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A nuclear-mitochondrial DNA interaction affecting hearing impairment in mice

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Abstract

The pathophysiologic pathways and clinical expression of mitochondrial DNA (mtDNA) mutations are not well understood. This is mainly the result of the heteroplasmic nature of most pathogenic mtDNA mutations and of the absence of clinically relevant animal models with mtDNA mutations. mtDNA mutations predisposing to hearing impairment in humans are generally homoplasmic, yet some individuals with these mutations have severe hearing loss, whereas their maternal relatives with the identical mtDNA mutation have normal hearing^{1,2}. Epidemiologic, biochemical and genetic data indicate that nuclear genes are often the main determinants of these differences in phenotype^{3–5}. To identify a mouse model for maternally inherited hearing loss, we screened reciprocal backcrosses of three inbred mouse strains, A/J, NOD/LtJ and SKH2/J, with age-related hearing loss (AHL). In the (A/J×CAST/Ei)×A/J backcross, mtDNA derived from the A/J strain exerted a significant detrimental effect on hearing when compared with mtDNA from the CAST/Ei strain. This effect was not seen in the (NOD/LtJ × CAST/Ei)×NOD/LtJ and (SKH2/J×CAST/Ei)×SKH2/J backcrosses. Genotyping revealed that this effect was seen only in mice homozygous for the A/J allele at the *Ahl* locus on mouse chromosome 10. Sequencing of the mitochondrial genome in the three inbred strains revealed a single nucleotide insertion in the *tRNA-Arg* gene (*mt-Tr*) as the probable mediator of the mitochondrial effect. This is the first mouse model with a naturally occurring mtDNA mutation affecting a clinical phenotype, and it provides an experimental model to dissect the pathophysiologic processes connecting mtDNA mutations to hearing loss.

Mouse models for mitochondrial disorders include nuclear gene mutants that lead to oxidative phosphorylation disorders^{6,7} or, in the case of the *Tfam*-deficient mouse, to mtDNA transcription-deficient mice^{8,9}. These models are potentially useful, especially because the defects can be expressed in specific tissues through the use of the loxP-Cre system⁹, but they are unlikely to represent good models to elucidate the pathogenetic pathways caused by human mtDNA disease mutations. Efforts to create an mtDNA defect in mouse recently led to the introduction of a chloramphenicol-resistant mitochondrial chromosome into the embryonic stem cells of mice, with the creation of heteroplasmic mice¹⁰. These mice have cataracts and retinal changes, and when the mutation is passed through the germ line, it is lethal *in utero* and in the perinatal period¹¹. Most recently, a similar approach led to a mouse with a mitochondrial deletion that, when transmitted maternally, causes multiple organ dysfunction and death before the age of 1 year, mostly because of renal failure¹². Although the technical hurdles to create

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GenBank accession numbers. mtDNA of the MilP and NZB/B1NJ mouse strains, L07096 and L07095.

true models of pathogenic mtDNA mutations remain formidable, the establishment of such models remains a very important and rate-limiting step in mitochondrial research.

We previously reported on the hearing sensitivity of 80 inbred strains of mice as assessed by auditory-evoked brainstem response (ABR) threshold measurements¹³. To assess the potential mitochondrial effects on AHL, reciprocal matings were made between each of three hearing-impaired laboratory inbred strains—A/J, NOD/LtJ and SKH2/J—and the wild-derived inbred strain CAST/Ei. The choice of the three strains was fortuitous in that it was the first step of a systematic search to identify putative maternal effects. CAST/Ei retains good hearing beyond 12 months of age and is genetically distinct from the laboratory inbred strains. Female and male mice from each hearing-impaired strain were mated with, respectively, male and female CAST/Ei mice to produce reciprocal F₁ hybrids. All F₁ hybrids retained good hearing, similar to the parental CAST/Ei mice, at three and six months of age. Because of the apparent recessive nature of this hearing loss, F₁ hybrids were backcrossed to the hearing-impaired strains (Fig. 1). We analyzed associations of maternal strain lineage with ABR thresholds in all three backcross populations at three and six months of age (Table 1). There was a statistically significant association of maternal origin with hearing loss in the A/J backcross with CAST/Ei, which was not seen in the NOD/LtJ and SKH2/J backcrosses (Table 1).

