemical evaluation indicates that the carboxyl-terminal region is essential for the interaction of T7 gp4 with T7 DNA polymerase (28). The carboxyl-terminal regions of the mtDNA helicases are not well conserved between species but do contain a substantial number of basic amino acids. However, high level overexpression of the d-mtDNA helicase Δ(597–613) mutant that lacks a basic tail failed to show a dominant negative phenotype and results in an increased mtDNA copy number, as was observed with the overexpression of wild type d-mtDNA helicase. The current findings are consistent with our recent biochemical studies of a human recombinant helicase lacking its extreme carboxyl terminus that show an

**FIGURE 4. Expression in Schneider cells of Drosophila mtDNA helicases carrying mutations in the carboxyl-terminal region.** Schneider cells containing no plasmid (control) or carrying pMt/WT/Hy (WT), pMt/K576A/Hy (K576A), pMt/R576A/Hy (R576A), pMt/Y577A/Hy (Y577A), pMt/D580A/Hy (D580A), pMt/E587A/Hy (E587A), pMt/F588A/Hy (F588A), pMt/K90A/Hy (K90A), or pMt/Y595A/Hy (Y595A) were cultured for 14 days in the presence of 0.2 mM CuSO$_4$. DNA was digested with XhoI, fractionated in a 0.7% agarose/Tris-buffered EDTA gel, and then blotted to a nylon membrane. The membrane was hybridized as described under “Experimental Procedures.”

**FIGURE 5. ATPase activities of human mtDNA helicase and its variants.** ATP hydrolysis was measured in the presence of the indicated amounts of wild type mtDNA helicase and mutant forms. Open circles, wild type helicase; closed circles, R609A; open triangles, F621A; closed triangles, F628A.

F621A and F628A mutants exhibit 10- and 4-fold decreases in activity, respectively (Fig. 5). These data indicate that in addition to an arginine finger at amino acid Arg-609 and a base stack at Phe-621, amino acid Phe-628 also serves a role in the nucleotide hydrolysis reaction. Structural modeling of the human

**Structure-Function of Mitochondrial DNA Helicase**
increase in DNA-dependent ATPase activity (12), suggesting that the carboxyl-terminal tail of the d-mtDNA helicase may not be required for its function in mtDNA replication. However, we note that a deletion of three carboxyl-terminal residues of the RepA protein abolishes RSF1010 replication while retaining helicase activity in vitro yet does not interfere with replication in the presence of wild type RepA in vivo (26). Because we overexpress the Δ(597–613) mutant in the presence of endogenous protein, this remains a possibility that warrants further study.

We constructed four carboxyl-terminal deletion mutants, but we could not establish high expression cell lines for the most amino-terminal Δ(572–613) and Δ(586–613) mutants. Because these showed a mtDNA depletion phenotype in cultured cells even upon low level expression, higher expression may induce lethality. Before induction, the cell lines overexpressing either the arginine finger or base stack mutants maintained mtDNA copy number at ~50% of that of wild type levels. In this situation the molar ratio of the endogenous to exogenous polypeptides is 1:2. By comparison, upon induction of the deletion mutant overexpressing cell lines, mtDNA copy number is also maintained at ~50% that of wild type, but the protein expression level is less than 50% that of the endogenous wild type. We conclude that the deletion mutants induce a more severe phenotype as compared with the amino acid substitution mutants. One possible explanation is as follows. Hexameric helicases have six NTPase pockets with the arginine finger and the base stack residue of each individual protomer contributing to adjacent NTPase pockets. The mutant Δ(572–613) lacks both an arginine finger and the base stack residue such that a hexameric helicase containing one Δ(572–613) protomer results in two adjacent deficient NTPase pockets. However, the Δ(586–613) mutant lacks only the base stack residue Phe-588 but shows similar results to Δ(572–613). Rather, deletion of amino acids 586–596 may cause a change that destabilizes the position of the arginine finger and/or other critical residues. Another possibility may be that the deletion of amino acids 586–596 causes rapid protein degradation.

