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Manipulating mitochondrial DNA heteroplasmy by a mitochondrially targeted restriction endonuclease

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Mutations in the mitochondrial DNA (mtDNA) can cause a variety of human diseases. In most cases, such mutations are heteroplasmic (i.e. mutated and wild-type mtDNA coexist) and a small percentage of wild-type sequences can have a strong protective effect against a metabolic defect. Because a genetic approach to correct mtDNA mutations is not currently available, the ability to modulate heteroplasmy would have a major impact in the phenotype of many patients with mitochondrial disorders. We show here that a restriction endonuclease targeted to mitochondria has this ability. A mitochondrially targeted *Pst*I degraded mtDNA harboring *Pst*I sites, in some cases leading to a complete loss of mitochondrial genomes. Recombination between DNA ends released by *Pst*I was not observed. When expressed in a heteroplasmic rodent cell line, containing one mtDNA haplotype with two sites for *Pst*I and another haplotype having none, the mitochondrial *Pst*I caused a significant shift in heteroplasmy, with an accumulation of the mtDNA haplotype lacking *Pst*I sites. These experiments provide proof of the principle that restriction endonucleases are feasible tools for genetic therapy of a sub-group of mitochondrial disorders. Although this approach is limited by the presence of mutation-specific restriction sites, patients with neuropathy, ataxia and retinitis pigmentosa (NARP) could benefit from it, as the T8399G mutation creates a unique restriction site that is not present in wild-type human mitochondrial DNA.

INTRODUCTION

It has been shown that most pathogenic mitochondrial DNA (mtDNA) mutations are present in a heteroplasmic state (i.e. co-existing with wild-type genomes) (1–3). In most cases, a very high percentage of mutated mtDNA is necessary to elicit phenotypic abnormalities, commonly associated with muscle and the central nervous system dysfunction. This has been exemplified by the variable clinical picture of different members of the same kindred (4,5) as well as by the different tissue involvement in patients (6). Intercellular phenotypic differences in a tissue have also been associated with small variations in the proportion of wild-type and mutated mtDNA in individual cells (7,8). This concept suggested that approaches to manipulate mtDNA heteroplasmy may have potential therapeutic applications (9). We attempted to manipulate mtDNA heteroplasmy by taking advantage of unique features of specific mtDNA haplotypes, namely the presence of a restriction endonuclease site that could differentiate two forms of the mitochondrial genome.

RESULTS

We were interested in testing the ability of a restriction endonuclease to alter mtDNA heteroplasmy as well as in the potential role of double-strand breaks in mtDNA recombination. For this reason, we chose to express the restriction endonuclease *Pst*I in mammalian mitochondria. The human and mouse mtDNA have two relatively close sites for *Pst*I, which could promote intramolecular recombination and create deletion mutants. *Pst*I could also distinguish between mtDNA haplotypes in rodent cell lines developed in our laboratory, as described below. Therefore, we synthesized a gene coding for the bacterial *Pst*I endonuclease (NCBI P00640) altering the codon usage to optimize expression in mammalian cells. The recoded *Pst*I gene was cloned downstream of a DNA fragment coding for a mitochondrial targeting sequence (Materials and Methods). The final construct was cloned into a mammalian expression vector (Fig. 1A) and transfected into human 293T and mouse NIH 3T3 cells. A polyclonal antibody, raised against the bacterial *Pst*I, was able to detect a polypeptide with a migration that was indistinguishable from the bacterial *Pst*I (Fig. 1B). This observation showed that the polypeptide was expressed and that the mitochondrial targeting sequence was removed from the mature polypeptide. Confocal microscopy showed that the *Pst*I protein co-localized with the mitochondrial marker CMX-ROS Mitotracker™ (Fig. 1C). The antibody also detected nuclear structures in both transfected and untransfected cells (Fig. 1C). The same structures were also observed in 143B cells not subjected to transfection with pMtPstI (not shown).

Transiently transfected 293T cells were analyzed for the presence of mtDNA fragmentation 48 h after transfection with the mitochondrial *Pst*I construct (pMtPstI). The human mtDNA harbors two restriction sites for *Pst*I (at positions 6914

