Selection of a mtDNA sequence variant in hepatocytes of heteroplasmic mice is not due to differences in respiratory chain function or efficiency of replication

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We have previously constructed lines of heteroplasmic mice from two inbred strains (NZB/BinJ and BALB/c) to investigate the mechanisms of segregation of mtDNA sequence variants. Analysis of the segregation behaviour of mtDNA in several tissues showed that the NZB genotype was invariably selected in liver/kidney and the BALB genotype in blood/spleen. Segregation was not significant in post-mitotic tissues. Here we have investigated this novel pattern of mtDNA segregation in isolated hepatocytes to determine the mechanism of selection. Polarographic measurements of respiratory chain function showed no difference between mitochondria containing either 0 or 91–97% NZB mtDNAs on a BALB nuclear background. Single-cell PCR analysis of mtDNA in isolated hepatocytes demonstrated that most hepatocytes eventually fix the NZB genotype. The rate of selection was constant with time and independent of the initial genotype frequency. Based on a mtDNA replication rate of 9.4 days, NZB mtDNA has an ∼14% selective advantage over BALB mtDNA; however, in vivo pulse labelling with BrdU demonstrated that this was not based on efficiency of replication. Surprisingly, when hepatocytes were cultured in vitro, the majority of independent colonies selected BALB mtDNA, even if they were nearly fixed for the NZB mtDNA genotype when initially plated. These data suggest that selection for NZB mtDNA in the liver of these mice is not based on respiratory chain function at the cellular or organellar level, or a simple replicative advantage, but on a factor(s) involved with mtDNA maintenance.

INTRODUCTION

Mammalian mtDNA is a small, double-stranded circular molecule of ∼16.5 kb that codes for 13 polypeptides in the mitochondrial respiratory chain and is strictly maternally inherited. There are several hundreds to thousands of copies of mtDNA in most somatic cells and multiple copies per mitochondrion (1–3). Although mtDNA is highly polymorphic in mammalian populations, normally a single sequence variant of mtDNA is transmitted through the female germ line to her offspring (mtDNA homoplasmy). New germ line or somatic cell mutations in mtDNA lead to the co-occurrence of two or more sequence variants in a cell (mtDNA heteroplasmy). Such sequence variants will segregate in the daughter cells during mitosis, or during mitochondrial turnover, a process called mitotic (or replicative) segregation (4). mtDNA copy number varies widely from cell to cell and is tightly regulated in a cell-specific fashion; however, replication of mtDNA is not tightly coupled to the cell cycle (5). Thus, during mitosis some templates may replicate more than once, others not at all. This behaviour, coupled with the random distribution of mtDNAs to daughter cells at cytokinesis provides a mechanism for mitotic segregation of mtDNA sequence variants. This is thought to be a stochastic process that depends primarily on the number of genomes segregating in the cell and the rate of mitochondrial turnover; however, it can be altered by sequence-specific differences in replication efficiency or selection pressures, leading to very different proportions of two mtDNA species in cell lineages derived from the same stem cells, even in the face of high mtDNA copy number (6,7).

Mutations in mtDNA have been shown to be an important cause of a heterogeneous group of multisystem disorders, usually associated with neurological or neuromuscular disease (reviewed in 8,9). Most patients with mtDNA mutations are heteroplasmic, likely because most pathogenic mtDNA mutants are lethal in the homoplasmic state. The relative proportion of mutant mtDNAs transmitted to offspring and their tissue distribution are important determinants of the severity and nature of the disease phenotype. Whereas this process appears to be primarily stochastic in the female germline (10,11), the factors that determine how wild-type and pathogenic mtDNAs segregate in different tissues after birth are not well understood. Considerable differences in the segregation patterns of particular pathogenic mutations have been documented despite the fact that all affect the same final common biochemical pathway—oxidative phosphorylation (6,7,12–15).

To investigate the mechanisms of transmission and segregation of mtDNA we previously constructed heteroplasmic mice segregating two polymorphic mtDNA sequence variants (NZB and BALB) (10,16). We observed an unexpected tissue-specific and age-related selection for different mtDNA
genotypes in the same animal; all animals selected the NZB genotype in liver/kidney and the BALB genotype in spleen/blood (16). This pattern of mtDNA segregation, which does not reflect the distribution of any tissue-specific gene known to be associated with respiratory chain (or other mitochondrial) function, suggests a novel nuclear–mitochondrial interaction.