We have shown that all 10 inbred strains with an elevated ABR threshold so far chosen for genetic analysis, including the three backcrosses described above, share at least one common gene (designated *Ahl*) on mouse chromosome 10 that has a major effect on AHL (ref. 13). We examined the combined effects on hearing impairment of this nuclear gene with maternal origin in A/J back-cross mice (Table 2). *Ahl* genotypes exhibit an extremely high association with ABR threshold variation, which increases with the age of the mice. The maternal effect on AHL in this backcross, albeit less than that exerted by *Ahl*, is statistically highly significant. Maternal effects on average ABR thresholds were highly significant in backcross mice that inherited two copies of the A/J *Ahl* allele, whereas a single copy of the CAST/Ei *Ahl* allele prevented AHL regardless of maternal origin (Table 2). These results show an interaction between the effects of the nuclear *Ahl* gene and maternal origin in determining hearing impairment in (A/J×CAST/Ei)×A/J backcross mice.

Although this maternal effect could be due to imprinting or to the placental transmission of an ototoxic teratogenic agent in A/J mice, mtDNA is by far the most probable cause of this effect. In humans, hearing loss is associated with a number of different mtDNA mutations², but not with imprinting or an ototoxic-specific congenital agent. Imprinting can also be directly excluded because back-cross mice derived from F₁ females with A/J mothers (77 mice) had significantly higher hearing thresholds than back-cross mice derived from F₁ females with CAST/Ei mothers (157 mice). At 3 months of age, the Mann–Whitney *P* value was 0.0023, and at 6 months of age, it was 0.01. These results are not consistent with imprinting because, for imprinting, all female F₁ hybrids are equivalent (as all alleles should imprint as females regardless of the sex of the parents).

As we observed the maternal effect only for the A/J backcross and not for the NOD/LtJ and SKH2/J backcrosses (Table 1), it seems likely that a difference in the mtDNA of these three inbred strains should account for this. Laboratory strains of mice have a high frequency of one type of *Mus domesticus* mtDNA, whose frequency in wild populations is only 0.04 (ref. 14). Nearly all of the ‘old’ inbred strains (established before 1922) were derived from females with this rare mtDNA type, which makes the number of expected sequence variations low (if any indeed occur).

In a search for putative differences between the mtDNA of the A/J mouse strain and the mtDNAs of the SKH2/J and NOD/LtJ mice, we sequenced the complete mitochondrial genome

of all three strains and compared its sequence with the originally published mouse mtDNA sequence¹⁵. A single nucleotide change was found in the A/J strain but not in the NOD/LtJ and SKH2/J strains (Table 3). The change is an insertion of an adenine in a repeat of nine adenines in *mt-Tr*. The sequence change was confirmed by sequencing this region of the mtDNA from eight backcross mice with mitochondria inherited from the two A/J founder females, the four NOD/LtJ founder females and the two SKH2/J founder females. The length of this repeat appears to be polymorphic in mice, varying between 8 and 10 adenines. NOD/LtJ and SKH2/J mice repeats contain nine adenines. The mtDNA of the A/J, as well as of the MilP and NZB/B1NJ strains, contains 10 adenines. The published reference sequence contains eight adenines, and when we sequenced the *tRNA-Arg* gene from the CAST/Ei strain used in the backcross experiments described above, the length of the adenine repeat was also eight. The adenine repeat is located between nt 14 and 22 in the D stem of the 69-bp mouse mitochondrial *tRNA-Arg* and is not highly conserved through evolution (Fig. 2).