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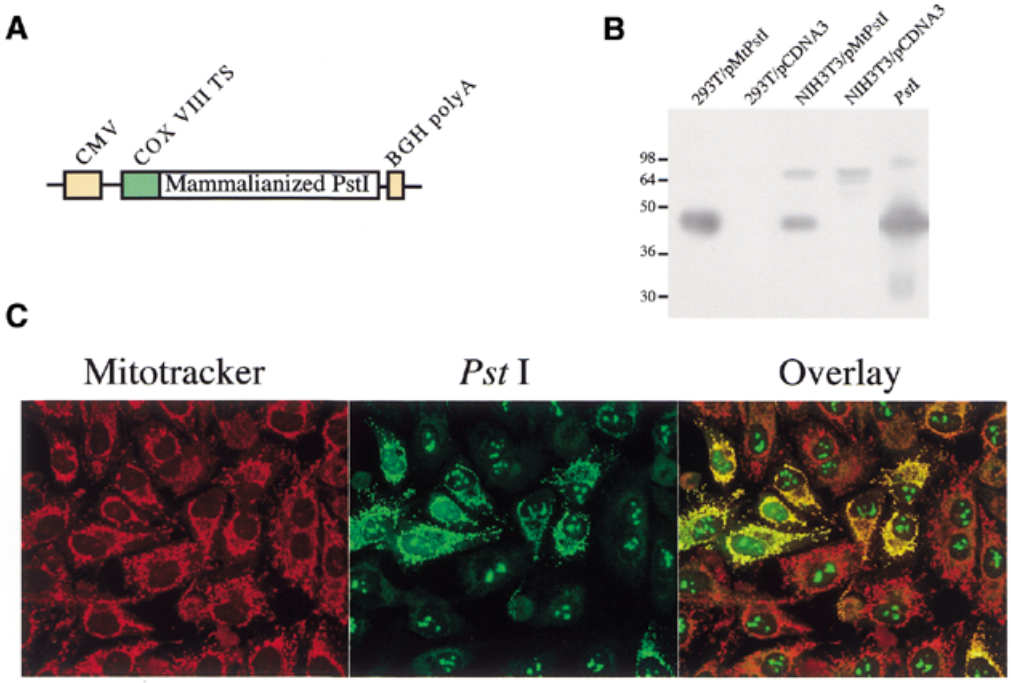

Figure 1. Engineering and expression of a mitochondrial *Pst*I. A gene coding for the *Pseudomonas stuartti Pst*I restriction endonuclease was synthesized *in vitro*, altering the codon usage to optimize expression in mammalian cells. A DNA fragment coding for the COX VIII targeting sequence (TS) was added to the 5′ end of the synthetic *Pst*I gene. This construct was cloned into pCDNA3 containing a CMV promoter (**A**). The final construct was transfected into human 293T and mouse NIH 3T3 cells. Forty-eight hours after transfection the cells were harvested, lysed and analyzed by western blot using an antibody directed against native *Pst*I. Expression of *Pst*I was readily detectable in transfected cells (**B**). The mitochondrial localization of *Pst*I was confirmed by confocal microscopy of HeLa cells transiently transfected with the pMtPstI plasmid. Co-localization of *Pst*I and mitotracker was evident in cells expressing pMtPstI (**C**).

and 9024) (10) (Fig. 2A). Therefore, we expected to observe the release of a 2.1 kb fragment from the mitochondrial genome that could be detected by a specific probe within this region (asterisk in Fig. 2A). We routinely have ~30–40% of 293T cells transiently transfected, what should have been enough to show the presence of a mtDNA fragment. Southern blot analysis showed that most of the mtDNA molecules were present as full-length circles. We could observe the presence of an extremely faint band of 2.1 kb, suggesting that only a very small fraction of the mtDNA pool was readily digested by the mitochondrial *Pst*I (Fig. 2B).

To study the effect of long-term mtDNA exposure to mitochondrial *Pst*I we obtained 143B human osteosarcoma cells stably expressing the mitochondrial *Pst*I gene. Four independent G418-resistant clones were obtained 15 days after pMtPstI transfection. These clones were expanded, tested for *Pst*I expression (Fig. 2C) and analyzed for the presence of intact or rearranged mtDNA. To detect the presence of mtDNA in these cells, we first amplified a fragment corresponding to the ND5 mitochondrial gene (Fig. 2A). The amplified product could be observed in two of the four clones, suggesting that a dramatic loss of mitochondrial genomes occurred in two of the mitochondrial *Pst*I-expressing clones (Fig. 2D). We also searched for the presence of rearranged molecules that could have originated by the recombination of free staggered ends created by *Pst*I digestion. This was performed by the amplification of a DNA fragment using oligonucleotide primers flanking the *Pst*I restriction sites (Fig. 2A). Amplification products were observed only with DNA from the same two clones that showed amplification of the ND5 gene (Fig. 2E). A recombination involving free *Pst*I-digested ends would give rise to amplification products of 200 bp, whereas amplification of the wild-type sequence would give rise to a 2.3 kb amplicon. The two clones that produced an amplicon showed exclusively the 2.3 kb fragment, suggesting that recombination between free *Pst*I-digested sites did not occur at any significant level (Fig. 2E).