Here we have investigated the mechanism for the selection of NZB mtDNA in the liver of these heteroplasmic mice to determine if it is based on respiratory chain function or a replicative advantage for NZB mtDNA. We show that the segregation of the NZB mtDNA in the liver is constant with time, independent of genotype frequency and the rate of mtDNA replication. In addition, we find no evidence for altered oxidative phosphorylation capacity in isolated mitochondria with different proportions of NZB mtDNA. Unexpectedly, the majority of hepatocytes proliferating in vitro reversed mtDNA selection and increased the proportion of BALB mtDNA. These results are consistent with the idea that the tissue- and cell-type specific selection for different mtDNA sequence variants involves an unknown factor(s) important in the maintenance of mtDNA.

RESULTS

Respiratory chain function in hepatocytes with BALB or NZB mtDNA

There are 15 predicted amino acid changes in the mtDNA-encoded polypeptides between BALB and NZB mtDNA (Table 1). Most of the changes are conservative substitutions (except the R→C change at amino acid 59 in ND1) at non-conserved positions in evolution; however, it is possible that these polymorphisms could affect interactions with nuclear-encoded respiratory chain subunits and alter oxidative phosphorylation function (17,18). To test whether functional differences exist in the maximum capacity for oxidative phosphorylation in mitochondria with different levels of NZB mtDNA, the respiratory chain capacity of isolated liver mitochondria was measured polarographically in aged-matched mice with high (91–97%) or low (0%) levels of NZB mtDNA. No significant differences were detected in the maximal activities (determined by substrate-specific oxygen consumption) of Complex I, Complex III or Complex IV in mitochondria with either high and low levels of NZB mtDNA. Additionally, no differences were observed in the respiratory control ratios (RCR = 3.4 ± 0.1 SEM versus 3.5 ± 0.2 SEM in mitochondria containing high versus low levels of NZB mtDNA).

NZB mtDNA selection in vivo

To test whether the NZB mtDNA genotype in the liver conferred a growth advantage at the level of the cell, the distribution of the relative proportion of NZB mtDNA in single hepatocytes was measured as a function of age. If a higher proportion of NZB mtDNA conferred a growth advantage to the cell, one would expect a skewing in the distribution of heteroplasmy, with an increasingly large fraction of cells containing predominantly NZB mtDNA. Single hepatocytes were isolated from the liver of 2-, 4-, 9- and 18–20-month-old mice and their mtDNA genotype was determined using single-cell PCR techniques. We initially compared mice with similar proportions of NZB mtDNA at birth (range 12–27% NZB mtDNA, mean 18.3 ± 1.0 SEM, n = 16), determined from tail biopsy. In the 2- and 4-month-old mice, the frequency of the

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Table 1. Amino acid differences in the mtDNA-encoded polypeptides of NZB and BALB mice compared to other vertebrate species

<table>
<thead>
<tr>
<th>AA a</th>
<th>Mouse</th>
<th>Rat</th>
<th>Gorilla</th>
<th>Human</th>
<th>Trout</th>
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<tbody>
<tr>
<td>NZB</td>
<td>C3H/AN</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Complex I</td>
<td></td>
<td></td>
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<tr>
<td>ND1</td>
<td>3 A I I L L P P</td>
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<tr>
<td>59 C R R K K R R</td>
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<td>ND2</td>
<td>7 T A T P P S T</td>
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<td>265 V I A L A F L</td>
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<td>294 T I T L L L L</td>
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<tr>
<td>ND4</td>
<td>263 M I I L L I L</td>
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<tr>
<td>ND4L</td>
<td>37 M V M M M L M</td>
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<tr>
<td>ND5</td>
<td>103 F L F F F F F</td>
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<tr>
<td>365 T I A A T S L</td>
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<td>568 I T I Q Q N Q</td>
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<tr>
<td>ND6</td>
<td>91 A I F A A I V</td>
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<tr>
<td>122 I V I G G G G</td>
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<td></td>
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<td>Complex III</td>
<td></td>
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<tr>
<td>Cyt b</td>
<td>24 T A A T T T A</td>
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<tr>
<td>Complex IV</td>
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<tr>
<td>CoxI</td>
<td>46 T A A N N T A</td>
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<tr>
<td>CoxIII</td>
<td>248 V I V V V V</td>
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aAmino acid position inferred from the nucleotide sequence of the C3H/AN mouse (48).