Several lines of evidence support the hypothesis that this insertion of an adenine in *mt-Tr* of the A/J strain is responsible for the worsening of the hearing deficit in mice homozygous for a susceptibility allele at the *Ahl* locus (Tables 1 and 2). First, this is the only difference in the sequence of the mtDNA of the A/J strain that is not present in the NOD/LtJ and SKH2/J strains. Second, we sequenced the mitochondrial genome of the A/J strain and the two similar substrains A/HeJ and A/WySnJ, which have significantly milder and later-onset hearing impairment (Fig. 3). The only difference in sequence was the lack of the adenine insertion in the two substrains (nine adenines). Third, when sequencing *mt-Tr* in the diabetic NOD/LtJ progenitor to the diabetes-resistant NOD.NON-*H2^{nb1}* congenic strain used in our experiments, NOD/LtJ had heteroplasmy for the 9-bp and 10-bp adenine repeat (10 being preponderant). Diabetes in humans is associated with several mtDNA mutations, and heteroplasmy is generally associated with pathogenic mutations (<http://www.gen.emory.edu/mito-map.html>). Fourth, in similarity to these mouse results, all the mtDNA mutations predisposing to hearing deficits in humans involve ribosomal or tRNA genes, require additional environmental or genetic factors for phenotypic expression and have relatively subtle biochemical effects^{2,4,16,17}. Fifth, the fact that this insertion has also been observed in other mouse strains without apparent clinical impact is not surprising, given that the clinical phenotype is subtle, dependent on the genotype of the *Ahl* locus and limited to an additional hearing loss of less than 20 db. Finally, a single base pair change in the D loop of *tRNA-Arg* has been shown to be functionally important. A base change from cytosine to adenine at position 20 of the wild-type yeast *tRNA-Arg* transcript increased sixfold the aminoacylation by *Escherichia coli* arginyl-tRNA synthetase¹⁸.

The interaction between the mitochondrial and nuclear genome described here mimicks in many ways the maternally inherited hearing deficits in humans^{2,5,16,17}. It is, however, necessary to verify the role of the mitochondrial genome by generating and characterizing additional crosses of inbred mouse strains. Once the interaction has been verified, several experimental approaches will provide opportunities to study the pathophysiological basis of the hearing deficit and the role of the mtDNA mutation in it. The identification of *Ahl* and of other nuclear modifier genes, as well as the biochemical analysis of the effects of the mtDNA change in different mouse tissues, may be an example of such approaches. In the interim, it is possible to speculate about the possible functional interactions, based on the known role of *Ahl* and its chromosomal location.

First, it has been shown that *Ahl* is the major gene causing non-syndromic AHL in at least 10 inbred mice strains¹⁹. Independently, it has also been shown that, compared with controls, at least a proportion of people with hearing loss associated with aging, that is, presbycusis, have a significant load of acquired mitochondrial DNA mutations in their auditory tissue^{20,21}. Our

data may link these two findings, allowing speculation that a common pathway of age-related hearing loss is oxidative phosphorylation dysfunction.

Second, the chromosomal location of *Ahl* is very close to the autosomal recessively transmitted waltzer mutation, which causes circling behavior and deafness^{22,23}. This has suggested possible allelism, especially because the cochlear pathology of hair cell loss and spiral ganglion cell degeneration observed in old mice from the inbred strains with AHL is similar but less severe than that seen in mice homozygous for waltzer^{22,24–26}. It should also be noted that the modifier of the deaf waddler gene in mice maps to the same region, and the recessive non-syndromic deafness gene DFNB12 and the Usher syndrome type ID gene map to the syntenic region on human chromosome 10q21–q22 (refs. 27–29). This suggests that *Ahl* may be involved in several forms of deafness and that mitochondrial mutations may be modifiers of phenotypic expression in several forms of hearing loss. It is hoped that additional mouse models for mtDNA disorders can be identified or experimentally induced, and that this will provide a basis for understanding and eventually treating these diseases.

Methods

Mice

All the mice used in these studies were reared and tested under modified barrier conditions in the Mouse Mutant Resource at the Jackson Laboratory. To alleviate husbandry difficulties encountered with diabetic mice, we used the resistant NOD.NON-*H2^{nb1}* congenic strain rather than its diabetic NOD/LtJ progenitor, but for brevity we have designated these mice as NOD/LtJ. The care and use of the animals reported on in this study were approved by the Animal Care and Use Committee of The Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Assessment of hearing in mice

Hearing in mice was assessed by ABR thresholds with equipment from Intelligent Hearing Systems using described methods and equipment¹³. The ABR thresholds of all the mice were measured for broadband clicks and pure-tone frequencies of 8 kHz, 16 kHz and 32 kHz. Evoked responses to the 16 kHz stimulus were used to assess hearing because the mouse ear is most sensitive to sounds at that frequency and because these responses showed the greatest elevation above normal values.