Southern blot analysis of the 143B clones stably expressing the mitochondrial *Pst*I confirmed that clones 2 and 4 had lost their mtDNA. The remaining two clones (clones 1 and 3) showed extensive mtDNA degradation observed as a smear as well as discrete low molecular weight bands (Fig. 2F). This was observed when total DNA was digested with either *Pst*I or *Pvu*II. This experiment also showed that the mtDNA molecules in clones 1 and 3 retained their original *Pst*I sites. Oxygen consumption assays confirmed the mtDNA depletion, as no detectable respiration was observed in clones 2 and 4 (not shown).

To test if the mitochondrial *Pst*I could modulate mtDNA heteroplasmy in intact cells, we produced a hybrid cell line containing both mouse and rat mtDNA. The mouse mtDNA harbors two restriction sites for *Pst*I (at positions 8420 and 12 232) (11) whereas the rat mtDNA harbors none. This hybrid cell line was produced by fusing a mouse LM(TK$^-$) to a rat NRK cell as described by Dey *et al.* (12). The original percentage of rat mtDNA in this cell line (termed LMNRK#6)

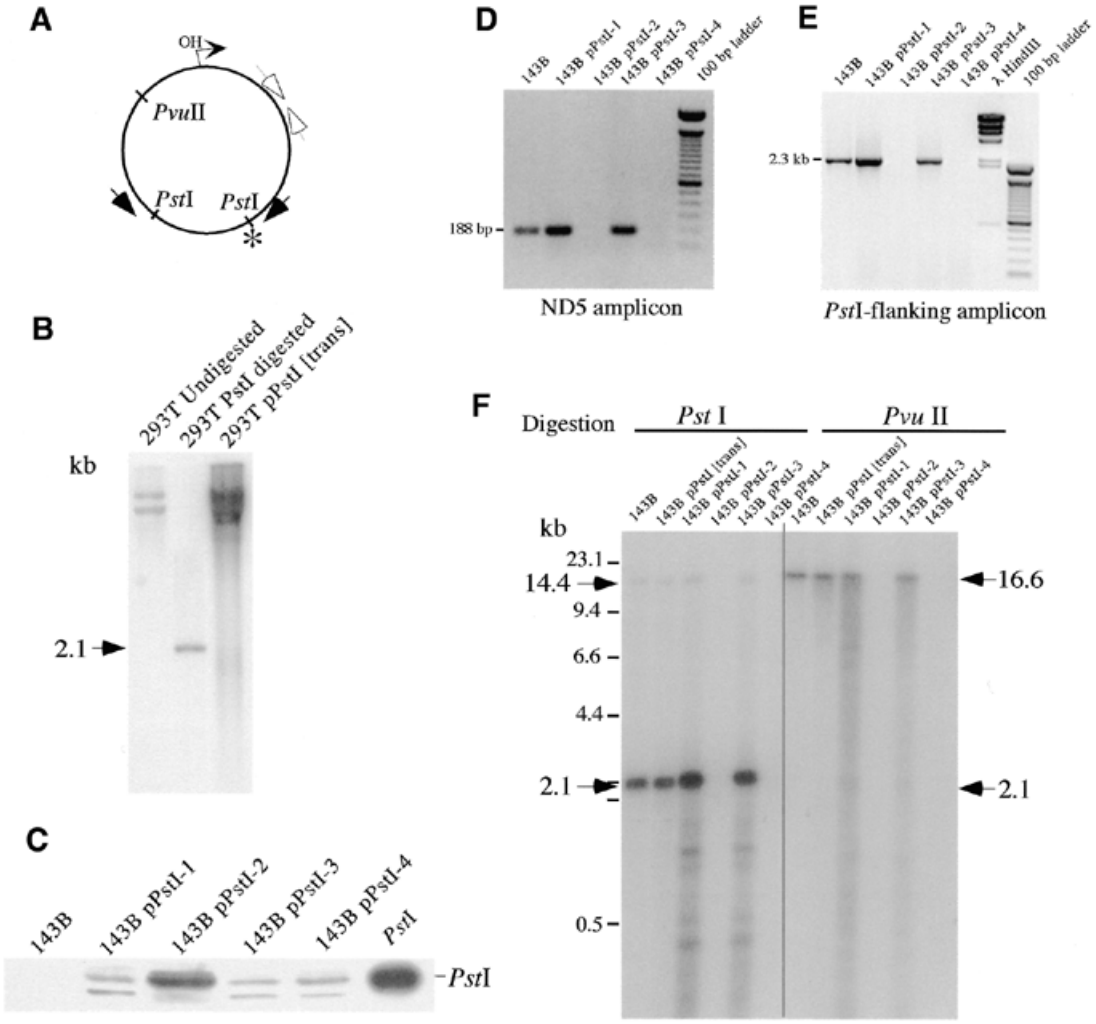

Figure 2. Mitochondrial *Pst*I leads to degradation and depletion of mtDNA. (**A**) The human mtDNA with the approximate location of *Pst*I, *Pvu*II sites and the origin of H-strand replication (D-loop). It also shows the approximate location of oligonucleotide primers used to amplify a region of the ND5 gene (open arrowheads) or a region flanking the *Pst*I sites (black arrowheads). The asterisk shows the position of the probe utilized in the different Southern blot analyses shown in the figure. Human 293T cells were transiently transfected with pMtPstI and their DNA extracted after 48 h. Both *Pst*I-digested and undigested DNA were analyzed by Southern blot using a probe depicted as an asterisk in (A). A very faint band was observed in the 2.1 kb region in the lane corresponding to pMtPstI-transfected DNA (**B**). 