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Figure 1. The respiratory chain capacity of liver mitochondria from heteroplasmic mice with high or low levels of NZB mtDNA. Maximal state 3 oxygen uptake was determined in isolated liver mitochondria from aged-matched heteroplasmic BALB mice with high and low proportions of NZB mtDNA (n = 4 for the high percentage NZB mice, n = 3 for the 0% NZB mice).
The proportion of NZB mtDNA in individual hepatocytes was normally distributed, with mean levels of heteroplasmy of $\sim 25$ and 62%, respectively (Fig. 2). By 9 months of age, the distribution was very slightly skewed and the majority of hepatocytes had $>80\%$ NZB mtDNA. At 18–20 months of age, most hepatocytes had $>98\%$ NZB mtDNA. Consistent with previous results (16) the rate of selection for NZB mtDNA was independent of the initial genotype frequency (data not shown). If selection were occurring at the level of the cell, hepatocytes with a higher proportion of NZB mtDNA would be fitter than those with a lower proportion, and the relative fitness (advantage) of the NZB mtDNA would increase with age. The relative fitness of the NZB mtDNA was calculated as a function of age using an equation that compares two asexual populations after $n$ generations (19), assuming that the half-life of mtDNA in the mouse liver is similar to that reported in the rat, $\sim 9.4$ days (20). The relative fitness of NZB mtDNA did not differ significantly in mice between 2 and 9 months of age and was $\sim 14\%$ per replication cycle (Table 2). In mice beyond 9 months of age, most hepatocytes were either fixed or nearly fixed for NZB mtDNA, regardless of the starting level of heteroplasmy, which precludes comparing the relative fitness values from these time points with those from younger mice. These data do not support a model of selection based on a growth advantage at the level of the cell.

In order to test whether the selective advantage of NZB mtDNA was due to the rate of mtDNA replication itself, or some other aspect of mtDNA maintenance, mice were injected with a bolus of BrdU to estimate the relative replication rates of NZB versus BALB mtDNA. DNA was extracted from isolated mitochondria derived from liver at 10, 20, 30, 40 and 50 h. mtDNA was digested with NheI, which cuts NZB mtDNA once producing a linear 16 kb fragment, and BALB mtDNA twice, producing 4.4 and 11.8 kb diagnostic fragments (Fig. 3A and B). These samples were subjected to a southern analysis using an anti-BrdU antibody to measure the relative incorporation of BrdU into mtDNA of both genotypes. The blots were then stripped and subjected to Southern analysis to measure the relative proportions of the two mtDNA genotypes. No significant differences were observed in the relative rates of incorporation of BrdU into the two different species of mtDNA (Fig. 3A and B). A standard curve generated by mixing mtDNAs containing known proportions BrdU-labelled BALB and NZB mtDNA over the range of heteroplasmy investigated in the experiments shows we would have been able to detect small differences in the relative rates of incorporation of BrdU into mtDNA of both genotypes. The blots were then stripped and subjected to Southern analysis to measure the relative proportions of the two mtDNA genotypes. No significant differences were observed in the relative rates of incorporation of BrdU into the two different species of mtDNA (Fig. 3A and B). A standard curve generated by mixing mtDNAs containing known proportions BrdU-labelled BALB and NZB mtDNA over the range of heteroplasmy investigated in the experiments shows we would have been able to detect small differences in the relative rates of incorporation of BrdU into the different genotypes (Fig. 3D). As a control, other tissues (brain, heart) that are neutral with respect to mtDNA selection were analysed with similar results (data not shown). Thus, the selective advantage of NZB mtDNA in hepatocytes cannot be attributed to a replicative advantage per se.