Genotyping the nuclear *Ahl* gene

DNA was extracted from spleen tissue of backcross mice. The closely linked microsatellite marker *D10Mit138* was used to deduce genotypes at the *Ahl* locus on chromosome 10. All backcross mice were typed for *D10Mit138* by standard PCR methods¹⁹. PCR studies were carried out for 30 cycles, the products being separated on 3% agarose gels (Metaphor; FMC BioProducts) and visualized by ethidium bromide staining. Primer pairs for *D10Mit138* were purchased from Research Genetics.

Statistical analyses

Probability values were calculated by non-parametric methods using the Mann–Whitney test for unpaired means, performed with the StatView computer program. Non-parametric statistics were used because of the non-normal distribution of ABR thresholds in these mice.

mtDNA sequencing

Synthetic oligonucleotides were designed using Oligo 6.3 program (Molecular Biology Insights) to amplify the whole mtDNA in 21 overlapping PCR products with an average size

of 914 bp. PCR reactions contained 400 ng DNA, 50 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs and 1 U *Taq* DNA polymerase (PE Applied Biosystems) in a total volume of 50 μl. The DNA was initially denatured at 94 °C for 5 min, followed by 35-step cycles of denaturing at 94 °C for 30 s, annealing at 49–53 °C (according to the conditions for each primer pair recommended by the Oligo program) for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min in the GeneAmp PCR System 9700 (PE Applied Biosystems).

PCR products were extracted from 1% agarose gel using Concert Rapid Gel Extraction System (Life Technologies) or directly purified using Concert Rapid PCR Purification System (Life Technologies).

The complete sequence was obtained by sequencing each of the PCR products in one direction with the primers previously used for the PCR amplifications and specifically designed internal sequencing primers. The dideoxynucleotide chain termination method was employed by using the dsDNA Cycle Sequencing System (Life Technologies) with γ-[³³P]-dATP (NEN). The mtDNA region around *mt-Tr* was sequenced in both orientations in all the subjects.

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Fig. 1.

Frequency distribution of auditory-evoked brainstem response (ABR) thresholds in (A/J x CAST/Ei) x A/J backcross progeny. Hearing loss occurs earlier in NOD/LtJ and SKH2/J backcross mice than in A/J backcross mice, but otherwise their ABR threshold frequency distributions are similar. Mice were tested successively at ages 3 months (light bars) and 6 months (dark bars). The vertical dashed line indicates division between normal and elevated ABR thresholds. Backcross matings were set up using males and females from both types of reciprocal F₁ hybrid. Maternal strain origins in N2 backcross mice were noted.

Fig. 2.

Evolutionary comparison and two-dimensional structure of mouse *mt-Tr*. **a**, Cloverleaf structure of mouse *mt-Tr*. The string of adenine residues in the D loop region is underlined. **b**, Sequence alignment of the *tRNA-Arg* from different species. Region corresponding to the adenines string in mouse is denoted by brackets.

ABR thresholds

Fig. 3.

Comparison of age-related hearing loss among A/J, A/HeJ and A/WySnJ inbred strains of mice. Averages and standard errors of ABR threshold measurements (16 kHz stimulus) are shown for each strain and time point. The number of mice tested per strain varied from 4 to 11 at 8 weeks, 8 to 18 at 12 weeks, 3 to 8 at 18 weeks and 8 to 14 at 36 weeks.