143B cells control and stably transfected with pMtPstI were analyzed by western blot using an anti-*Pst*I antiserum raised against the bacterial protein (**C**). MtDNA from these four clones were analyzed by PCR using primers corresponding to the ND5 gene region depicted in (A) (open arrowheads). We were unable to amplify a fragment of DNA from two of the four clones (**D**). A second set of primers was used to amplify across the *Pst*I sites to attempt to detect recombination between the released *Pst*I ends [black arrowheads in (A)]. This analysis confirmed the absence of amplification products from two clones and could not detect recombination events involving the *Pst*I sites (**E**). Southern blot analysis of these clones after digestion with either *Pst*I or *Pvu*II using the same probe shown in (A) (asterisk) showed that mtDNA was fragmented in two of the clones and absent in the remaining two (**F**). Again, no DNA degradation was observed in 143B transiently transfected ([trans]).

was 33%. Because heteroplasmy fluctuations are known to occur during clonal analyses, we transfected LMNRK#6 cells with either the pMtPstI or pCDNA3. Seventy-two hours after transfection, cells were subjected to selection with G418. After 15 days under selection, 12 individual clones were isolated from both pMtPstI and pCDNA3 transfections. PCR/RFLP analyses of mtDNA haplotypes showed that pCDNA3-transfected cells had an average of 25% rat mtDNA (Fig. 3A). Although heteroplasmy fluctuations were observed, a significant number of clones (6/12) had extremely low levels of rat mtDNA, suggesting a bias toward maintaining the mouse mtDNA. This bias may be related to the preferential loss of rat chromosomes in this cell line. On the other hand, clones transfected with pMtPstI had significantly higher levels of rat mtDNA (53%, Fig. 3A and B). However, three of the pMtPstI-transfected clones showed a predominance of mouse mtDNA. Western blot analysis of all clones showed that these three pMtPstI-transfected clones were the only ones with undetectable levels of *Pst*I, demonstrating that mitochondrial *Pst*I expression can effectively modulate mtDNA heteroplasmy in a predictable direction. The difference in percentage rat mtDNA between pCDNA3 and pMtPstI transfections, when the three

