In Vivo Functional Analysis of the Human Mitochondrial DNA Polymerase POLG Expressed in Cultured Human Cells*

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The human gene POLG encodes the catalytic subunit of mitochondrial DNA polymerase, but its precise roles in mtDNA metabolism in vivo have not hitherto been documented. By expressing POLG fusion proteins in cultured human cells, we show that the enzyme is targeted to mitochondria, where the Myc epitope-tagged POLG is catalytically active as a DNA polymerase. Long-term culture of cells expressing wild-type POLG-myc revealed no alterations in mitochondrial function. Expression of POLG-myc mutants created dominant phenotypes demonstrating important roles for the protein in mtDNA maintenance and integrity. The D198A amino acid replacement abolished detectable 3′-5′ (proofreading) exonuclease activity and led to the accumulation of a significant load (1:1700) of mtDNA point mutations during 3 months of continuous culture. Further culture resulted in the selection of cells with an inactivated mutator polymerase, and a reduced mutation load in mtDNA. Transient expression of POLG-myc variants D890N or D1135A inhibited endogenous mitochondrial DNA polymerase activity and caused mtDNA depletion. Deletion of the POLG CAG repeat did not affect enzymatic properties, but modestly up-regulated expression. These findings demonstrate that POLG exonuclease and polymerase functions are essential for faithful mtDNA maintenance in vivo, and indicate the importance of key residues for these activities.

Human mitochondrial DNA (mtDNA) is a 16.5-kb circular double-stranded DNA molecule (1) that depends on many nuclear-coded proteins for its maintenance and expression. A working hypothesis for the mechanism of mtDNA replication was published almost two decades ago (2), yet little is known about the key enzymes involved in this essential cellular process. Synthesis of mtDNA is apparently asymmetrical, with two-thirds of the leading strand being synthesized before the lagging strand initiates. The displaced parental strand is believed to be protected from intra-strand recombination and illegitimate initiation events by the mitochondrial single-stranded DNA-binding protein, mtSSB (3).

The human POLG gene encodes the catalytic subunit of what is believed to be the only DNA polymerase active in mitochondria, polymerase γ, POLG (4–6). However, clear evidence that this enzyme functions in mtDNA replication in vivo remains lacking. POLG and homologous enzymes, such as Mip1p in yeast (7), are related to the Family A DNA polymerases, which include Pol I from Escherichia coli and phage T7 DNA polymerase (4, 5, 8, 9). The regions of highest similarity between POLG and the Family A polymerases include an NH2-terminal 3′-5′ exonuclease domain involved in proofreading, and a COOH-terminal region that in eubacterial members of the family is essential for polymerase activity. Residues essential for DNA polymerase activity have not yet been identified in any mitochondrial enzyme. A feature unique to human POLG is the presence of a repeat of 13 glutamines, partly encoded by a run of 10 CAG codons near to the NH2-terminus of the coding region. Population analysis implies that active selection maintains the CAG tract at or near 10 repeats (10), which may indicate a stringent dependence of catalytic activity on glutamine tract-length.

In Saccharomyces cerevisiae, specific Mip1p residues have been shown to be critical for exonuclease activity (11–13). In recombinant human POLG the double mutant D198A/E200A is also exonuclease deficient in vitro (14). Mutations that abolish Mip1p exonuclease activity in yeast result in the accumulation of base substitutions in mtDNA, and in some cases also in a significantly increased frequency of generation of rho− cells carrying defective mtDNA (12, 13, 15). Exonuclease-deficient mip1 mutations are at least partially dominant (13), meaning that even in the presence of wild-type Mip1p, mutations accumulate in mtDNA. However, a role for the exonuclease domain of POLG in maintaining the integrity of mtDNA in humans has not been demonstrated. A mitochondrial mutator for human cells would be an invaluable tool for studying the mechanisms involved in the generation, accumulation, and phenotypic effects of human mtDNA mutations, which are an important cause of human disease.

DNA replication requires the combined action of many proteins, including those involved in nucleotide metabolism, DNA unwinding, priming of DNA synthesis, decatenation, and sta-
bilization of intermediary structures. Such proteins are predicted to assemble in a large replication complex with DNA polymerase, but few of them have been identified in vertebrate mitochondria. Two examples are the mtSSB, which can stimulate POLG activity in vitro (16–19) and the accessory (β) subunit of DNA polymerase γ (20), that enhances processivity in vitro (21) and has been proposed to play a role in primer recognition, on the basis of structural considerations (22).

Thus far, human POLG has been characterized only in vitro, via access to the purified or recombinant enzyme (5, 14, 16–18, 21, 23–25). We therefore set out to analyze the functions of the protein and its constituent domains in vivo, using an episomal expression system in cultured cells. In this study we confirm, using reporter constructs, that POLG is targeted in vivo to the inner face of the inner mitochondrial membrane and is functional as a DNA polymerase. Long-term overexpression does not, however, affect mtDNA levels, indicating that the polymerase is not limiting for the overall rate of mtDNA synthesis. Via the expression of mutated versions of the enzyme, we further demonstrate that the D198A mutation abolishes detectable proofreading exonuclease activity in mitochondrial extracts and promotes the accumulation of mtDNA mutations, leading to selection in long-term cell culture. Mutations at either of 2 aspartate residues in the COOH-terminal domain abolish DNA polymerase activity, inhibit the endogenous enzyme, and cause mtDNA depletion. Catalytic activity is unaffected by deletion of the CAG repeat, although expression is modestly up-regulated. These results represent the first demonstration of the functional roles and importance of POLG in the maintenance and integrity of mtDNA in human cells.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 µg/ml uridine, and 10% fetal bovine serum, at 37 °C in a humidified atmosphere with 8.5% CO2 in air. No antibiotics were added. HEK293T cells were routinely detached by pipetting alone, and split 1:10–1:20. HeLa cells were split after detachng the cells by a 5-min incubation in 10 mM EDTA. Cells were transfected in 6-well culture plates with 1–3 days prior to transfection at 40–70% density. Transfection routinely used 1 µg of DNA and 10 µl of LipofectAMINE (Life Technologies, Inc.), diluted in 1 ml of Opti-MEM (Life Technologies, Inc.) according to the manufacturer’s protocol. Five hours following transfection, 2 ml of fresh medium was added and, when necessary, was replaced 24 h after transfection. Transfection efficiency for HEK293T cells was routinely high (approximately 50%), based on co-transfection with a β-galactosidase-encoding construct, but for HeLa cells was less than 1%. Transfections in 100-mm plates were successively replated to eliminate untransfected cells. Geneticin-resistant clones were picked by ringcloning, regrown in multwell plates, and subsequently tested for the presence of an expressed transgene by reverse transcriptase-PCR or by Western blot analysis. Whole cell oxygen consumption was measured essentially as described previously (26).