### In vitro reversal of NZB mtDNA selection

Rat hepatocytes turn over very slowly in vivo (20). When cultured in vitro, many of the cells that initially plate down die; however, those that survive de-differentiate and enter a proliferative period of growth, but are still able to express hepatocyte markers under appropriate culture conditions (21). To test whether the state of differentiation/growth affected the selection of NZB mtDNA, hepatocytes were isolated by collagenase perfusion and cultured in vitro. In the majority of independent in vitro cultures, proliferating hepatocytes dramatically reverse the selection for NZB mtDNA selection, and strongly select BALB mtDNA (Fig. 4). However, there was one exception. In

### Table 2. Relative fitness of the NZB mtDNA genotype in hepatocytes with age

<table>
<thead>
<tr>
<th>Age</th>
<th>Relative Fitness</th>
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<tr>
<td>2 months</td>
<td>1.13 (0.03)</td>
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<tr>
<td>4 months</td>
<td>1.16 (0.01)</td>
</tr>
<tr>
<td>9 months</td>
<td>1.12 (0.01)</td>
</tr>
<tr>
<td>18 months</td>
<td>1.09–1.08</td>
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Values are expressed as a mean (SEM) ($n = 5$ for 4 and 9 month animals; $n = 4$ for 2 month animals). Relative fitness is calculated using $(p_n / q_n) = (w / w_1)^n (p_0 / q_0)$, where $p$ and $q$ represent NZB and BALB; $n$, the number of days; $w / w_1$, relative fitness.
Cells proliferating in vitro in growth medium were of variable size with a typical flat, epithelial cell-like morphology (Fig. 6A). Cells with a spindle-shaped morphology, characteristic of fibroblasts, were never observed, nor did we find evidence for any focal outgrowth of contaminating cells (Fig. 6A). No differences in the relative proportions of BALB or NZB mtDNA were observed in cells of different sizes obtained by fluorescence activated cell sorting (data not shown). To confirm that these cells were hepatocytes, they were cultured in the presence of Matrigel and hepatocyte growth factor (HGF), factors known to induce hepatocyte differentiation (21). The expression of albumin mRNA was evaluated by RT–PCR, and albumin protein by immunofluorescence. In differentiation medium the cells formed highly ordered structures consisting of relatively uniform islands of cells with an epithelial morphology (Fig. 6B) that express albumin mRNA (Fig. 7). Staining for albumin in these islands showed a heterogeneous distribution of albumin-positive cells after 10 days in differentiation medium that did not correlate with cell size (Fig. 6C). Similar heterogeneous patterns of albumin staining, have been documented previously, in both rat liver in vivo and in hepatocytes grown in vitro (22–25). This series of experiments suggests that the cells we have cultured in vitro are of hepatocyte origin, have the potential to differentiate into cells that express hepatocyte markers under appropriate conditions and are not due to the outgrowth of a contaminant cell type.

To calculate the relative fitness of BALB mtDNA selection in vitro, we measured the proportion of the BALB genotype in proliferating hepatocytes after several population doublings, reasoning that mtDNA turnover could be estimated by cell doubling time in a proliferating cell population. In vitro, the mean relative fitness for the BALB mtDNA genotype from independent cultures of proliferating hepatocytes was 1.32 ± 0.12 SEM, which is a little more than twice the relative fitness of NZB mtDNA in vivo.

One mechanism that could account for the more rapid selection for mtDNA in vitro, from a mean NZB level of 95% to <5% (Fig. 4), could involve a reduced mtDNA copy number (bottleneck) upon proliferation in vitro. To test this hypothesis the level of mtDNA was compared to that of the nuclear 18S gene in both in vitro hepatocyte cultures and freshly isolated liver samples. Culturing hepatocytes in vitro resulted in a 2-fold reduction in the mean level of mtDNA relative to 18S (ratio of mtDNA:18S in vitro, 8.9 ± 0.7 SEM, n = 14; in vivo, 17.9 ± 2.5 SEM, n = 12). This reduction in mtDNA copy number could contribute to the more rapid segregation of mtDNA as the cells enter a proliferative state in vitro, but cannot of course account for the reversal in selection, from NZB to BALB mtDNA.