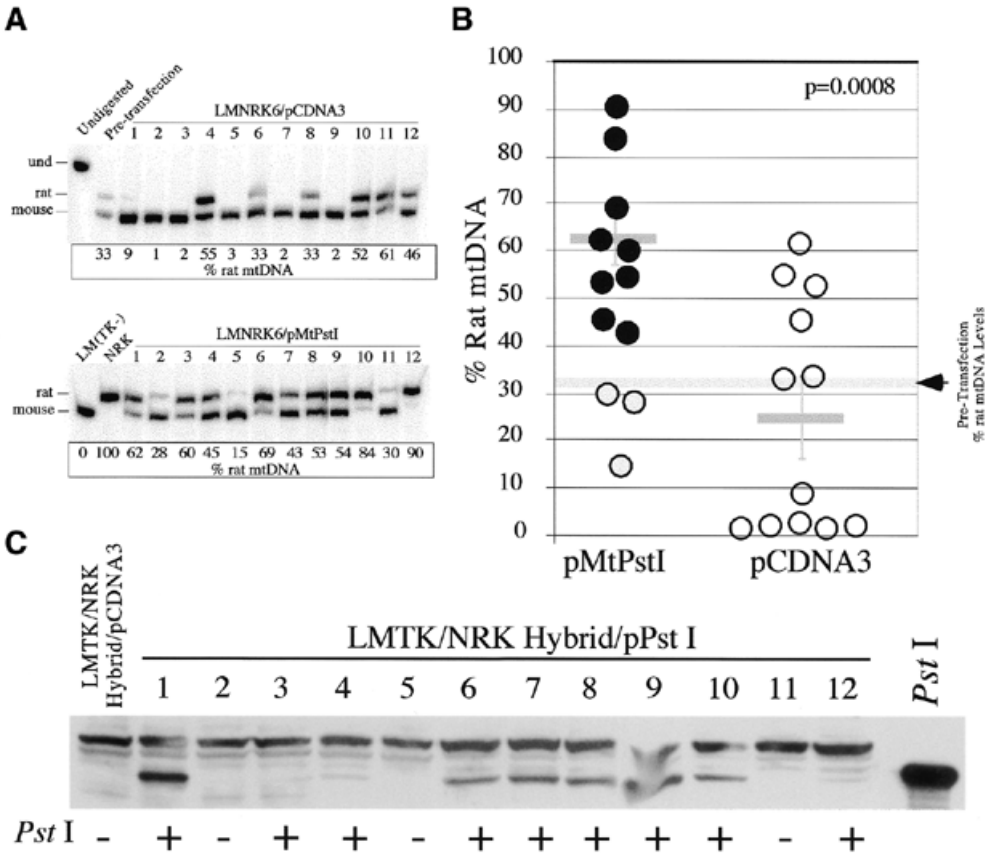

Figure 3. Mitochondrial *Pst*I can modulate mtDNA heteroplasmy in intact cells. A hybrid cell (LMNRK#6) containing mouse and rat mtDNA was transfected with pCDNA3 or pMtPstI. Twelve G418-resistant clones from each transfection were analyzed for the percentage of rat mtDNA by PCR/RFLP (last-cycle hot) (**A**). pCDNA3-transfected cells had predominantly mouse mtDNA, whereas the pMtPstI-transfected cells had predominantly rat mtDNA. This difference was highly significant [$P = 0.008$ (**B**)]. Three clones transfected with pMtPstI had high levels of mouse mtDNA [(B) gray circles]. Western blot analysis showed that these three clones were the only ones with undetectable levels of *Pst*I protein (**C**).

non-expressing clones were removed from the pMtPstI group, was highly significant ($P = 0.0008$). The mean percent rat mtDNA was also increased to 62%. We did not observe a direct correlation between the levels of *Pst*I expression and the percentage of rat mtDNA, possibly because heteroplasmy was not stable in these cells and showed some fluctuations, as observed in the pCDNA3-transfected cells.

DISCUSSION

The ability to manipulate the mitochondrial genome in mammalian cells would provide a powerful approach both to create mutants and modulate heteroplasmy. Unfortunately, the location of the mtDNA and its association with mitochondrial proteins and membranes make this task extremely difficult. Although modification of mitochondrial genes in yeast have been accomplished (13), no such successes have followed with mammalian cells. Because of this limitation, the field of mammalian mitochondrial genetics has relied on naturally occurring mutations for the study of altered genes. Moreover, the association of mtDNA mutations with human diseases have underscored the need for an approach that could lead to the manipulation of mitochondrial genomes.

The protective effect of low levels of wild-type mtDNA in patients with mtDNA mutations was first observed more than a decade ago (3,14,15). Pathogenic mtDNA mutations are usually heteroplasmic, and are associated with deleterious phenotypic changes only if their levels are extremely high (5,8,16). Therefore, the ability to manipulate mtDNA heteroplasmy could lead to therapeutic approaches to most mitochondrial diseases. Such approaches are being developed, including the use of antisense peptide nucleic acids (9). However, these have not yet been shown to work in intact cells. Taivassalo *et al.* (17) showed that patients performing concentric exercise training repopulated damaged muscle cells with regenerating satellite cells that contained lower levels of mutated mtDNA, an alternative approach that also operates by heteroplasmy shifting.

We explored the usefulness of expressing a polypeptide that could target specific mtDNA haplotypes. A bacterial restriction endonuclease has the required specificity to perform such a task. Although the expression of bacterial genes in mammalian cells have the potential to exert desirable biochemical

functions, translational codon bias can be a difficult obstacle to overcome in order to obtain high levels of expression. Because of this, we chose to synthesize a gene coding for the *Providencia stuartii Pst*I endonuclease that was optimized for translation in mammalian ribosomes. We chose this gene because we were also interested in the mechanisms associated with mtDNA recombination. The human (and the mouse) mtDNA has two relatively close sites for *Pst*I, which could recombine and in theory create deletion mutants.