Plasmid DNA Constructs and in Vitro Mutagenesis—Site-directed mutagenesis of single residues of the POLG cDNA (4) was carried out using the Altered Sites™ II mutagenesis system (Promega), essentially according to the manufacturer’s protocols. For this purpose, full-length POLG cDNA originally cloned in pcDNAI/Amp (4) was recloned in the pAlter-1 vector, and subjected to site-directed mutagenesis and ampicillin selection. Appropriate POLG fragments derived from selected plasmids were re-inserted into full-length POLG in pBluescript SK- (Stratagene) and sequenced to confirm the presence and integrity of the CAG repeats, and to ensure that no other mutations had occurred during the procedure. Deletion of the CAG repeats was effected using a PCR-based method, and the resulting PCR product was also recloned into the pBluescript SK- POLG construct. All constructs were finally recloned in pCDNA3.1 (−)/Myc-His A (Invitrogen) using a high-fidelity PCR-based method. These final constructs contain the full coding sequence plus 61 nucleotides from the 5′-untranslated region, cloned in-frame with the Myc-His tag sequence using the EcoRI and BamHI restriction sites of the vector. The resulting fusion protein contains the 1239 amino acids of the POLG precursor, followed by an additional 31 amino acids of the Myc-His tag, with a predicted total molecular mass of 148 kDa. A similar fusion was created for TUFM cDNA (27). GFP fusion proteins were constructed by recloning cDNAs for POLG, TUFM, and mtTFA (kind gift of Dr. R. Wiesner) in the pEGFP-N3 vector (CLONTECH). All constructs were confirmed by DNA sequencing.

Monitoring of GFP Reporter Gene Expression by Fluorescence Microscopy—Cells were washed with Dulbecco’s modified Eagle’s medium 24 h after transfection and incubated in medium containing 100 µg/ml Mitotracker Red (Molecular Probes) for 10–15 min. The cells were washed twice in PBS, after which normal medium was replaced and were incubated for a further 2 h at 37 °C. They were then washed twice in PBS and fixed in 4% formaldehyde, 5% sucrose in PBS for 10 min at 37 °C. After two final PBS washes the coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories) and visualized using an Olympus BX-50 microscope, with appropriate filters for Mitotracker Red (U-MWG, wavelength 510–550 nm) and enhanced green fluorescent protein (U-MWB, wavelength 450–480 nm) fluorescence.

Polyacylamide Gel Electrophoresis and Western Blotting—SDS-PAGE used 7.5–12% polyacrylamide (Laemmli) gels run under standard conditions (20). All samples were heated prior to electrophoresis for 5 min in SDS-PAGE sample buffer (29) containing dithiothreitol (50 µl Tris-HCl, pH 6.8, 12% glycerol, 4% SDS, 0.01% Serva Blue G, 0.1 mM dithiothreitol). Wbetooging to Hybond™-C extra nitrocellulose membrane (Amersham Pharmacia Biotech) was carried out at 100 V for 1 h at 4 °C (30). Blots were blocked for 1 h at room temperature in TBS-T (0.1% Tween) containing 5% freeze-dried fat-free milk powder, washed several times with TBS-T, and reacted with primary antibody in TBS-T overnight at room temperature. Primary antibodies and dilutions used were: mouse anti-Myc monoclonal 9E10 (Roche Molecular Biochemicals), 1:15,000 dilution of a 5 mg/ml stock, rabbit anti-mtSSB (kind gift of Dr. M. Zeviani), 1:1000, rabbit anti-β-subunit of DNA polymerase γ (kind gift of Dr. P. Lestienne), 1:5000, rabbit anti-human mtTFA (kind gift of Dr. R. Wiesner), 1:10,000, rabbit anti-complex IV (31, 32), 1:10,000, and a mouse anti-COXIIi monoclonal (kind gift of Dr. R. Capaldi), 1:1000. Blots were washed 2 × 10 s, 1 × 15 min, and 2 × 5 min in TBS-T, and then incubated for 1 h at room temperature with peroxi- dase-conjugated goat or horse secondary antibody (anti-mouse IgG (Bio-Rad or Vector Laboratories, Inc.), 1:10,000, or anti-rabbit IgG (Vector Laboratories, Inc.)). mss was re-washed as above and finally washed in 0.1% SDS, 0.009% H2O2, 0.1 M Tris-Cl, pH 6.8) and 50 µl of enhancer solution (1.1 mg/ml para-hydroxycoumaric acid (Sigma) in dimethyl sulfoxide) were mixed and incubated on each blot for 1 min. Film (Kodak Biomax™ ML) was exposed for periods from 15 s to 45 min, as necessary. Silver staining was carried out as described by Morrissey (33). Other gels were stained for 30 min in 0.1% Coomassie Brilliant Blue (Sigma) in 40% methanol, 10% acetic acid followed by 10% methanol, 10% acetic acid.

Cell Lysis, Subcellular Fractionation, and Immunoprecipitation—Cell pellets, obtained by centrifugation at 1200 × g for 1 min, were lysed in PBS containing 1.5% (w/v) lauryl maltoside and 2.5 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min, then centrifuged at 16,000 × g, for 1 min at 4 °C. Supernatant and pellet were either stored at −80 °C or used immediately for SDS-PAGE. Finer subcellular fractionation used a standard procedure for mitochondrial isolation (34). Essentially, cells were washed once with PBS, dislodged from the plate by pipetting up and down in ice-cold NKM (1 mM Tris-HCl, pH 7.4, 0.13 mM NaCl, 5 mM KCl, 7.5 mM MgCl2) and centrifuged at 400 × g for 3 min at 4 °C. The cell pellet was resuspended by gentle pipetting in 2 volumes of ice-cold 0.1% homogenization buffer (4 mM Tris-HCl, pH 7.8, 2.5 mM NaCl, 0.5 mM MgCl2), kept on ice for 5 min, then homogenized in a glass homogenizer with 20 strokes of a tight-fitting pestle. Disruption of the cells was monitored by microscopy. One-ninth of 10 × homogenization buffer was added and nuclei and cell debris were pelleted by two sequential centrifugations at 1,200 × g for 3 min at 4 °C. Mitochondria from the post-nuclear supernatant were recovered by centrifugation at 4000 × g for 1 min. Mitochondrial pellets from 1 ml of cytoplasm were washed once with 1 ml of ice-cold PBS and either directly processed further or frozen at −80 °C. Submitochondrial fractionation was carried out essentially as described elsewhere (35, 36). Protease resistance of imported mitochondrial proteins was verified as follows. A mitochondrial pellet obtained from one or two 90% confluent, 175 cm2 cell culture flasks was resuspended in

