Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice

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We report a method for introducing mtDNA mutations into the mouse female germ line by means of embryonic stem (ES) cell cybrids. Mitochondria were recovered from the brain of a NZB mouse by fusion of synaptosomes to a mtDNA-deficient (ρ−) cell line. These cybrids were enucleated and the cytoplasts were electrotossed to rhodamine-6G (R-6G)-treated female ES cells. The resulting ES cell cybrids permitted transmission of the NZB mtDNAs through the mouse maternal lineage for three generations. Similarly, mtDNAs from a partially respiratory-deficient chloramphenicol-resistant (CAP3) cell line were also introduced into female chimeric mice and were transmitted to the progeny. CAP3 chimeric mice developed a variety of ocular abnormalities, including congenital cataracts, decreased retinal function, and hamaratomas of the optic nerve. The germ-line transmission of the CAP3 mutation resulted in animals with growth retardation, myopathy, dilated cardiomyopathy, and perinatal or in utero lethality. Skeletal and heart muscle mitochondria of the CAP3 mice were enlarged and atypical with inclusions. This mouse ES cell cybrid approach now provides the means to generate a wide variety of mouse models of mitochondrial disease.

The use of embryonic stem (ES) cells to generate transgenic animals has revolutionized the study of gene action in vivo as well as provided animal models for studying pathophysiology and therapies. Strategies for modification of the nuclear genome have become sophisticated, allowing not only for the inactivation of a resident gene, but also for the introduction of more subtle genetic changes, such as the modification of a single base within the genome or modulation of gene expression. Recently, transgenic mouse models for mitochondrial disease have been generated by modifying nuclear-encoded mitochondrial genes (1–6). Although these mice exhibit certain phenotypes characteristic of mitochondrial disease, they cannot recapitulate the unique genetic features of mtDNA mutations, such as maternal inheritance, heteroplasmacy, and bioenergetic threshold expression, which are central to understanding mitochondrial diseases (7, 8).

Initial efforts to introduce mtDNA mutations into whole-animal systems have focused on the mtDNAs from cultured mouse cells resistant to the mitochondrial ribosome inhibitor, chloramphenicol (CAP3). CAP3 resistance in a number of mouse cell lines results from a T to C transition at nucleotide pair (np) 2433 (m.2433T>C) near the 3′ end of the 16S ribosomal RNA (rRNA) gene (9). Although chimeric mice have been obtained with varying levels of CAP-resistant (CAP8) and wild-type mtDNAs, a state known as heteroplasmacy, transmission through the maternal germ line has not been achieved until recently (10–13).† This barrier has been overcome in our laboratory by fusion of female mouse ES cells to cytoplasts carrying mutant mtDNAs, and then using the mtDNA mutant ES cells to generate chimeric female mice that transmit the mtDNA mutations to subsequent generations (11).††

Microinjection of cytoplast containing foreign mtDNA into oocytes has resulted in chimeric embryos, but the mutant DNA appears to have been rapidly lost by segregation in early preimplantation development (14). Heteroplasmic mice also have been achieved by fusion of cytoplasts to mouse one cell embryos, permitting introduction of mtDNAs with either naturally occurring polymorphisms (15, 16) or deletions (17).

To demonstrate the versatility of the female ES cell cybrid transfer technique for producing transgenic mice with mutant mtDNAs, we now report the introduction and maternal transmission of mtDNAs harboring polyomavirus variants or deleterious mutations. This has permitted us to make a more detailed analysis of the transmission of both heteroplasmic and homoplasmic mutations and to assess their in vivo consequences. This experimental approach will facilitate production of a variety of mouse models of mtDNA disease.

Methods

ES Cell Culture and Cybrid Preparation. The mouse female CC9.3.1 ES cell line and its derivatives were cultured on mitomycin C-inactivated SNL76/7 feeder cells (18). CC9.3.1 cells were derived from a 129SvEv-GpiF embryo (A. Bradley, personal communication). Pilot experiments demonstrated that this line would contribute to the female germ line and produce normal fertile females.