The modified *Pst*I gene, engineered to have its polypeptide product targeted to mitochondria, was efficiently expressed in human and mouse cells and was correctly targeted to mitochondria. We did not observe any change in morphological or growth features associated with mitochondrial *Pst*I expression. Mitochondrial targeting was efficient, and we could not detect *Pst*I in the nucleus. The expression of this protein in mitochondria had an unequivocal biological effect, namely the degradation of human mtDNA, in some cases leading to its complete loss. It is interesting to note that cleaved mtDNA fragments seemed to be rapidly degraded by mitochondrial nucleases, mostly by exonucleases as we observed a smeary pattern of degradation. Discrete bands were clearer when the DNA was digested with *Pst*I, suggesting that many fragmented molecules had one common end, possibly one of the *Pst*I sites. This pattern of fragmentation contrasts with the Southern blot pattern of yeast mtDNA exposed to *Eco*RI targeted to mitochondria. Even though these cells were exposed for a shorter period of time to the endonuclease, the predicted fragmentation pattern was observed (18). It is also possible that mtDNA molecules are protected by the nucleoid structure (19) and became exposed to *Pst*I only during specific periods (e.g. certain replicative or organelle division stages), but as soon as a endonucleolytic cleavage occurs, the molecule is rapidly degraded. The absence of recombined *Pst*I sites suggested that these staggered ends were not available for long periods of time to engage in recombination/repair. However, recombined molecules would still be susceptible to *Pst*I degradation, unless the site was somehow destructed. Therefore, the fact that we could not observe re-ligation of *Pst*I sites does not exclude the possibility that these sites could be re-joined if *Pst*I was not present, as observed in yeast (18).

We showed that *Pst*I could effectively reduce the levels of a mtDNA haplotype harboring *Pst*I sites. In a heteroplasmic environment, a mtDNA haplotype lacking *Pst*I sites was preferentially maintained, causing a significant shift in heteroplasmy. Although there was a highly significant correlation between the presence of *Pst*I and the percent of rat mtDNA ($P = 0.0008$), we did not observe a strict correlation between the relative levels of *Pst*I expression and the percentage of rat mtDNA. However, none of the expressing clones showed an accumulation of mouse mtDNA, the most common outcome of pCDNA3-transfected cells (6/12 of pCDNA3-transfected clones). This latter observation can be explained by the fact that chromosomal changes in these hybrid cells may confer a preferential maintenance to different haplotypes. In fact, although most of the pCDNA3-transfected clones showed a strong bias towards maintaining the mouse mtDNA, a few clones showed an accumulation of rat mtDNA. We believe that, even though this fluctuation would prevent a strict correlation between *Pst*I levels and rat mtDNA percentage, it would make it harder to obtain clones with predominantly rat mtDNA as observed in the pMtPstI-transfected cells.

The usefulness of mitochondrially targeted restriction endonucleases in modulating mtDNA heteroplasmy is limited by the presence of an appropriate restriction site. However, a mitochondrial disease caused by a T→G transversion in the mitochondrial ATP6 gene (at mtDNA position 8399) creates a unique *Sma*I–*Xma*I site that could be the target for such approach. This mutation has been associated with neuropathy, ataxia and retinitis pigmentosa (NARP) and maternal inherited leigh syndrome (MILS) (2,5,20,21). In most patients, the mutation is heteroplasmic and the severe MILS phenotype differs from the milder NARP by the presence of relatively higher levels of wild-type mtDNA. Family members with mtDNA harboring the T8399G mutation in less than 60–90% of their mtDNA can be completely asymptomatic (5,16,21). Therefore, the approach described here may be applicable to genetic therapy of a sub-group of patients with mitochondrial diseases. Potential problems associated with the expression of a bacterial protein in human mitochondria are still unknown, but inducible promoters may help minimize this problem.

In conclusion, we believe that mitochondrially targeted restriction endonucleases can provide unique tools to modulate mtDNA heteroplasmy, with applications to both basic research and genetic therapy.

MATERIALS AND METHODS

Construction of a mitochondrial *Pst*I gene

A synthetic gene coding for the *Pst*I restriction endonuclease (NCBI P00640) was synthesized by IDT Technologies (Coralville, IA). The gene was constructed with a codon bias towards mammalian ribosome usage. The following codons were used: Ala (GCC), Arg (CGC), Asn (AAC), Asp (GAC), Cys (TGC), Gln (CAG), Glu (GAG), Gly (GGC), His (CAC), Ile (ATC), Leu (CTG), Lys (AAG), Met (ATG), Phe (TTC), Pro (CCC), Ser (TCC), Thr (ACC), Trp (TGG), Tyr (TAC) and Val (GTG). We included a unique *Hin*dIII site flanking the methionine start codon for cloning downstream of a cytochrome oxidase subunit VIII mitochondria targeting sequence (22). The final construct was cloned into *Eco*RI–*Xba*I sites of pCDNA3 (Invitrogen, Carlsbad, CA), sequence verified and named pMtPstI.

Cell lines and culture conditions

Human osteosarcoma 143B, human embryonal kidney 293T, mouse LM(TK), NIH 3T3 and rat NRK (normal rat kidney) were obtained from the American Type Culture Collection (ATCC CRL-8303, CCL1.3, CRL1658 and CRL6509, respectively). HeLa cells were also used for co-localization studies. Cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml sodium pyruvate and 50 µg/ml uridine. The heteroplasmic hybrid cell LMNRK#6 was obtained as described previously and characterized for nuclear and mitochondrial markers (12).