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PBS. An equal volume of 4 mg/ml digitonin (heat solubilized in PBS), was added and samples were vortexed vigorously, split into three equal portions, and incubated on ice for 5 min. An additional 9 volumes of ice-cold PBS were added and samples were centrifuged at 16,000 × gmax for 3 min at 4 °C, then washed twice in 1 ml of PBS. The resulting mitochondria were recovered in PBS by resuspension of the pellets, incubated for 10 min at room temperature either without any further additions, with 50 μg/ml trypsin (Fukua), or with 0.15% lauryl maltoside plus 50 μg/ml trypsin. 2 × SDS-PAGE sample buffer was added directly to the last sample. The first two samples were pelleted, washed three times with PBS, and finally resuspended in SDS-PAGE sample buffer. Immunoprecipitations were carried out essentially as described elsewhere (36). In brief, mitochondrial pellets containing up to 1 mg of protein were lysed in PBS, 1.5% lauryl maltoside, 2.5 mM phenylmethylsulfonyl fluoride, incubated for 30 min on ice, and centrifuged at 16,000 × gmax for 1 min at 4 °C. The supernatant was incubated on a rotary shaker at 4 °C for 2 h or overnight with 20–100 μl of 10% Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech), prepared in PBS, 1 mg EDTA, 1 mg/ml bovine serum albumin, and preincubated for 15 min with the appropriate antibody. The beads were pelleted at 14,000 × gmax for 10 s, washed once with 10 ml Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM NaCl and three times with 10 ml Tris-HCl, pH 8.0, 1 mM EDTA, 0.05% lauryl maltoside. The final pellet was resuspended in 1–2 volumes of SDS-PAGE sample buffer.

**Polymerase and Exonuclease Assays—**POLG activity was measured by means of an RNA-dependent DNA polymerase (reverse transcriptase) assay, adapted from Longley et al. (14). Freshly isolated mitochondria were lysed in 25 mM Hepes-KOH, pH 8.0, 100 mM NaCl, 1% Triton X-100 so that the final protein concentration was between 2.5 and 10 mg/ml. After incubation on ice for 30 min and centrifugation at 16,000 × gmax for 1 min at 4 °C, activity was assayed in 10-μg aliquots of lysate in a final volume of 50 μl containing 25 μM Hepes-KOH, pH 8.0, 100 mM NaCl, 2.5 mM 2-mercaptoethanol, 10 μg/ml acetylated bovine serum albumin, 0.5 mM MnCl₂, 2.5 μg of poly(rA)oligo(dT) (Amersham Pharmacia Biotech), and 50 μg/ml Aphidicolin. Reactions containing either no further inhibitor, or else ddTTP as indicated, were preincubated for 5 min at room temperature before the addition of 5 μl of a mixture of [α-32P]dTTP (Amersham Pharmacia Biotech, 3000–6000 Ci/mmol) diluted 0.6:10 with 100 μM ddTTP. Samples were then incubated for 15 min at 37 °C, after which 10 μl was spotted on a GFC filter, air-dried, washed three times with 5% trichloroacetic acid then once with 70% ethanol, air-dried and counted for POLG activity (24). Samples were incubated at 37 °C and 10-μl aliquots were taken and directly added to 10 μl of formamide sample buffer (95% formamide, 0.6% SDS, 25 mM EDTA, 0.05% w/v bromphenol blue and 0.05% w/v xylene cyanol), at various time points varying from 0 to 20 min. Samples were boiled for 2 min and run on a 15% polyacrylamide focusing gel. The gels were fixed in 10% acetic acid, 15% ethanol, dried, and autoradiographed. Control reactions were carried out without the addition of mitochondrial lysate.

**Protein and Peptide Analysis—**For amino-terminal sequence analysis, proteins separated by gel electrophoresis and electroblotted on polyvinylidene difluoride, the membranes were sequenced by automatic Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer (model 477A). At the end of each cycle of Edman degradation, the phenylthiohydantoin-derivative was separated from reaction by-products using an Applied Biosystems HPLC apparatus (model 120A) on a Browle C18 reverse phase column (Spheri-5 phenylthiohydantoin, 5 mm, 220 × 2.1 mm). Data were analyzed with the Applied Biosystems Data Analysis System software (version 1.2). For MALDI analysis, protein-containing gel slices were S-alkylated, digested with trypsin (Roche Molecular Biochemicals, sequencing grade), and extracted according to Shevchenko et al. (37). Only the peptides eluted with 20 mM NH₄HCO₃ were used in the MALDI analysis. After drying in a vacuum centrifuge the peptides were dissolved in 0.1% acetic acid, 0.5 μl of the solution (final volume, 0.1 μl), were mixed with 0.5 μl of a 10 mg/ml o-phenylenediamine solution. Before dissolving the o-phenylenediamine acid was briefly washed with 1/10 of the final volume of acetone. 0.5 μl of the mixture was spotted on the target and allowed to dry at room temperature. MALDI spectra were acquired on a Microflex TOF Spec 2E (Micromass, Wythenshawe, UK), equipped with a 2 GHz LeCroy LSA-100 digitizer (LeCroy Corp., Chestnut Ridge, NY). The resulting peptide spectra were used to search a nonredundant protein sequence data base (SWISS-PROT/TREMBL) using the Proteinprotease program.

**Miscellaneous DNA and RNA Manipulations—**Total cellular DNA was isolated, EcoRI digested, and subjected to Southern analysis as described elsewhere (38, 39). Total cellular RNA was isolated using Trizol reagent (Life Technologies, Inc.) by the manufacturer’s recommended procedure and Northern blots were carried out as described previously (40). Northern hybridization probes for POLG and for glyceraldehyde-3-phosphate dehydrogenase (CLONTECH) were synthesized by random-primed labeling of coding region PCR products in the presence of [α-32P]dCTP (Amersham Pharmacia Biotech, 3000–6000 Ci/mmol). Hybridization and final washes were at 65 °C. Autoradiographic images were analyzed using a PhosphorImager (Molecular Dynamics). DNA sequencing was performed using either DuPont Sequenase or dye-terminator chemistry on the Perkin-Elmer ABI 310 Genetic Analyzer, with kit reagents supplied by the manufacturer and vector- or POLG-specific primers.