A crystal structure of the T7 gp4 established that Arg-522 acts as an arginine finger that interacts with the γ-phosphate of the bound nucleotide, and Tyr-535 acts as the base stack that contacts the nucleotide base (15, 18, 19). We demonstrate that the overexpression of a putative arginine finger mutant, R576A in *Drosophila*, that is analogous to the arginine finger in T7 gp4, results in a dose-dependent depletion of mtDNA. Similarly, the analogous recombinant human helicase mutant, R609A, exhibits loss of ATPase activity *in vitro*, confirming the previous proposal by Ziebarth et al. (12) that this residue acts as the arginine finger. Similar results were reported for the arginine finger mutants of T7 gp4, which showed reduced ATPase activity and failed to support T7 replication *in vivo* (17). We also examined the ATP hydrolysis activity of a putative, human base stack mutant residue, F621A, and found it to catalyze only 7% of the ATPase activity of a putative, human base stack mutant overexpressing cell lines, mtDNA copy number is 50% that of wild type levels. In this situation the molar ratio of the endogenous to exogenous polypeptides is 1:2. By comparison, upon induction of the deletion mutant overexpressing cell lines, mtDNA copy number is also maintained at ~50% that of wild type, but the protein expression level is less than 50% that of the endogenous wild type. We conclude that the deletion mutants induce a more severe phenotype as compared with the amino acid substitution mutants. One possible explanation is as follows. Hexameric helicases have six NTPase pockets with the arginine finger and the base stack residue of each individual protomer contributing to adjacent NTPase pockets. The mutant Δ(572–613) lacks both an arginine finger and the base stack residue such that a hexameric helicase containing one Δ(572–613) protomer results in two adjacent deficient NTPase pockets. However, the Δ(586–613) mutant lacks only the base stack residue Phe-588 but shows similar results to Δ(572–613). Rather, deletion of amino acids 586–596 may cause a change that destabilizes the position of the arginine finger and/or other critical residues. Another possibility may be that the deletion of amino acids 586–596 causes rapid protein degradation.

A crystal structure of the T7 gp4 established that Arg-522 acts as an arginine finger that interacts with the γ-phosphate of the bound nucleotide, and Tyr-535 acts as the base stack that contacts the nucleotide base (15, 18, 19). We demonstrate that the overexpression of a putative arginine finger mutant, R576A in *Drosophila*, that is analogous to the arginine finger in T7 gp4, results in a dose-dependent depletion of mtDNA. Similarly, the analogous recombinant human helicase mutant, R609A, exhibits loss of ATPase activity *in vitro*, confirming the previous proposal by Ziebarth et al. (12) that this residue acts as the arginine finger. Similar results were reported for the arginine finger mutants of T7 gp4, which showed reduced ATPase activity and failed to support T7 replication *in vivo* (17). We also examined the ATP hydrolysis activity of a putative, human base stack mutant residue, F621A, and found it to catalyze only 7% of the ATPase activity of the wild type human enzyme. Moreover, we determined that overexpression of the d-mtDNA helicase analog of the human mutant, F588A, results in the depletion of mtDNA in Schneider cells, revealing a loss of helicase activity *in vivo* as well. Phe-588 is analogous to the base stack, Tyr-535, in T7 gp4. Although a crystal structure of T7 gp4 revealed that
Tyr-535 contacts the base of the bound nucleotide, no biochemical or genetic data are available (15, 18, 19). Our results indicate that Phe-588 likely acts as the base stack in d-mtDNA helicase, and this finding may be applicable to many of the SF4 helicases such as T7 gp4 and *E. coli* DnaB.

We also constructed new mutants, K574A, Y577A, and Y595A, and found that K574A and Y595A show loss of function *in vivo*, and Y577A shows a moderate loss of function. Lys-574 and Tyr-577 lie adjacent to the putative arginine finger, Arg-576. The T7 gp4 crystal structure shows that the loop containing residues Lys-520—Gly-525 that flanks the arginine finger, Arg-522, contributes to the edge of the nucleotide pocket (18). Our physiological data argue that Lys-574 and Tyr-577 in mtDNA helicase most likely contribute to nucleotide stabilization as well. Interestingly, Lys-574 is well conserved not only in hexameric helicases but also in the ATP-dependent recombinase RecA. This suggests that this lysine residue may be critical for the RecA/DnaB superfamily (30). Tyr-595 is conserved in the mtDNA helicase of the mosquito and is replaced by a phenylalanine in the frog, human, and DnaB helicases but is not conserved in T7 gp4. Our biochemical analysis of the human F628A mutant, analogous to Y595A in *Drosophila* mtDNA helicase, showed a defect in nucleotide hydrolysis similar to the base stack and arginine finger mutants, indicating that F628A contributes to NTP hydrolysis in the human enzyme. Our homology model of the human mtDNA helicase predicts that Phe-628 interacts directly with Phe-621, the base-stacking residue, and an interior residue, Phe-424, suggesting that the mutation in Phe-628 may result in a defect in nucleotide binding (Fig. 6). Similarly, in DnaB, residue Phe-437 interacts with the base-stacking residue Phe-430 and another interior residue, Phe-424 (Fig. 6). These residues are well conserved in both mtDNA helicases and in DnaB helicases, and, hence, our finding may be applicable to SF4 helicases in general (20–22). Our model also predicts that residues Lys-607, Arg-609, and Phe-610, which yield dominant negative phenotypes in the fly protein, point to the intermolecular cleft in which nucleotide would be bound, and where the conserved helicase motifs are clustered for their involvement in nucleotide binding and hydrolysis. In contrast, three additional mutants, D580A, E587A, and K590A, which are well conserved in mtDNA helicase and T7 gp4 but not in DnaB, did not show the dominant negative phenotype. Interestingly, in our homology model, the side chains of these residues do not face the NTPase pocket (Fig. 6). Taken together, our data argue that these three residues do not contribute to NTP binding and hydrolysis in mtDNA helicase.