To generate female ES cell cybrids, CC9.3.1 cells were plated at a density of 2 × 106 per cm2, and 24 h later were treated with 0.75 to 1.25 μg/ml of the mitochondrial toxin R-6G (dissolved in 3% ethanol) for up to 72 h. During the R-6G treatment period, the media were supplemented with 1 mM pyruvate and 50 μg/ml uridine. The cells were subsequently washed with PBS and resuspended in 0.3 M mannitol fusion medium for electrofusion.

To recover mtDNAs from the NZB mouse strain, brain synaptosomes were prepared and fused to the mouse ρ−, thymidine kinase-deficient (TK−). L cell line LMEB4 (19). LMEB4 (mtNZB) cybrids were selected in 30 μg/ml BrdUrd in the absence of uridine.

To generate donor cytoplasts for the ES cell fusions, either 4 × 107 LMEB4(mtNZB) or 4 × 106 TK− CAP8 cells (20) were harvested, resuspended in 12.5% Ficoll and 20 μg/ml cytochalasin B, layered onto Ficoll step gradients, and enucleated by centrifugation at 77,000 × g for 1 h at 31°C (21). The cytoplast band was recovered and washed with DMEM, then washed with 0.3 M mannitol fusion medium at pH 7.2. A total of 1 × 107 cytoplasts were mixed with 1 × 106 R-6G-treated ES cells and fused by electric current delivered as a 20-s alignment at 50-V alternating current (AC), followed by two 20-msec pulses of 800-V direct current (DC) (2.5 kV/cm) without a postfusion AC field using a BTX-Genetronics ECM200 (San Diego). After a 2-min

Abbreviations: ES, embryonic stem; CAP, chloramphenicol; np, nucleotide pair; CAP3, CAP-resistant; TK−, thymidine kinase-deficient.

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recovery time, the cells were plated onto fresh feeders and continued to receive pyruvate and uridine supplementation for 24 h. The cells then were exposed to selection in DMEM with hypoxanthine/aminopterin/thymidine (HAT), without further pyruvate or uridine supplementation. Individual colonies were visible 5 days after fusion and were picked for further expansion and analysis at days 7–9.

**Production and Genotyping of Chimeric and Transgenic Mice.** Cells that had evidence of the donor cell mtDNA were injected into day 3.5 postcoitum (p.c.) C57BL/6J (B6) blastocysts and the embryos were transferred into day 2.5 p.c. pseudopregnant B6CBAF1 females (22). All experiments using animals were conducted in accordance with the procedures of the Emory University School of Medicine Division of Animal Resources as reviewed by the Emory University Institutional Animal Care and Use Committee and the Research and Education Institute at Harbor-UCLA.

To detect the NZB mtDNA in cybrids and transgenic mice, we took advantage of a naturally occurring *BamHI* polymorphism. The primers CGGCCCATTCGGCGTTATTC and AGGTTGAGTA-GAGTGAAGGA were used to PCR-amplify a 1,127-bp fragment over 30 cycles using successive 30-s denaturation (94°C), annealing (55°C), and extension (72°C) steps with *Taq* polymerase (Roche Applied Science). The NZB mtDNA lacks the *BamHI* recognition site at np 4277 such that *BamHI* digestion produces 502-bp fragments. N is the NZB mtDNA. W is the common haplotype (wild type) mtDNA from the 129SvEv ES cell line CC9.3.1. N is the NZB mtDNA.

**Pathological Analysis.** Tissues were fixed in buffered formalin, embedded in paraffin, sectioned at 6–7 μm, and stained with hematoxylin and eosin. Eyes were removed, immersed in cold 1% paraformaldehyde and 2% glutaraldehyde fixative, washed in Karnovsky buffer, embedded in methacylate histoestores, and sectioned for staining (24).