**Human mtDNA Polymerase Expression—**We measured POLG activity by an assay adapted from Longley et al. (14). Freshly isolated mitochondria were lysed in 25 mM Hepes-KOH, pH 8.0, 100 mM NaCl, 1% Triton X-100 so that the final protein concentration was between 2.5 and 10 mg/ml. After incubation on ice for 30 min and centrifugation at 16,000 × gmax for 1 min at 4 °C, activity was assayed in 10-μg aliquots of lysate in a final volume of 50 μl containing 25 μM Hepes-KOH, pH 8.0, 100 mM NaCl, 2.5 mM 2-mercaptoethanol, 10 μg/ml acetylated bovine serum albumin, 0.5 mM MnCl₂, 2.5 μg of poly(rA)oligo(dT) (Amersham Pharmacia Biotech), and 50 μg/ml Aphidicolin. Reactions containing either no further inhibitor, or else ddTTP as indicated, were preincubated for 5 min at room temperature before the addition of 5 μl of a mixture of [α-32P]dTTP (Amersham Pharmacia Biotech, 3000–6000 Ci/mmol) diluted 0.6:10 with 100 μM ddTTP. Samples were then incubated for 15 min at 37 °C, after which 10 μl was spotted on a GFC filter, air-dried, washed three times with 5% trichloroacetic acid then once with 70% ethanol, air-dried and counted using liquid scintillation counting. For each lysis a control assay without template was used to measure the background trichloroacetic acid soluble counts. A method for mitochondrial nuclelease activity purification was adapted from existing protocols (12, 14, 24), using primer-activated single-stranded M13mp18 (+) (Amersham Pharmacia Biotech). 50 pmol of paired or 3′-nucleotide-mismatched oligo (paired, AB353: GTAAAAACGACCGGACT; mismatched, AB354: GTAAAAACGAGGCGCAGA) were 5′-labeled using [γ-32P]ATP (Amersham Pharmacia Biotech, 3000–6000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s recommended procedure. POLG activity was measured using random-primed labeling of coding region PCR products in the presence of [α-32P]dCTP (Amersham Pharmacia Biotech, 3000–6000 Ci/mmol). Hybridization and final washes were at 65 °C. Autoradiographic images were analyzed using a PhosphorImager (Molecular Dynamics). DNA sequencing was performed using either DuPont Sequenase or dye-terminator chemistry on the Perkin-Elmer ABI 310 Genetic Analyzer, with kit reagents supplied by the manufacturer and vector- or POLG-specific primers.

**RESULTS**

**Tagged POLG Is Targeted to Mitochondria in Vivo—**To confirm that the POLG protein is targeted to mitochondria we constructed reporter gene fusions of full-length POLG, fused at the COOH terminus to a Myc tag (POLG-myc), or to green fluorescent protein (POLG-GFP). Transient expression by liposome-mediated transfection of POLG-GFP into a variety of human cell lines showed a specific green fluorescent staining pattern typical of mitochondria (Fig. 1). This was confirmed by co-staining with Mitotracker Red (a mitochondrial-specific dye). Similar staining patterns were observed with equivalent reporter constructs for several known mitochondrial proteins, including the mitochondrial transcription factor mtTFA, and mitochondrial translational elongation factor EF-Tu, TUFM.
(not shown). No nuclear staining was observed in any of the experiments. Mitochondrial green fluorescence was uniform, i.e. no subfraction of mitochondria, such as in a perinuclear location, showed more intense or specific fluorescence. Transfection with the unmanipulated GFP vector resulted in uniform, diffuse staining in the cytosol, with no evidence of specific localization to mitochondria (data not shown).

HEK293T cells stably expressing POLG-myc (see below) were used in subcellular and submitochondrial fractionation studies, with analysis by Western blotting against the anti-Myc monoclonal antibody that recognizes the epitope tag. Lauryl maltoside lysis revealed that POLG-myc, like the LacZ-myc control, was mainly cytoplasmic rather than nuclear (Fig. 2). Further fractionation (Fig. 3) showed that the majority of POLG-myc co-purifies with mitochondria, being found mainly in the inner mitochondrial membrane fraction, together with COXII (subunit II of cytochrome c oxidase), but absent from the matrix fraction, which is heavily enriched for glutamate dehydrogenase. A small proportion of the POLG-myc was also detected in the outer membrane fraction, a pattern similar to that exhibited by endogenous mtTFA, mtSSB, and also the putative β-subunit of DNA polymerase γ (Fig. 3a).

The POLG-myc found in intact mitoplasts was resistant to trypsin digestion, but lysis of either mitochondria or mitoplasts with low concentrations of lauryl maltoside rendered it trypsin-sensitive (Fig. 3b). These results imply that POLG-myc is imported into mitochondria and localized mainly on the inner face of the inner membrane. Similar results were obtained for TUFM-myc (data not shown). Expressed POLG-myc was immunoprecipitated from mitochondrial lysates as a 140-kDa polypeptide whose identity was confirmed by MALDI mass spectrometry. NH2-terminal sequencing failed, however, indicating that the amino terminus is blocked. Parallel analysis of POLG-myc containing the polyglutamine repeat deletion (ΔQ16-myc), plus vector, TUFM-myc, and LacZ-myc controls (Fig. 2). In two additional cases, clonal cell lines expressed an internally deleted variant of POLG-myc. These were not studied further. Expression of POLG-myc transgenes was verified by Western and Northern blotting (Fig. 2), and was stably maintained in all cell lines over a period of at least 3 months.

Transfection of constructs encoding the D890N-myc and D1135A-myc variants did not give rise to any clones expressing the transgene, despite repeated attempts to isolate such clones. We analyzed 50 geneticin-resistant colonies from several transfections using each of the mutant constructs, but found none positive for expression. This was not due to any inherent problem in the expression of these variants, such as a frameshift mutation not detected by DNA sequencing, since they were successfully expressed transiently, at approximately the same level as wild-type POLG-myc (Fig. 4c). However, expression of the mutant transgenes declined sharply during the 2 weeks following transfection, even under geneticin selection.