Mitochondrial DNA analyses

Human cell lines expressing the mitochondrial *Pst*I were analyzed by PCR and Southern blot. For Southern blot analysis 10 µg of total DNA from each cell line was digested with *Pst*I or *Pvu*II, electrophoresed through a 0.8% agarose gel, and transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). For a probe, we used [α-^{32}P]dCTP-labeled PCR fragment corresponding to the mtDNA small region between *Pst*I sites (Fig. 2A). The presence of mtDNA in pMtPstI-transfected cells was also investigated by PCR using the following two sets of primers: ND5 (12 400–12 422F and 12 588–12 566B) and flanking *Pst*I sites (6800–6823F and 9110–9087B).

To determine mtDNA haplotypes in rodent cells, we amplified a mtDNA region containing the *COX I* gene, using primers Rod-F [corresponding to nucleotides 5559–5578 in the mouse mtDNA (11) and nucleotides 5540–5559 in the rat mtDNA (23)] and Rod-B (nucleotides 6259–6278 in the mouse mtDNA and nucleotides 6240–6259 in the rat mtDNA). We labeled the PCR products with [α-^{32}P]dCTP in the last cycle of the reaction to avoid the detection of heteroduplexes (i.e. the formation of duplexes containing one strand from mouse and one from rat). The details of this procedure, termed 'last cycle hot PCR', have been reported previously by Moraes *et al.* (24). The radiolabeled fragments were digested with *Msp*I, electrophoresed through a 12% polyacrylamide gel and analyzed in a Cyclone Phosphor Storage system (Packard Instruments, Meriden, CT).

DNA transfections

The cell line LMNRK#6 was plated at ~70% confluence in six-well tissue culture plates (Costar, Cambridge, MA) and transfected (with pMtPstI or pCDNA3) using the cationic lipid LipofectAMINE Plus™ (Life Technologies) as described by the manufacturer, using 1.5 µg of plasmid DNA, 4 µl of LipofectAMINE reagent and 6 µl of PLUS reagent. After 72 h, cells were selected in medium containing 1 mg/ml Geneticin™ (G418; Life Technologies). Stable transfectants were isolated 15 days after selection was applied. HeLa cells were transfected by the same procedure.

Immunodetection of mitochondrial *Pst*I

Approximately 20 µg of total cell proteins were resolved by SDS–PAGE (15% gels) and transferred onto polyvinylidene difluoride membranes (NEN Life Science Products, Carlsbad, CA). Blots were blocked with 5% milk and probed with an anti-*Pst*I polyclonal antibody. The antibody was produced by injecting a purified *Pst*I protein (a kind gift from New England Biolabs, Beverly, MA) with Freund's complete adjuvant in rabbits. The antibody production was performed by Pocono Farms Inc. (Canadensis, PA). After washes, the membrane was treated with a secondary anti-rabbit antibody conjugated to horseradish peroxidase. Detection was performed using a Phototope-horseradish Peroxidase Western Blot Detection Kit (New England Biolabs).

Transiently transfected HeLa cells were used for subcellular localization of *Pst*I. Cells were grown on coverslips to 60% confluency and transfected as described above. After 48 h cells were incubated with 200 nM CMX-ROS Mitotracker™ (Molecular Probes, Eugene, OR) for 20 min. The cells were washed with PBS and fixed with 4% paraformaldehyde. After washes, the preparations were permeabilized with –20°C methanol for 5 min and incubated with the polyclonal anti-*Pst*I antiserum (1:100) for 12 h at 4°C. After washes with PBS the coverslips were incubated with anti-rabbit FITC (1:200, Sigma) for 2 h. After washes, the coverslips were mounted with ProLong™ (Molecular Probes) and imaged on a confocal Axiovert 100M microscope with a LSM510 scanning module (Carl Zeiss).

Computer and statistical analysis

Experimental data were analyzed using the Excel Statistical Package (Microsoft). The Student's *t*-test was used for comparison of two groups. Results are expressed as mean ± SEM.