We determined that the overexpression of the mutants K574A, R576A, F588A, and Y595A reduces mtDNA copy number in a dose-dependent manner. These data are very similar to our findings with Walker A and Walker B motif mutants (13), suggesting their involvement in nucleotide binding and hydrolysis. This is consistent with our observation that the efficiency of hexamer formation *in vivo* is similar to that of wild type for all of these mutants as examined by velocity sedimentation and in cross-linking analyses. Before induction, ~50% of the mtDNA copy number is maintained in cells where the typical molar ratio of endogenous to exogenous mtDNA helicase polypeptides is 1:2. If the mutants form hexamers with the same efficiency as the wild type polypeptide, then less than 0.2% of the hexamers should contain six wild type protomers, whereas hexamers containing 5 wild type and 1 mutant protomers would comprise only 1.6%, and hexamers with 4 wild type and 2 mutant protomers would constitute 8.2% of the total. Based on our results, it appears that these low abundance heterohexamers maintain some helicase activity *in vivo*. Under induced conditions, the typical molar ratio of endogenous to exogenous d-mtDNA helicase polypeptides is 1:19. In the case of K574A, R576A, F588A, and Y595A, mtDNA copy number is less than 15% that of the control cells, indicating that these mutants lose helicase activity *in vivo*. In contrast, the mutant Y577A retains half of its mtDNA as compared with the control cells, arguing it possesses substantial helicase function. Recently, an autosomal recessive mutation T457I has been reported in patients with mtDNA depletion syndrome, and this residue was speculated to lie in the NTPase pocket (2). Very recently, autosomal dominant progressive external ophthalmoplegia mutations in the linker region were studied in biochemical assays and by structural modeling (35). Interestingly, these mutations also showed defects in ATPase activity. However, the positions of these mutations were not localized to the nucleotide binding pocket. Rather, the structural modeling analysis suggests that one of the mutant amino acids, Leu-381, interacts with motif H1a, which contributes to NTP hydrolysis. Moreover, because the linker region is involved in interaction with neighboring subunits, mutants in the linker region might affect positioning of amino acids that lie within the nucleotide binding pocket such as the arginine finger. Similarly, mutations in the extreme carboxyl-terminal region may be expected to cause mtDNA depletion syndrome or progressive external ophthalmoplegia in humans. Numerous mutations in the mtDNA helicase have been documented to cause progressive external ophthalmoplegia. Although these are nearly equally distributed within the amino-terminal, linker, and helicase domains, none of the >20 mutations has been shown to map to the carboxyl terminus. Thus, forward mutational studies of mtDNA helicase are important not only to understand helicase function but may also yield new insights to understand human mitochondrial disease.

**Acknowledgment—**We thank Tawn Ziebarth for critical reading of the manuscript.

**REFERENCES**

1. Spelbrink, J. N., Li, F. Y., Tiranti, V., Nikali, K., Yuan, Q. P., Tariq, M., Wannooij, S., Garrido, N., Comi, G., Morandi, L., Santoro, L., Toscano, A., Fabrizi, G. M., Somer, H., Croxen, R., Beeson, D., Poulton, J., Suomalainen, A., Jacobs, H. T., Zeviani, M., and Larsson, C. (2001) *Nat. Genet.* **28**, 223–231

2. Sarzi, E., Goffart, S., Serre, V., Chretien, D., Slama, A., Munnich, A., Spelbrink, J. N., and Rotig, A. (2007) *Ann. Neurol.* **62**, 579–587

3. Hakonen, A. H., Isohanni, P., Paetau, A., Herva, R., Suomalainen, A., and Lonnoqvist, T. (2007) *Brain* **130**, 3032–3040