The pattern of mtDNA segregation is not altered by differentiation of hepatocytes in vitro or liver regeneration in vivo

The above results suggested that selection for one mtDNA genotype or the other might depend on the growth/differentiation state of the cell. To test whether the direction of mtDNA selection could be altered by the growth or differentiation state of the cell, we tried to reverse BALB mtDNA selection in vitro by inducing a differentiated state, and NZB mtDNA selection in vivo by stimulating proliferation.
To investigate whether the selection of BALB mtDNA in vitro could be reversed, a series of experiments was designed to induce hepatocyte differentiation in the presence or absence of various growth factors, cell adhesion matrix, with or without glucose in the medium. Cells grown without a fermentable carbon source (glucose) would be forced to rely on oxidative phosphorylation for all their ATP. The following growth/adhesion factors were investigated alone or in combination: Matrigel, a cell adhesion matrix that is reported to promote hepatocyte differentiation in vitro (21); HGF, a known stimulator of both initial hepatocyte proliferation and later differentiation (26) and epidermal growth factor (EGF), commonly used to grow hepatocytes in vitro. After 10 days under culture conditions designed to promote differentiation, hepatocytes expressed albumin mRNA, a marker of the differentiated state; however, urokinase mRNA expression, a marker of the proliferative state, was never down regulated compared to a β-actin control (Fig. 7). Expression of albumin mRNA was much slower when EGF or HGF were used alone without Matrigel (Fig. 7). None of the growth factor/extracellular matrix/carbon source combinations altered this pattern and selection of the BALB mtDNA was unchanged. Similar experiments involving the two hepatocyte cultures that maintained NZB mtDNA selection in vitro also showed unaltered selection (data not shown).

Hepatocytes can be induced to regenerate in vivo after a partial hepatectomy or large-scale necrosis due to toxic insult (26,27). Approximately 70% of the liver’s mass is restored 72 h after losing two-thirds of the organ (28). To test whether this rapid cell division in vivo during regeneration would be sufficient to induce a shift in the NZB mtDNA genotype frequency in single cells, mice were subjected to a two-thirds hepatectomy (29). This was achieved by removing the majority of the left and median lobes of the liver, from which single hepatocytes were isolated. Three days later single hepatocytes were isolated from the remaining right and caudal liver lobes,
which had clearly enlarged in size. Hepatocytes from before and after the two-thirds hepatectomy were genotyped to compare the distribution of NZB mtDNA in single cells. No significant shift in the proportion of NZB mtDNA was observed at any level of NZB heteroplasmy (Fig. 8). Thus, the cell division that accompanies liver regeneration does affect mtDNA segregation significantly.

**DISCUSSION**

The tissue-specific pattern of segregation of the NZB and BALB mtDNAs in the heteroplasmic mice we constructed is clearly not stochastic, nor can it be correlated with, or explained by, any known pattern of expression of respiratory chain or other mitochondrial proteins (16). We have investigated hundreds of animals on several different nuclear backgrounds (DBA, 129Sv, C57Bl6, C3H, NZB) in backcross experiments (unpublished data) and we have never observed an exception to the pattern of selection for NZB mtDNA in the liver/kidney and BALB mtDNA in the blood/spleen. In this study we focused our investigations on the phenomenon in the liver and (i) sought to test whether selection is likely to occur at the level of the cell, organelle or mitochondrial genome and (ii) attempted to modify the pattern of selection to gain some insight into the molecular basis for the segregation behaviour.

Our evidence strongly suggests that the advantage of the NZB mtDNA genotype in liver does not derive from an overall growth advantage to cells containing a high proportion of this polymorphic sequence variant. This is based on the observation that the rate of selection of NZB mtDNA in liver is more or less constant with age and independent of genotype frequency. If NZB mtDNA conferred a growth advantage to the cell, cells with a higher proportion of NZB mtDNAs would be expected to leave more daughters than those with less, and the rate of selection would be non-linear. This would be reflected in a skewing of the frequency distribution of heteroplasmy ratios with age in single hepatocytes. In fact, these frequency distributions could be fitted to a normal distribution,
and the relative fitness of NZB mtDNA, determined using a population genetic model for selection in asexual populations (19), showed a constant 14% advantage of NZB over BALB mtDNA at all ages up to 9 months, the time by which most selection had occurred.