Table 1

Effects of mitochondrial origin on ABR thresholds of 3- and 6-month-old N2 mice from three backcrosses

		ABR thresholds		
Mitochondrial origin	Number of N2 mice	Average	s.d.	Probability values Mann–Whitney
(A/J×CAST/Ei) F ₁ ×A/J backcross				
3-month test age				
A/J	135	30.7	25.4	0.0043*
CAST	157	21.1	17.2	
6-month test age				
A/J	130	38.9	31.5	0.012*
CAST	151	29.2	23.4	
(NOD/LtJ×CAST/Ei) F ₁ ×NOD/LtJ backcross				
3-month test age				
NOD	144	37.8	34.4	0.27
CAST	156	40.9	34.6	
6-month test age				
NOD	133	44.4	37.4	0.31
CAST	141	47.5	36.9	
(SKH2/J×CAST/Ei) F ₁ ×SKH2/J backcross				
3-month test age				
SKH	141	33.3	30.2	0.30
CAST	74	35.6	29.6	
6-month test age				
SKH	141	45.3	34.9	0.80
CAST	74	45.9	34.1	

* Denotes significant results.

Table 2

Effects of the Chr 10 *Ahl* gene (as marked by *D10Mit138*) and mitochondrial origin on ABR thresholds of 3- and 6-month old N2 mice from the (A/J×CAST/
Ei) F₁×A/J backcross

<i>D10Mit138</i> Chr 10 <i>Ahl</i>	Mitochondrial origin	Number of N2 mice	ABR thresholds		Probability values Mann–Whitney
			Average	s.d.	
3-month test age					
AA	–	125	40.0	26.7	<0.0001*
AC	–	167	14.7	5.8	
–	A/J	135	30.7	25.4	0.0043*
–	CAST	157	21.1	17.2	
AA	A/J	68	47.3	26.8	0.0002*
AA	CAST	57	31.3	24.0	
AC	A/J	67	13.8	3.4	0.55
AC	CAST	100	15.7	6.9	
6-month test age					
AA	–	120	56.1	28.9	<0.0001*
AC	–	161	17.0	8.7	
–	A/J	130	38.9	31.5	0.012*
–	CAST	151	29.2	23.4	
AA	A/J	64	63.1	28.7	0.0005*
AA	CAST	56	48.1	27.4	
AC	A/J	66	15.4	6.3	0.14
AC	CAST	95	18.1	9.9	

* Denotes significant results.

MtDNA sequence variation between the three inbred mouse strains and the published mouse sequence

Table 3

Position (bp)	Published sequence	A/J	NOD/LtJ	SKH2/J	Gene/RNA	Amino acid substitution
1767	A	G	G	G	<i>mt-Rnr2</i>	
3815	T	del T	del T	del T	<i>mt-Tg</i>	
3824		ins T	ins T	ins T	<i>mt-Tg</i>	
4012	A	G	G	G	<i>mt-Nd2</i>	none
4794	T	C	C	C	<i>mt-Nd2</i>	Ile→Thr
5734	C	T	T	T	<i>mt-CoI</i>	Pro→Leu
5737	T	C	C	C	<i>mt-CoI</i>	Val→Ala
6238	G	A	A	A	<i>mt-CoI</i>	Cys→Tyr
9348	A	A	A	G	<i>mt-Co2</i>	Ile→Val
9553		ins AAA	ins AAA	ins AAA	<i>mt-Nd3</i>	
9826		ins A	ins A	ins A	<i>mt-Tr</i>	
9827		ins A			<i>mt-Tr</i>	
9856	C	A	A	A	<i>mt-Tr</i>	
10059		ins CAT	ins CAT	ins CAT	<i>mt-Nd4</i>	
12042	C	T	T	T	<i>mt-Nd5</i>	Leu→Phe
15823	T	delT	delT	delT	D-loop	
16119	A	C	C	C	D-loop	
16241	A	delA	delA	delA	D-loop	

A total of 18 changes from the originally published mouse mtDNA sequence were identified¹⁵. All appeared homoplasmic within the level of detection of the sequencing gels. Sixteen of the changes were found in all three mouse strains. Fifteen of them, with the exception of the insertion of an adenine at position 9826 in *mt-Tr*, represent most likely errors in the published sequence. A BLAST search of the mouse EST database and non-redundant database revealed that all available ESTs, as well as two previously sequenced mouse strains, MtlP and NZB/BlNJ (ref. 30), contained the same sequence changes. The A9348G sequence change was found only in the SKH2/J mouse. It appears to be a polymorphism, as the MtlP and NZB/BlNJ strains, as well as 40 of the mouse ESTs, contained a guanine, whereas nine ESTs contained an adenine.