4. Copeland, W. C. (2007) *Annu. Rev. Med.* **59**, 131–146

5. Spinazzola, A., and Zeviani, M. (2005) *Gene (Amst.)* **354**, 162–168

6. Kaguni, L. S. (2004) *Annu. Rev. Biochem.* **73**, 293–320

7. Suomalainen, A., Majander, A., Wallin, M., Setala, K., Kontula, K., Leinonen, H., Salmi, T., Paetau, A., Haltia, M., Valanne, L., Lonnoqvist, J., Peltonen, L., and Somer, H. (1997) *Neurology* **48**, 1244–1253
8. Suomalainen, A., Majander, A., Haltia, M., Somer, H., Lonnqvist, J., Savontaus, M. L., and Peltonen, L. (1992) J. Clin. Invest. 90, 61–66
9. Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S., and DiDonato, S. (1989) Nature 339, 309–311
10. Singleton, M. R., Dillingham, M. S., and Wigley, D. B. (2007) Annu. Rev. Biochem. 76, 23–50
11. Patel, S. S., and Picha, K. M. (2000) Annu. Rev. Biochem. 69, 651–697
12. Ziebarth, T. D., Farr, C. L., and Kaguni, L. S. (2007) J. Mol. Biol. 367, 1382–1391
13. Matsushima, Y., and Kaguni, L. S. (2007) J. Biol. Chem. 282, 9436–9444
14. Farge, G., Holmlund, T., Khvorostova, J., Rofougaran, R., Hofer, A., and Falkenberg, M. (2008) Nucleic Acids Res. 36, 393–403
15. Donmez, I., and Patel, S. S. (2006) Nucleic Acids Res. 34, 4216–4224
16. Crampton, D. J., Guo, S., and Richardson, C. C. (2006) Mol. Cell 21, 165–174
17. Crampton, D. J., Guo, S., Johnson, D. E., and Richardson, C. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4373–4378
18. Singleton, M. R., Sawaya, M. R., Ellenberger, T., and Wigley, D. B. (2000) Cell 101, 589–600
19. Sawaya, M. R., Guo, S., Tabor, S., Richardson, C. C., and Ellenberger, T. (1999) Cell 99, 167–177
20. Wang, G., Klein, M. G., Tokonzaba, E., Zhang, Y., Holden, L. G., and Chen, X. S. (2008) Nat. Struct. Mol. Biol. 15, 94–100
21. Bailey, S., Eliason, W. K., and Steitz, T. A. (2007) Science 318, 459–463
22. Bailey, S., Eliason, W. K., and Steitz, T. A. (2007) Nucleic Acids Res. 35, 4728–4736
23. Hamdan, S. M., Johnson, D. E., Tanner, N. A., Lee, J. B., Qimron, U., Tabor, S., van Oijen, A. M., and Richardson, C. C. (2007) Mol. Cell 27, 539–549
24. Lee, S. J., Marintcheva, B., Hamdan, S. M., and Richardson, C. C. (2006) J. Biol. Chem. 281, 25841–25849
25. Hamdan, S. M., Marintcheva, B., Cook, T., Lee, S. J., Tabor, S., and Richardson, C. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 5096–5101
26. Ziegelin, G., Niedenzu, T., Lurz, R., Saengker, W., and Lanka, E. (2003) Nucleic Acids Res. 31, 5917–5929
27. Kong, D., and Richardson, C. C. (1998) J. Biol. Chem. 273, 6556–6564
28. Notarnicola, S. M., Mulcahy, H. L., Lee, J., and Richardson, C. C. (1997) J. Biol. Chem. 272, 18425–18433
29. Wanrooij, S., Goffart, S., Pohjoismaki, J. L., Yasukawa, T., and Spelbrink, J. N. (2007) Nucleic Acids Res. 35, 3238–3251
30. Lei, D., Aravind, L., Grishin, N. V., and Koonin, E. V. (2000) Genome Res. 10, 5–16
31. Matsushima, Y., Adan, C., Garesse, R., and Kaguni, L. S. (2005) J. Biol. Chem. 280, 16815–16820
32. Matsushima, Y., Garesse, R., and Kaguni, L. S. (2004) J. Biol. Chem. 279, 26900–26905
33. Niedenzu, T., Roleke, D., Bains, G., Scherzinger, E., and Saenger, W. (2001) J. Mol. Biol. 306, 479–487
34. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
35. Korhonen, J. A., Pande, V., Holmlund, T., Farge, G., Pham, X. H., Nilsson, L., and Falkenberg, M. (2008) J. Mol. Biol. 377, 691–705