NZB mtDNA also does not appear to enhance the capacity for oxidative phosphorylation at the level of the organelle. Maximum rates of oxygen consumption with a variety of substrates were no different in mitochondria containing 0 or 97% NZB mtDNA. In this context it is interesting to note that no differences in respiratory chain function could be measured in hybrid cells constructed from two different mouse species, Mus musculus domesticus or Mus spretus (30,31). This suggests a high degree of compatibility between sequence variants in the nuclear- and mtDNA-encoded subunits of the respiratory chain complexes in mice. Hepatocytes grown in the absence of glucose, which would be forced to rely on aerobic ATP production, also did not alter mtDNA selection. These data would seem to eliminate selection for aerobic energy production at the level of the organelle; however, we cannot eliminate the possibility of more subtle effects on mitochondrial respiratory chain function or factors that might alter the turnover of organelles containing a preponderance of one mtDNA genotype or the other.

Another factor arguing against selection for respiratory chain function is the lack of measurable segregation of mtDNA in highly aerobic tissues like heart, brain and skeletal muscle. These are precisely the tissues in which segregation of human pathogenic mtDNA mutations is particularly evident, but still poorly understood. The first reports of large-scale mtDNA deletions (32) demonstrated that mtDNA deletions were abundant in muscle cells, but undetectable by Southern blot analysis in peripheral blood. Longitudinal studies of patients harbouring large-scale deletions showed an increase in the frequency of deletions in muscle with age (12). This led to the suggestion that the deleted molecules had a replicative advantage in post-mitotic cells, perhaps because of their smaller size, but were selected against in rapidly dividing cells because they conferred a growth disadvantage. However, in long-term cultured cells heteroplasmic for large-scale deletions of mtDNA there was no selection for or against the deleted

Figure 6. Differentiation of hepatocytes in vitro. (A) Cells grown to confluency in growth medium display a typical epithelial morphology, but with some variation in cell size. (B) Cells grown in growth medium with a Matrigel overlay and the addition of HGF form highly ordered structures consisting of islands of cells with epithelial morphology (arrow). (C) Heterogenous production of albumin is detected by indirect immunofluorescence in the flat epithelial cells shown in (B).

Figure 7. Differentiation of hepatocytes in vitro does not reverse selection for BALB mtDNA. An expression time course of hepatocyte differentiation in vitro with different combinations of growth factors and extracellular matrix [(A) with Matrigel; (B) without Matrigel]. RT–PCR of albumin mRNA expression was used as a marker of differentiation; urokinase, for proliferation; and β-actin, as a control. The change in the proportion of NZB mtDNA in the cultures is shown below the gels.
mtDNA (33). Moreover, some mtDNAs carrying pathogenic tRNA point mutations clearly increase with age in human skeletal muscle (6,7) so genome size alone cannot account for the pattern of segregation. For other tRNA point mutations there is evidence to suggest no change in the frequency of the mutant allele with age (13,34) or a decrease in the proportion of the mutant with age (14,15). The pattern of segregation of pathogenic mtDNA mutations in dividing cells is also extremely variable (35). Since all of the above mutants are thought to produce mitochondrial protein translation defects, it is not at all clear why such different patterns of segregation are observed. What one can conclude from these data is that there is no simple relationship between respiratory chain function and the tissue-specific pattern of mtDNA segregation.

Taken together, our data support a model in which selection is occurring at the level of the genome itself. The fact that proliferating versus differentiated hepatocytes select opposite mtDNA genotypes suggests that the phenomenon is not simply due to a trivial difference in sequence between the two genotypes, but rather has some biological significance. The sequences that are thought to be important in the regulation of mtDNA replication (36) are apparently identical in both the BALB and NZB mtDNA genotypes (16). Interestingly, the strong selection for BALB mtDNA over NZB in vitro has been documented earlier in experiments involving fusion of a BALB-derived plasmocytoma cell line with NZB spleenocytes (37). The ND1 protein from NZB mtDNA can act as a maternally inherited minor histocompatibility antigen on the cell surface (38), but these immunological properties of the different mtDNA genotypes do not explain the tissue-specific patterns of selection or genetic drift seen in our mice (16).