Overexpressed POLG-myc Is Catalytically Active as a Mitochondrial DNA Polymerase—Mammalian mtDNA polymerase activity is routinely measured by its rate of incorporation of radioactive dTTP on a primed RNA template, poly(rA):oligo(dT), i.e. by its reverse transcriptase activity, in the presence of aphidicolin. Crude mitochondrial fractions were prepared from stable or transiently transfected cell lines using hypotonic lysis. Mitochondrial DNA polymerase activity is routinely measured by its rate of incorporation of radioactive dTTP on a primed RNA template, poly(rA):oligo(dT), i.e. by its reverse transcriptase activity, in the presence of aphidicolin.
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![Western blot analysis of submitochondrial localization of POLG-myc fusion protein.](Image)

**FIG. 3.** Western blot analysis of submitochondrial localization of POLG-myc fusion protein. Blots from SDS-PAGE gels were probed using the anti-Myc monoclonal antibody, polyclonal antibodies against human mtTFA, mtSSB, the putative β-subunit of DNA polymerase γ or glutamate dehydrogenase, or a monoclonal antibody against COXII. a, proteins from submitochondrial fractions as indicated (OM, outer membrane; IM, inner membrane), separated on a 7.5% polyacrylamide gel (each lane represents an equal amount of the starting material prior to fractionation). Blots probed with the anti-Myc antibody and, after stripping, with the anti-glutamate dehydrogenase antibody. For successive detection of mtTFA, mtSSB, COXII, and the putative β-subunit the proteins were separated on a 12% polyacrylamide gel (IM lane represents twice as much unfractionated starting material as the other lanes). Membrane localized proteins such as COXII are not found in the matrix fraction (data not shown). b, protease accessibility of POLG-myc in mitochondria and mitoplasts (see "Materials and Methods"). Analysis was via a 7.5% polyacrylamide gel, probed by Western blotting with the anti-Myc monoclonal.

**FIG. 4.** DNA polymerase activity in mitochondrial extracts. a, POLG RNA-dependent DNA polymerase (reverse transcriptase) activity (arbitrary units AU, normalized to template-dependent 32P incorporation in extracts from control cells transfected with episomal vector). All assays contained the same amount of mitochondrial protein. b, sensitivity of RNA-dependent DNA polymerase activity to inhibition by ddTTP. c, Western blot of lauryl maltoside extracts from HEK293T cells transiently transfected with the vector only (293T-myc), or POLG-myc variants, prepared either 2 days after transfection (2d), or after 2 weeks of culture in the presence of genetin (2w). The Myc-tagged POLG polypeptide is indicated by an arrow. d, POLG RNA-dependent DNA polymerase activity, as in panel a, of mitochondrial protein extracts prepared from transiently transfected cells 2 days after transfection. The higher polymerase activity seen in ΔQ10-myc extracts reflects a higher concentration of the protein in this extract, confirmed by immunoprecipitation from the extract, followed by SDS-PAGE and Coomassie staining (not shown).

The D198A Mutation Abolishes Detectable POLG Proofreading Exonuclease Activity—Proofreading (3′-5′) exonuclease activity was studied in the same extracts from stably transfected cells (Fig. 5), using a primed M13 DNA substrate, with either a perfectly matched or a 3′ terminal mismatched primer. Significant activity was not detected in extracts from vector-transfected cells, but was easily detected in extracts from cells expressing wild-type POLG-myc or ΔQ10-myc. As predicted for the POLG-associated 3′-5′ exonuclease, the reaction yielded a ladder of products whose mean size declined with time (Fig. 5c). In addition, activity was greater on a 3′ mismatched than a perfectly base paired substrate (Fig. 5b), and, as expected from previous studies of purified POLG (14, 24) was inhibited by 100 mM NaCl (Fig. 5b). High salt did appear to activate another nuclease activity already present in lysates from control cells, that did not give a ladder of products (Fig. 5b and other data not shown). The enhanced 3′-5′ exonuclease activity was not found in mitochondrial extracts of two independent clonal cell lines expressing D198A-myc, consistent with the prediction that this mutation alone results in exonuclease deficiency.

In summary, POLG-myc expressed episomally at high levels had the catalytic properties expected of mitochondrial DNA polymerase. The stably expressed D198A-myc mutant was de-
Control cells, although cells expressing studied. The values were generally within the range of variation. Fig. 6 shows the results of these experiments, in whether mitochondrial function was affected by transgene expression. Several months following transfection, in order to determine base paired to M13 ssDNA.

Mitochondrial extracts, assayed by degradation of labeled oligonucleotides base paired to M13 ssDNA. Reaction products were analyzed on 7 m urea, 15% polyacrylamide gels. a, adjacent narrow lanes of a gel loaded with ice-quenched reactions representing a time course for each extract, using the two 17-nucleotide (nt) substrates (arrows) described under “Materials and Methods.” A separate time course for the wild-type POLG-myc extract is shown in panel c, indicating the ladder of shorter products. Note that the enhanced activity of the ΔQ10-myc variant reflects its greater expression at the protein level. b, salt inhibition of the exonuclease activity. Reactions containing 100 mM NaCl (10 min incubation with each lysate, loaded in adjacent narrow lanes): compare with panel a. A large excess of the unreacted substrate is also shown in panel b, to indicate that the ladder of products is not generated except when mitochondrial lysate from transfected cells is present.

Exonuclease activity in mitochondrial extracts, assayed by degradation of labeled oligonucleotides base paired to M13 ssDNA. Reaction products were analyzed on 7 m urea, 15% polyacrylamide gels. a, adjacent narrow lanes of a gel loaded with ice-quenched reactions representing a time course for each extract, using the two 17-nucleotide (nt) substrates (arrows) described under “Materials and Methods.” A separate time course for the wild-type POLG-myc extract is shown in panel c, indicating the ladder of shorter products. Note that the enhanced activity of the ΔQ10-myc variant reflects its greater expression at the protein level. b, salt inhibition of the exonuclease activity. Reactions containing 100 mM NaCl (10 min incubation with each lysate, loaded in adjacent narrow lanes): compare with panel a. A large excess of the unreacted substrate is also shown in panel b, to indicate that the ladder of products is not generated except when mitochondrial lysate from transfected cells is present.