**Electroretinography.** After 2 h of dark adaptation, mice were anesthetized by i.p. injection of 15 μg/g Ketamine and 7 μg/g xylazine. Electroretinograms (ERGs) were recorded from the corneal surface of one eye after pupil dilation (1% atropine sulfate) using a gold loop corneal electrode together with a mouth reference and tail ground electrode. Stimuli were produced with a Grass Photic Stimulator (PS33 Plus; Grass Instruments, Quincy, MA) affixed to the outside of a highly reflective Ganzfeld dome. Response signals were amplified (CP511 AC amplifier; Grass Instruments), digitized (PCI-1200; National Instruments, Austin, TX) and computer-analyzed (24).

Rod responses were recorded to short-wavelength (Wratten 47A; λ_max = 470 nm) flashes of light over a 3.0-log unit range of intensities (in 0.6-log unit steps) up to the maximum allowable by the photic stimulator (0.668 cd-s/m^2). Mixed rod and cone responses were obtained with white flashes incremented in 0.3-log unit steps up to the maximum allowable (8.46 cd-s/m^2). Cone dominated responses were recorded in response to an intensity series of white flashes (in 0.3 steps) on a rod-saturating background (32 cd/m^2).

To analyze the rod-mediated responses to blue light, the intensity versus b-wave amplitude response curves were fitted to the Naka-Rushton function, V/V_max = I/(I + k), where V = rod peak-to-peak amplitude, V_max = maximum rod amplitude, I = retinal illuminance, and k = retinal illuminance at half amplitude. The rod ERG threshold (2.0 μV criterion) was derived
from the Naka-Rushton parameters, such that log threshold = log k + 0.3 - log(V̄max) - 2. Similarly, the cone ERG threshold (2.0 μV criterion) was derived from the fit of a linear regression (slope constraint of 1.0) to the intensity-response series obtained to white light on a rod-saturating background.

In these ophthalmological studies, we used 129S4 mice as controls. These differ slightly from the 129SvEv-derived CC9.3.1 cells used to make the chimeras. However, the 129Sv ERG data are representative of that seen for related inbred strains and hence should provide a valid comparison.

Results

Transfer of NZB mtDNA from Brain Tissue into ES Cells. To develop a reliable procedure for introducing exogenous mtDNAs into the mouse germ line, we took advantage of the naturally occurring mtDNA polymorphisms that differentiate mtDNAs of the NZB mouse strain from mtDNAs of most inbred strains that harbor a “common mtDNA haplotype.” Inbred mouse strains with the

common haplotype (129/Sv, B6, C3H, BALB/c) are thought to have been derived from a single female lineage (25). The NZB and the common haplotype mtDNAs differ by 108 nt, and these polymorphic differences have been used to monitor the segregation of heteroplasmic populations of mtDNAs created by the fusion of cytoplasts from NZB/BINJ with BALB/c single-cell embryos (15, 16).

To establish the NZB mtDNA in cultured mouse L cells, we homogenized the brain of a NZB/BINJ mouse and isolated the synaptosomes by Percoll density gradient centrifugation. The resulting synaptosomes contained one to several mitochondria (19). These synaptosomes were fused by electric field pulse to the ρ0 cell line LMEB4 (19). Synaptosome cybrids having the LMEB4 nucleus and the NZB mtDNA [LMEB4 (mtNZB)] were isolated by selection in medium containing BrdUrd but lacking uridine. The LMEB4 (mtNZB) cybrids were then enucleated and the cytoplasts were electrofused to the mouse ES cell line, CC9.3.1.

The CC9.3.1 ES cell line was selected after screening several candidate ES cell lines for the presence or absence of a Y chromosome by PCR of Sry (26). CC9.3.1 has a karyotype of 39X, with a normal chromosome G banding pattern (data not shown). Unlike the 45,X Turner syndrome in humans, the 39X karyotype in mouse gives rise to viable and fertile females. Hence, the CC9.3.1 (mtNZB) cybrids have the potential for transmitting the NZB mtDNA through oocytes of the female germ line.