Tissue-specific segregation patterns of mtDNA have also been reported in other heteroplasmic mice constructions. In mice heteroplasmic for C57BL/6 (B6) and RR mtDNA, RR mtDNA has a selective advantage over the B6 mtDNA in all tissues, independent of nuclear background (39), although the heart and brain were shown to be more heteroplasmic (containing more B6 mtDNA) than other tissues. The D-loop

**Figure 8.** A liver hepatectomy is insufficient to alter the distribution of the NZB mtDNA genotype in single hepatocytes. The frequency distribution of NZB mtDNA in pre- (A) and post-hepatectomy (72 h later) (B) hepatocytes.
region differs at 43 sites between these strains and it is possible that the absolute advantage of the RR mtDNA genotype in these animals reflects a difference in replication efficiency. A similar pattern of tissue specificity has been reported in heteroplastic mice produced from Mus musculus molossinus and M. m. domesticus in which mtDNA from the latter could only be detected in brain, heart and spleen (40). These diverse patterns of mtDNA segregation suggest complex interactions between the nuclear and mitochondrial genomes.

We have ruled out a mechanism based strictly upon a difference in replication efficiency between the two genotypes in our mice, as there is no replicative advantage for NZB mtDNA in vivo. The fact that the pattern of segregation cannot be altered by partial differentiation in vitro or acute regeneration in vivo suggests that selection for NZB in mature liver might involve some aspect of long-term mitochondrial genome maintenance. Such maintenance factors could include proteins that affect mtDNA structure (degree of supercoiling, assembly into nucleoids), expression or replication (polymerases or accessory factors) or perhaps even components of the DNA repair system that might affect mtDNA turnover. Identification of the factor(s) responsible for mtDNA selection in the liver of these heteroplastic mice should help in the understanding of the mechanisms that determine the segregation patterns of pathogenic mtDNA mutations in humans.

MATERIALS AND METHODS

Isolation of hepatocytes

Hepatocytes were isolated from heteroplastic mice, carrying both BALB and NZB mtDNA on a BALB nuclear background (10), by a two-step collagenase perfusion of the liver via the inferior vena cava (41). The liver was initially perfused with 50 ml of Ca2+ and Mg2+ free Hanks balanced salt solution (HBSS; Gibco), followed by 50 ml of collagenase (Sigma) dissolved in HBSS. Viable cells were separated by isodensity Percoll (Pharmacia) centrifugation and tested for viability by Trypan blue exclusion. For PCR analysis, single hepatocytes were selected and prepared according to Li et al. (42).

mtDNA genotype analysis

mtDNA was genotyped using one of two methods: RFLP or solid-phase mini-sequencing assay. For the RFLP, an Rsal site at position 3691 in the ND1 gene present in NZB but absent in BALB mtDNA was used to genotype individual animals. The mtDNA genotype analysis method was designed to anneal adjacent to a polymorphism between NZB and BALB at nt 3932. The mini-sequencing solution, 10 pmol nested primer, 0.25 U Tag DNA polymerase (Gibco), 1× Gibco PCR buffer, 1.5 mM MgCl2, and either 0.2 µCi 3H-dTTP or 3H-dCTP, was incubated in a well at 50°C for 20 min. The single base extension of the primer should incorporate 3H-CTP in the case of the BALB allele and 3H-TP in the case of the NZB allele. Each well was then washed three times with 40 mM Tris–HCl, pH 8.8, 1 mM EDTA, 50 mM NaCl and 0.1% Tween 20; the primer was eluted after incubation in 50 mM NaOH for 5 min. The separate incorporation of both 3H-dNTPs into the primer was counted using a liquid scintillation counter. To determine the proportion of NZB to BALB in each unknown sample, the ratio of c.p.m. incorporated into each detection primer was determined. This value was compared to a standard curve constructed using known proportions of NZB and BALB mtDNA.

mtDNA copy number

Total genomic DNA was isolated from hepatocytes grown in vitro or from freshly isolated liver. An aliquot of DNA (4 pg) was digested with Ncol, which cuts both NZB and BALB mtDNA once at the same position, producing a 16.3 kb linear fragment. Digested DNA was separated on a 0.7% agarose gel, transferred to a Zeta-probe (BioRad) nylon membrane and UV cross-linked. The membrane was hybridized overnight at 65°C (44) with either a [32P]CTP labelled full-length mouse mtDNA probe or a human 18S fragment (45). Signals were detected and quantified on PhosphorImager and ImageQuant software (Molecular Dynamics).