Stable Overexpression of POLG-myc Does Not Disturb Mitochondrial Function—Cell lines stably expressing POLG-myc, D198A-myc, and ΔQ10-myc were analyzed over a period of several months following transfection, in order to determine whether mitochondrial function was affected by transgene expression. Fig. 6 shows the results of these experiments, in which mtDNA content and whole cell oxygen consumption were studied. The values were generally within the range of variation seen over comparable time periods or for sister clones of control cells, although cells expressing ΔQ10-myc had a relatively low mtDNA content (Fig. 6b). Oxygen consumption for two lines expressing D198A-myc was at the low end of the normal range. Two-dimensional blue native-SDS-PAGE (42–44) did not reveal any difference in the amount, composition, or assembly state of the mitochondrial redox complexes in stably transfected cells expressing wild-type POLG-myc, D198A-myc, or ΔQ10-myc (data not shown). Western blots (Fig. 3, combined with more data not shown) indicated that there was no up-regulation of the expression of mtTFA, mtSSB, or the putative β-subunit of DNA polymerase γ.

However, two observations suggest that cell clones expressing D198A-myc were less healthy than POLG-myc, ΔQ10-myc, or control cells. First, the cells grew significantly more slowly (approximately 30% longer doubling times) than the other cell lines, and also appeared to acidify their media more rapidly. Second, they were more sensitive to etidium bromide treatment, which is routinely used to deplete cultured human cells of their mtDNA (45). These observations are consistent with a mild impairment of mitochondrial metabolism in D198A-myc cells.

Polymerase-deficient POLG-myc Variants Inhibit mtDNA Replication in vivo—Because the D890N-myc and D1135A-myc variants could not be obtained as stably expressing cell lines, they were studied by transient expression in HEK293T cells. The amounts of expressed protein in these extracts was comparable with that from wild-type POLG-myc-transfected cells (Fig. 4c), indicating that the mutant proteins are stable. Crude mitochondrial extracts prepared from cells 48 h after transfection with wild-type POLG-myc showed approximately 5-fold enhancement of polymerase activity compared with vector-transfected cells (Fig. 4d). By contrast, extracts from cells transfected with the D890N-myc and D1135A-myc mutant proteins consistently showed inhibition of the endogenous polymerase activity comparable with that produced by ddTTP. Mixing experiments (data not shown) indicated that the inhibition was indirect, i.e. that synthesis by a given amount of wild-type POLG-myc extract was not directly inhibited by extracts from D890N-myc or D1135A-myc transfected cells, other than as a result of dilution. The inhibition of endogenous activity in extracts from D890N-myc or D1135A-myc transfected cells is therefore most likely due to the fact that expression from the mutant constructs overwhelms that of the endogenous enzyme and competes with it for recruitment into mitochondrial DNA synthesis complexes.

Compared with vector-transfected cells, cells transfected with D890N-myc or D1135A-myc also showed a reproducible and sustained decrease in mtDNA levels, over the 96 h following transfection (Fig. 7). Relative mtDNA levels fell progressively to approximately 60–70% of control values. Given that about half of all cells in such cultures were successfully transfected, this must indicate a complete or almost complete inhibition of mtDNA replication in cells expressing the polymerase-deficient POLG-myc variants. This is also consistent with the polymerase activity measurements at 48 h, indicating an inhibition of endogenous activity.

Exonuclease-deficient POLG-myc Promotes Accumulation of mtDNA Mutations in Vivo—If overexpressed POLG-myc is functional in mtDNA replication or repair in vivo, long-term expression of the D198A-myc variant that lacks proofreading exonuclease activity is expected to result in the accumulation of mtDNA mutations. Cells expressing D198A-myc showed evidence of only a very mild impairment of mitochondrial function. Nevertheless, we reasoned that the lack of a clear mitochondrial phenotype in cells expressing D198A-myc could be masked if most mtDNA mutations are functionally recessive and hence complemented by wild-type sequences present in other mtDNA molecules. We therefore analyzed a short region of the mitochondrial genome, by means of a carefully controlled high-fidelity PCR approach using Pfu DNA polymerase. We amplified through 20 cycles a region of mtDNA spanning portions of the 16 S rRNA, tRNA-leu(UUR), and ND1 genes from bulk cultures of cells expressing D198A-myc, wild-type POLG-myc, and 293T-myc (vector only), grown continuously for 3 months. The PCR products were cloned, and randomly selected insert-containing clones sequenced. To estimate the maximum rate of PCR-induced mutation, we amplified a previously sequenced clone of the same fragment using the same procedure,
and picked and sequenced a set of clones.

After correcting for the background of PCR-induced mutations (approximately 1 mutated base per 20 kb of sequence), there was no significant mtDNA diversity in the cell lines transfected either with vector or with wild-type POLG-myc (Table I). By contrast, two independent clones of D198A-myc cells grown for 3 months had each accumulated a significant mutation load in mtDNA, equivalent to 1 mutated base every 1700 bp. Assuming an upper limit for the background of PCR-induced mutations in our assay of 1 per 15 kb, Poisson statistics indicate that the chance of finding 37 or more mutations in 61 kb of sequence would be less than 10^{-22}. All three deviations from the reference sequence detected in the clone control or in vector-transfected cells were transversions, whereas mtDNA mutations in the D198A-myc expressing cells (Table II) were mainly transitions (29 out of 37), with half of all mutations representing the replacement of heavy strand C residues by T. Only one was a deletion (of 1 bp). Four mutations were found twice, all others just once only, and none has previously been reported as a polymorphism. The frequency and pattern of mutations in the two cell clones were very similar.

**Cells Expressing the POLG Mutator Undergo Selection after 3 Months in Culture**—We reasoned that a continuing accumulation of mtDNA mutation at this rate would eventually lead to a frank impairment of mitochondrial respiration and a selective crisis in the cell population. This can be predicted on the assumption that a deleterious mutation would eventually be introduced into every copy of at least one mitochondrial gene in each mitochondrial genetic unit. We therefore cultured the cells for longer times, periodically re-examining the mutation spectrum and the status of the D198A-myc transgene. In one experiment, a culture of line D198A-myc3 manifested a drop in growth rate after 4 months and eventually stopped growing. Other cultures of the same cell line were still growing after 6 or 8 months. A culture of clone D198A-myc2 also survived at least 6 months.