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REFERENCES

1. Goto, Y., Nonaka, I. and Horai, S. (1990) A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature*, **348**, 651–653.
2. Holt, I.J., Harding, A.E., Petty, R.K. and Morgan-Hughes, J.A. (1990) A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. *Am. J. Hum. Genet.*, **46**, 428–433.
3. Shoffner, J.M., Lott, M.T., Lezza, A.M., Seibel, P., Ballinger, S.W. and Wallace, D.C. (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. *Cell*, **61**, 931–937.
4. Moraes, C.T., Ciacci, F., Silvestri, G., Shanske, S., Sciacco, M., Hirano, M., Schon, E.A., Bonilla, E. and DiMauro, S. (1993) Atypical clinical presentations associated with the MELAS mutation at position 3243 of human mitochondrial DNA. *Neuromusc. Disord.*, **3**, 43–50.
5. Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J.T., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. and Robinson, B.H. (1992) Heteroplasmic mtDNA mutation (T–G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. *Am. J. Hum. Genet.*, **50**, 852–858.
6. Schon, E.A., Bonilla, E. and DiMauro, S. (1997) Mitochondrial DNA mutations and pathogenesis. *J. Bioenerg. Biomembr.*, **29**, 131–149.
7. Sciacco, M., Bonilla, E., Schon, E.A., DiMauro, S. and Moraes, C.T. (1994) Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. *Hum. Mol. Genet.*, **3**, 13–19.
8. Petruzzella, V., Moraes, C.T., Sano, M.C., Bonilla, E., DiMauro, S. and Schon, E.A. (1994) Extremely high levels of mutant mtDNAs co-localize with cytochrome c oxidase-negative ragged-red fibers in patients harboring a point mutation at nt 3243. *Hum. Mol. Genet.*, **3**, 449–454.
9. Taylor, R.W., Chinnery, P.F., Turnbull, D.M. and Lightowlers, R.N. (2000) *In vitro* genetic modification of mitochondrial function. *Hum. Reprod.*, **15** (Suppl. 2), 79–85.
10. Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F. *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.
11. Bibb, M.J., Van Etten, R.A., Wright, C.T., Walberg, M.W. and Clayton, D.A. (1981) Sequence and gene organization of mouse mitochondrial DNA. *Cell*, **26**, 167–180.
12. Dey, R., Barrientos, A. and Moraes, C.T. (2000) Functional constraints of nuclear-mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *J. Biol. Chem.*, **275**, 31520–31527.

13. Butow, R.A., Henke, R.M., Moran, J.V., Belcher, S.M. and Perlman, P.S. (1996) Transformation of *Saccharomyces cerevisiae* mitochondria using the biolistic gun. *Methods Enzymol.*, **264**, 265–278.
14. Boulet, L., Karpati, G. and Shoubridge, E.A. (1992) Distribution and threshold expression of the tRNA(Lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red fibers (MERRF). *Am. J. Hum. Genet.*, **51**, 1187–1200.
15. Moraes, C.T., Ricci, E., Petruzzella, V., Shanske, S., DiMauro, S., Schon, E.A. and Bonilla, E. (1992) Molecular analysis of the muscle pathology associated with mitochondrial DNA deletions. *Nat. Genet.*, **1**, 359–367.
16. Tsao, C.Y., Mendell, J.R. and Bartholomew, D. (2001) High mitochondrial DNA T8993G mutation (<90%) without typical features of Leigh's and NARP syndromes. *J. Child Neurol.*, **16**, 533–535.
17. Taivassalo, T., Fu, K., Johns, T., Arnold, D., Karpati, G. and Shoubridge, E.A. (1999) Gene shifting: a novel therapy for mitochondrial myopathy. *Hum. Mol. Genet.*, **8**, 1047–1052.
18. Donahue, S.L., Corner, B.E., Bordone, L. and Campbell, C. (2001) Mitochondrial DNA ligase function in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **29**, 1582–1589.
19. Jacobs, H.T., Lehtinen, S.K. and Spelbrink, J.N. (2000) No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. *Bioessays*, **22**, 564–572.
20. Tatuch, Y., Pagon, R.A., Vlcek, B., Roberts, R., Korson, M. and Robinson, B.H. (1994) The 8993 mtDNA mutation: heteroplasmy and clinical presentation in three families. *Eur. J. Hum. Genet.*, **2**, 35–43.
21. Uziel, G., Moroni, I., Lamantea, E., Fratta, G.M., Ciceri, E., Carrara, F. and Zeviani, M. (1997) Mitochondrial disease associated with the T8993G mutation of the mitochondrial ATPase 6 gene: a clinical, biochemical and molecular study in six families. *J. Neurol. Neurosurg. Psychiatry*, **63**, 16–22.
22. Rizzuto, R., Brini, M., Bastianutto, C., Marsault, R. and Pozzan, T. (1995) Photoprotein-mediated measurement of calcium ion concentration in mitochondria of living cells. *Methods Enzymol.*, **260**, 417–428.
23. Gadaleta, G., Pepe, G., De Candia, G., Quagliariello, C., Sbisa, E. and Saccone, C. (1989) The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. *J. Mol. Evol.*, **28**, 497–516.
24. Moraes, C.T., Ricci, E., Bonilla, E., DiMauro, S. and Schon, E.A. (1992) The mitochondrial tRNA(Leu(UUR)) mutation in mitochondrial encephalomyopathy, lactic acidosis and strokelike episodes (MELAS): genetic, biochemical and morphological correlations in skeletal muscle. *Am. J. Hum. Genet.*, **50**, 934–949.