Before cytoplast fusion, the CC9.3.1 cells were depleted of their resident mitochondria by treatment with R-6G. R-6G is a lipophilic mitochondrial poison that inhibits oxidative phosphorylation (OXPHOS), causing an irreversible collapse of
the mitochondrial membrane potential rendering the cells respiration-deficient (23, 27).

The R-6G-treated CC9.3.1 ES cells were electrofused to LMEB4(mtNZB) cytoplasts and the ES cell cybrids were selected in HAT medium lacking uridine and pyruvate. Individual CC9.3.1 (mtNZB) cybrids were picked and expanded for DNA analysis (28), and the NZB origin of the mtDNA was confirmed by using the polymorphism at np 4277 in the mtDNA ND1 gene. Most cybrids harbored predominantly NZB mtDNAs (Fig. 1).

**Figure 8.** Dilated cardiomyopathy in a CAPR neonate on the first day of life. (A) A wild-type B6,129S4 control heart. (B) The CAPR neonatal heart.

The CC9.3.1 (mtNZB) ES cell cybrids were injected into B6 blastocysts, and chimeric animals were obtained. Females were mated with B6 males having mtDNAs with the common haplotype and a founder female with the ES cell-encoded agouti coat color was obtained. Mitochondrial genotyping of this animal revealed that she was heteroplasmic, harboring both the NZB and the common haplotype mtDNAs (Fig. 2). Mating this individual with a different B6 male resulted in 7 and 10 offspring, respectively, all of which were heteroplasmic for the NZB and common haplotype mtDNAs. Mating of one of the progeny females with a B6 male again resulted in the maternal transmission of the heteroplasmic mtDNAs. In contrast, no transmission of the heteroplasmic NZB mtDNA was observed in offspring after mating of a heteroplasmic male with two different B6 females (Fig. 2). Analysis of mtDNA in multiple tissues derived from all three germ layers (ectoderm, mesoderm, and endoderm) revealed heteroplasmacy throughout the animals. None of the heteroplasmic mice had an overtly abnormal phenotype. Thus, the NZB mtDNA, recovered from the clones containing NZB mtDNA. A typical nuclear cataract of a CAPR mouse together with a control, as revealed by slit lamp biomicroscopy, is shown in Fig. 3.

To determine whether the presence of CAPR cells affected retinal function, we performed ERGs on 19 CAPR chimeric, CB11-derived, male mice with substantial agouti coat color. These were compared with six 129S4 controls. Representative ERGs for the CAPR chimeric and 129S4 control mice are shown in Fig. 4A. ERG b-wave amplitudes for the chimeric mice were reduced 50–60% with respect to the controls, with both rods and cones being similarly affected (Fig. 4B). The timing of peak responses was similar for the two groups of mice, although there was a trend for prolonged latencies for the CAPR chimeric mice.

Further characterization of the intensity-response series of both rods and cones, the blue and white flash series were fitted to the Naka-Rushton function. For the rods (blue-intensity series) the maximum saturated b-wave response (Vmax) of the CAPR chimeras was an average of 0.40 log units smaller than that of the 129S4 control mice; the rod ERG threshold intensity was elevated by 0.51 log units in the CAPR chimeric mice, and the retinal illuminance at half amplitude (k) was elevated by 0.003 log units. Similarly, the cone ERG threshold intensity of the CAPR chimeras was elevated 0.37 log units as compared with the control 129S4 mice.

Although both a- and b-waves were decreased in the CAPR chimeric mice, there was a generally greater deficit in the b-wave. To explore this further, the a- to b-wave amplitude ratio, b/a, was calculated for each mouse eye. The b/a ratio (±1 SD) was 2.6 ± 0.4 for the CAPR chimeras and 3.6 ± 0.7 for the 129S4 controls. Hence, there is a marked decrease in the b-wave amplitude, indicating that the CAPR chimeras have a marked reduction in both rod and cone function.