Using the same procedure as earlier, we re-sampled the mtDNA mutation spectrum in these cultures (Table III). In line D198A-myc3, the mutational load, as represented by the total frequency of deviations from the Cambridge reference sequence, was lower at both 4 and 8 months than at 3 months, suggesting that selection was operating. This hypothesis was supported by studies of the POLG transgene. Western blotting showed that transgene expression at the protein level in line D198A-myc3 had fallen to low levels by 5.5 months (Fig. 8a).
and also in a parallel culture analyzed at 8 months (not shown). Complete sequence analysis of the POLG transgene revealed the presence of a novel mutation (Fig. 8b), causing a non-conservative amino acid substitution (R832S). All vertebrate sequenced have arginine or lysine at this position. At the 3-month time point, the R832S substitution was already evident by direct sequencing (Fig. 8b) but at a lower level. No other transgene mutations were found.

These observations are consistent with inactivation of the mutant polymerase via a selection for a mutant form of the enzyme with greatly reduced stability. In support of this, POLG DNA polymerase activity in mitochondrial extracts from D198A-myc3 cells at the 5.5-month time point had fallen almost to the same level as in vector-transfected (293T-myc) cells (Fig. 8c). Transgene expression was undetectable by Western blotting in 9 out of 22 individual subclones of D198A-myc3 cells derived at 4 months, whereas all 23 subclones of wild-type POLG-myc cells analyzed at this time point remained positive. Subclones of D198A-myc3 cells that were negative for transgene expression (e.g. subclone D3–2, Fig. 8a) also carried the R832S mutation, and had low levels of POLG DNA polymerase activity (Fig. 8c), whereas subclones still positive for transgene expression at 5.5 months (e.g. subclone D3–1) were wild-type at this position and retained polymerase activity.

Analysis of mtDNA from line D198A-myc2 at the 4-month time point also suggested a decline in mutation load (Table III). In this cell line, expression of the transgene at the protein level was retained in the bulk culture when tested at 5.5 months (Fig. 8c), although 2 out of 19 subclones isolated at 4 months no longer expressed it (data not shown). However, POLG DNA polymerase activity was low in one out of two randomly selected subclones from line D198A-myc2 that did continue to express the transgene at the protein level (Fig. 8, a and c).

### DISCUSSION

The experiments presented here provide confirmation, via a reporter gene approach, that POLG encodes a mitochondrially localized protein that is functional in mtDNA synthesis in vivo. POLG variants create dominant-negative cellular phenotypes, including a mtDNA mutator that indicates the limits of mtDNA mutation load that mammalian cells can tolerate.

**Highly Expressed POLG-myc Is Functionally Active inside Mitochondria—**High level, episomal expression of the POLG-myc transgene is associated with greatly enhanced DNA polymerase and 3′-5′ exonuclease activity in mitochondrial extracts. These activities have characteristics that resemble those of the purified or recombinant POLG enzyme and are abolished by point mutations that alter key conserved residues analogous to those found previously to be essential for catalysis in other members of the Family A DNA polymerases. Inside mitochondria, the protein is localized to the inside face of the inner membrane, which is believed to be the compartment in which mtDNA is also located. As in yeast (15), enzymatic activities were not impaired by COOH-terminal tagging.

Enhanced polymerase activity does not alter mtDNA copy number during long-term culture. Therefore, availability of POLG is not a limiting factor for mtDNA synthesis, consistent with observations that the gene is unregulated developmentally (46), and that POLG gene dosage does not affect mtDNA levels (47). Other proteins involved in mtDNA maintenance, e.g. mtTFA (48, 49) or mtSSB (46), must regulate copy number.

**Expression of POLG-myc Variants Creates Dominant Phenotypes in Vivo—**The main aim of this study was to test the functional effects in vivo of expressing POLG variants mutated in each of three different regions, namely the proofreading exonuclease domain, the putative polymerase domain, and the polyglutamine repeat. By overwhelming the expression of the endogenous enzyme, we were able to evaluate both the enzymatic properties of the mutated polypeptides, as well as other dominant effects on cell phenotype. We infer that the DNA polymerase activity of POLG is essential for mtDNA maintenance, and that its exonuclease activity is essential for mtDNA integrity. By contrast, the polyglutamine repeat does not contribute to the enzymatic properties of POLG.

The D198A Substitution Creates a Mitochondrial Mutator in Vivo—**Previous in vitro analysis of recombinant POLG carrying the D198A mutation in combination with an additional substitution (E200A) indicated a loss of 3′-5′ exonuclease activity (14). Our results indicate that the D198A substitution alone is sufficient to abolish detectable proofreading exonuclease activity in mitochondrial extracts. In yeast, the analogous substitution D171G causes a 104-fold drop in 3′-5′ exonuclease activity, and a 100-fold increase in the rate of accumulation of point mutations in yeast mtDNA (15).
Overexpression of the D198A-myc variant for 3 months in culture generated a much higher frequency of mtDNA mutations than the minimal level found in control cultures, or the background of “PCR noise.” The pattern of substitutions, being mainly G-C to A-T transitions, was also distinct from that caused by *Pfu* polymerase on a mtDNA template, which in our hands and elsewhere seems to give mainly transversions (Table II, Ref. 50). Expression of wild-type POLG-myc alone did not create a mtDNA mutator. We can exclude the possibility that nuclear pseudogenes were being detected, since most mutations detected were unique, and occurred on different individual clones. A nuclear pseudogene of this region (GenBank accession number AF047836) can be detected by PCR, but it can be detected in D198A-myc2 and D198A-myc3 cells after 3 months of continuous culture. Only the 1-strand is shown. The reference sequence exhibits two differences (denoted by asterisks) from the original Cambridge reference sequence (1), namely a C deletion at np 3106, now designated as a 1-bp deletion. The boundaries of tRNA-leu(UUR) and of the ND1 coding sequence are shown.

![Image](https://example.com/image.png)

**Fig. 8. Consequences of long-term expression of D198A-myc.** a, Western blots as in Fig. 2a, using extracts from cells cultured continuously for 5.5 months, with equal amounts of protein (10 μg) loaded in each track. Compare relative signals with those in Fig. 2a, which shows extracts prepared shortly before the 3-month time point. Extracts were analyzed from bulk cultures of lines D198A-myc2 and D198A-myc3, plus two subclones of each (D2-4, D2-6 and D3-1, D3-2), isolated at 4 months, and verified to have retained the D198A mutation. b, sequencing traces for a region of the POLG transgene (np 2629–2621, antisense strand, GenBank U60325) from POLG-myc cells (wild type sequence), plus D198A-myc3 cells after 3 and 8 months of continuous culture. Arrow denotes the mutation that accumulated in the cells, causing amino acid substitution R832S. c, POLG DNA polymerase activities in mitochondrial extracts (as in Fig. 4a) of bulk cultures and subclones of mutator cells at 5.5 months. Subclone D3-1 was 100% wild type at residue R832, whereas subclone D3-2 was 100% mutant for the R832S substitution. Subclone D198A-myc2 was already detectable at 3 months, whereas another survived till 8 months. The selection that has occurred in these cultures makes it difficult to extrapolate an exact or absolute rate of mtDNA mutation. The fact that the R832S transgene mutation was already detectable at 3 months strongly suggests that significant selection was already occurring. Since such selection must limit the mtDNA diversity of the cell population, the accumulation of a mutation load of approximately 1:1700 over 3 months is therefore a minimum estimate of the mutation rate. Given that selection was already significant at 4 months, the maximum tolerable mutation load in mtDNA may also not be much greater than 1:1700 bp.

The simplest hypothesis is that selection was operating at the level of respiratory metabolism. However, such selection can clearly take more than one course. In line with D198A-myc3, one culture of cells appeared to enter a terminal crisis after 4 months in culture, whereas another survived till 8 months, with concomitant loss of transgene expression and a modest reduction in mutation load. This can be interpreted as the emergence, within the population, of a cell clone in which transgene expression was lost relatively late, i.e. in which significant mtDNA mutations had already accumulated, but were presumably not sufficient to compromise growth rate.

Further analysis will be required to characterize precisely the outcome of selection in line D198A-myc2. The mtDNA mutation load was again lower at 4 months than at 3, and this

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was also accompanied by transgene inactivation. However, in this cell line it appears to have involved at least two different mechanisms: loss of expression (in a minority of cells) and loss of polymerase activity in some others. Based on previous analyses of cultured heterplasmic cells (38, 51), random mitotic segregation could not alone have brought about the decline in mutation load observed in these cultures.

The mutation load observed at 3 months in both mutator clones implies that each mtDNA molecule should carry typically about 10 mutations, at least some of which are expected to be deleterious. This is strongly implied by the pattern of substitutions (Fig. 8d), in which first, second and third base pairs of codons within the ND1 coding sequence are equally targeted including 4 amino acid substitutions and one frameshift. Four out of 5 mutated residues in tRNA-leu(UUR) are phylogenetically conserved among all primates and virtually all mammals. However, this mutation load does not entail a severe respiratory phenotype. It seems reasonable that intramolecular complementation is able to sustain respiratory function, until a higher threshold of mutation load is crossed. This could represent the emergence of functionally dominant mutations that compromise protein synthesis or respiratory enzyme assembly and function, or simply the loss of all functional copies of at least one gene within each mitochondrial genetic unit.

Loss of POLG Exonuclease Does Not Account for Mutational Patterns in mtDNA—The pattern of human mtDNA sequence polymorphisms reported in the MITOMAP data base shows an enormous preponderance of transitions (88%) over transversions, with a nucleotide bias approximating that of the genome (i.e. the genome is 56% A + T, and 58% of the transitions convert an A-T pair to a G-C). Similar figures apply to the pathological mutations currently reported in the data base (90% transitions, 56% of them affecting an A-T pair). The mutations introduced by the POLG mutator are also mainly transitions (29 out of 37), but the pattern of substitutions is quite different, with C-G pairs replaced about 10 times more often by A-T pairs than vice versa (26 as against 3). L-strand C residues were more frequently replaced than those on the H-strand, although this may reflect the base bias of the region analyzed (see legend to Table II). The gene for tRNA-leu(UUR) is commonly claimed to be a hotspot for mutation, but this does not apply to the changes introduced by the POLG mutator. The mutational patterns of human mtDNA are therefore not obviously attributable to faulty POLG proofreading. The base- and possible strand-bias of the mutations we observed may reflect fundamental properties of the POLG enzyme, or aspects of the unusual, strand-asymmetric mode of mtDNA replication in mammalian cultured cells (2). It may be that the wild-type proofreading exonuclease has a better ability to handle some types of mutations than others and that the mutator superimposes a new set of mutations over a background of errors that even the wild-type editing activity handles poorly. Alternatively, the exonuclease activity of POLG may also play a role in DNA repair, as suggested by in vitro studies (25). Intriguingly, the mutational pattern that we found is similar to that introduced into mtDNA by the chemical mutagen MNNG (52).

Two Polymerase-deficient POLG Variants Inhibit mtDNA Synthesis and Cause Depletion—The polymerase-deficient substitutions in POLG-myc affect conserved aspartate residues of the polymerase domain of the enzyme, located in the carboxy-terminal half of the polypeptide. The corresponding residues Asp<sup>705</sup> and Asp<sup>882</sup> in <i>E. coli</i> Pol I are essential for catalysis (41, 53). Transient expression of D890N-myc or D1135A-myc inhibited endogenous POLG activity and caused a reproducible drop in the level of mtDNA during the 96 h following transfection. During a period when cell number increased through several cell doublings, the amount of mtDNA relative to nuclear DNA decreased progressively by up to 40%, whereas in parallel cultures transfected with vector or with wild-type POLG-myc there was no mtDNA depletion. Given typical transfection efficiencies of 50%, the kinetics of the effect are consistent with a total or almost total cessation of mtDNA replication, even without invoking a negative effect on cell growth. We postulate that the mutant proteins, expressed at much higher levels than the endogenous POLG, were incorporated into “dead-end” replication complexes, thus blocking mtDNA synthesis.

The dominant-negative phenotype suggests that POLG is required for mtDNA replication, although it need not be the only DNA polymerase involved. Two distinct DNA polymerases appear to be found, for example, in trypanosome (54) and bovine (55) mitochondria and, as already noted, POLG could also serve a dual purpose as a DNA repair enzyme (25). The repeated failure to isolate cell clones stably expressing the polymerase-deficient variants, and the decline of their expression in the weeks following transfection, even in cultures under selection, furthermore, implies that their expression has a significant negative effect on cell growth or survival, presumably via respiratory selection. Given that high level expression of the polymerase-deficient mutants appears to create a drastic phenotype, it may be profitable to attempt lower level regulated expression, which might have a milder dominant-negative effect on mtDNA metabolism.

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