**Histology of CAPR Mutants.** The light microscopy retinal histology was normal in the CAPR chimeric CB11 mice, and no evidence of...
retinal degeneration was observed despite the ERG amplitude loss of rods and cones. The pigment epithelium (PE) showed vacuoles throughout on all CAPR chimeric specimens examined (Fig. 5A). However, the most striking abnormality was hamartomatous-like changes of the optic nervehead, whose substance protrudes into the intraocular space (Fig. 5C). A gliotic membrane emanating from the surface of the optic nervehead covered the inner retinal surface (Fig. 5C).

Germ-Line Transmission and Lethality of the CAPR Mutation. Chimeric females generated from two independent CC9.3.1 (mt501-1) cybrid clones transmitted the CAPR mutation to their progeny. The genotypes of the first four mice born harboring the CAPR mutation in all of the cells of the body are shown in Fig. 6. Both homoplasmic (Fig. 6, 1-1 and 2-1) and heteroplasmic (Fig. 6, 3-1 and 3-2) CAPR mice were born. Of the two heteroplasmic pups, pup 3-1 harbored 44% and pup 3-2 harbored 52% CAPR mtDNAs, as measured in tail tissue biopsy.

The mutant mice showed marked muscle degeneration at birth. The average weight of the newborn homoplasmic mutant pups was 0.9 g compared with 1.4 g for mice born in our facility with identical nuclear backgrounds. Moreover, with the exception of pup 2-1, all mice born with CAPR mtDNA died within the first 12 h of birth. Genotypic analysis of embryos recovered from pregnant chimeric females revealed that additional CAPR animals died in utero. Growth-retarded, necrotic embryos, homoplasmic for the CAPR mutation, were observed at both embryonic day 15.5 and 17.5.

Fig. 9. Ultrastructural abnormalities of CAPR mouse skeletal muscle and heart. (A–C) Skeletal muscle samples. (D and E) Heart. (A) Wild-type control skeletal muscle at ×4,400. (B–E) CAPR mutant specimens, B at ×3,400, C at ×1,100, D at ×1,950, and E at ×10,500.

A single CAPR pup (2-1) survived beyond the first day of life. This pup was notably smaller than the other littermates at birth (Fig. 7). Although the development of hair was delayed, this pup ultimately developed the agouti hair color derived from CC9.3.1 ES cells. The pup continued to exhibit severe growth retardation throughout life and died at 11 days of age, having a weight of only 2.3 g compared with a mean weight of 6 g for four littermates.

Myopathy and Cardiomyopathy in CAPR Mutant Mice. Mutant mice displayed a variety of degenerative changes in both skeletal muscle and heart on gross and microscopic examination. The skeletal muscle from CAPR pups revealed severe mitochondrial myopathy. Light microscopy revealed degenerative changes and striking loss of muscle fibers and highly abnormal mitochondria. The pathological analysis of the heart of a CAPR neonate, examined 6 h after birth, revealed a dramatic dilated cardiomyopathy. Thinning of the atrial walls, and a marked enlargement of the atria, was observed with the atria exceeding the volume of the normally larger ventricles (Fig. 8).

Ultrastructural analysis confirmed the skeletal muscle and heart pathology. The skeletal muscle showed marked muscle fiber degeneration. Normal muscle cells have highly organized contractile arrays with mitochondria arranged at regular intervals adjacent to Z bands (Fig. 9A). By contrast, in the mutant muscle, there was a striking proliferation of mitochondria which splayed, displaced, or replaced contractile elements and 0.5- to 1-μm inclusions were observed within the cells (Fig. 9B). Some muscle fibers appeared as “ghost” cells in which only the nucleus and filament remnants occupied the intracellular space. The prevalence of gaps within the muscle indicated that many muscle fiber cells had been completely lost (Fig. 9C). Abnormal enlarged mitochondria were prominent and contained internal annular bodies and electron dense granules.

Ultrastructural analysis of the heart revealed a paucity of normal sarcomeres and an abundance of atypical mitochondria (Fig. 9D). There was a proliferation of abnormal mitochondria, some of which were enlarged and others had internal inclusion bodies (Fig. 9E).

In summary, the CAPR mtDNA was transmitted through the female germ line and some animals with a significant proportion of CAPR mtDNA developed to term. However, the presence of this mutation was incompatible with life beyond the neonatal period. The severe mitochondrial myopathy and cardiomyopathy observed in the CAPR mouse is reminiscent of the phenotype seen in humans with the most severe mtDNA protein synthesis mutations (8).

Discussion

We have introduced two different mtDNAs from cultured mouse cell lines into the mouse female germ line: one derived from the synaptosomes of a NZB mouse and another from a CAPR mouse cell line. Although the mice with the NZB mtDNAs appeared normal, those with the CAPR mtDNAs produced a phenotype not only in the germ-line progeny, but also in the chimeric parents.

The CAPR chimeras developed an ocular phenotype including congenital cataracts and functional retinopathy. This is reminiscent of the retinal defects seen in humans with mtDNA disease. For example, a portion of Kearn–Sayres syndrome patients have mild loss of ERG function with granular subretinal pigmentedary changes on ophthalmoscopy (31). Retinitis pigmentosa and macular degeneration have been also observed in patients harboring the heteroplasmic T8993G ATP6 gene mtDNA mutation (32) and retinitis pigmentosa, cataracts, and glaucoma have been reported in a patient with a heteroplasmic np 2971T deletion, which disrupts the anticodon loop of the tRNA1-Leu(UUR) (33).

It is currently unknown why the CAPR mutation induces cataract formation. The lens is a highly complex structure consisting of numerous types of crystalline proteins that form the lens sequentially during development (34). It is possible that an
energetic defect alters the development of the lens fibers during the fetal stage of lens development. The CAPR mutation also could result in increased mitochondrial production of reactive oxygen species throughout the development of the early eye altering the lens crystalline proteins, as has been suggested for the formation of cataracts in the elderly.

The functional retinopathy, associated with the depression of both rod and cone function and degenerative changes in the retinal pigment epithelium, suggests a metabolic defect as there is no evidence of degeneration of the photoreceptor cells. Loss of ERG amplitude in mouse models normally is associated with obvious proportionate degeneration of the retina on histology. However, the ERG findings in the CAPR mice may be similar to the alterations in retinal function seen in patients who harbor the NARP O9883T ATPase6 gene mutation (35).

The growth retardation of the CAPR mice is associated with mitochondrial cardiomyopathy and myopathy, analogous to that observed in a large human family harboring a high percentage of the tRNA\textsubscript{Leu(UUR)} A\textsuperscript{3243}G MELAS mutation. The mother of the family showed lactic acidosis and growth retardation. She had 11 children, who also exhibited lactic acidosis and growth retardation, most of which died in their teens or 20s of either cardiomyopathy or status epilepticus (36). Hence, the CAPR mice seem to represent a more severe form of some of the classical manifestations of mtDNA protein synthesis mutations.

With our method, it will now be feasible to introduce a wide variety of mouse mtDNAs into the mouse female germ line via somatic cell genetics. These could be naturally occurring variants from different strains or species of mice and rodents or additional mutants resistant to mitochondrial OXPHOS inhibitors such as rotenone, antimycin A, and mycycin (13, 37–41). In addition, the recovery of somatic mtDNA mutations that accumulate during normal aging by clonal expansion of brain mtDNAs in synaptosome cybrids (19) also may permit the introduction of naturally occurring deleterious somatic mtDNA mutations into mice. Such transgenic mice would allow the exploration of mtDNA changes in complex genetic processes such as aging.

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