In vitro culture of hepatocytes

Single hepatocytes were plated at a density of 4 × 10^3/cm^2 on collagen (rat tail) coated plates (Roche) or at low density to promote the formation of cell colonies. Cells were grown in DMEM:F12 (Gibco), supplemented with the following: 5% fetal bovine serum (FBS); 13 mg/l proline; 55 mg/l pyruvate; 20 mg/l asparatate; 1 g/l galactose; 10 mM nicotinamide; 20 ng/ml EGF (Becton Dickinson); 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (ITS) (Roche); 10⁻⁶ M dexamethasone. For differentiation experiments, Matrigel (Becton Dickinson) was diluted 1:3 with medium and added on top of cells. Cells were also grown in low glucose or glucose-free medium. For this purpose we used glucose-free DMEM (Gibco), supplemented with the

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following: 5% FBS, 26.6 mg/l aspartate; 30 mg/l proline; 110 mg/l pyruvate; 1 g/l galactose; 10 mM nicotinamide; 10⁻⁷ M dexamethasone; 20 ng/ml EGF; ITS. In the low glucose medium, glucose came only from the FBS (concentration ~0.5 mM); for the glucose-free medium, glucose was removed from FBS by spin dialysis.

Markers of hepatocyte differentiation and proliferation

Albumin and urokinase mRNA expression were used as markers of a differentiated or proliferative state of hepatocytes. Total RNA was isolated with a RNeasy Mini Kit (Qiagen). RT product was generated by incubating 1 µg of RNA, 30 mg/ml random primers, 1× MMLV buffer (Promega) and 0.5 µl of RNAsInhibitor (Roche) at 65°C for 5 min. Samples were put on ice for 5 min; then 0.5 mM dNTPs and 200 U of MMLV-RT (Promega) were added to the sample and incubated at 37°C for 45 min. PCR products were amplified from 1.5 µl of the PCR product using standard conditions with the following primers and cycles for each marker: albumin, forward 5′-TAT GCC CCG GAA CTC CTT TTC G-3′, reverse 5′-CCG CCC TGT CAT CAG CAC ATT C-3′, 94°C, 30 s, 55°C, 30 s, 72°C, 30 s, for 30 cycles; urokinase, forward 5′-CTG ACA ACC AGA AGC GAC CCT-3′, reverse 5′-CTT TGG GAT TTG AAT GAA GCA GTG T-3′, 94°C, 30 s, 56°C, 30 s, 72°C, 30 s, for 30 cycles; and β-actin as a control (46), forward 5′-TCA CCC ACA CTG TGC CAT TC-3′, reverse 5′-GAG TAC TTC GTG CCG TCA GGA GGA GC-3′, 94°C, 30 s, 62°C, 30 s, 72°C, 30 s, for 24 cycles. To each sample 1.5 µCi of [α-32P]CTP was added prior to the first PCR cycle. PCR products were run out on a 10% polyacrylamide gel and scanned on a Storm PhosphorImager. The signal intensity of the PCR products was linear with cycle number for each product.

Partial hepatectomy

The protocol for a two-thirds hepatectomy was adapted from that of Schaeffer et al. (29). Mice were anesthetized with avertin (0.014 ml/g body weight) and a mid-abdominal incision was made. The left and median liver lobes were isolated and hemoclips applied at the base of each lobe. The left and median lobes were then removed by cautery with an electrocautery unit. Single hepatocytes were isolated in a collagenase solution from the resected lobes.

Polarographic studies

Mitochondria were isolated from the liver by homogenization with a teflon pestle in 0.25 M sucrose, 10 mM HEPES pH 7.4, 0.1 mM EGTA and 5 mg/ml BSA. The liver suspension was centrifuged at 800 × g for 10 min at 4°C. The supernatant was centrifuged at 9000 g for 10 min at 4°C. The resulting mitochondrial pellet was washed (2×) with 0.25 M sucrose, 10 mM HEPES pH 7.4, 0.1 mM EGTA and centrifuged again at 9000 g for 10 min at 4°C. Mitochondrial oxygen uptake was measured polarographically (47) with a Clarke type electrode interfaced with Vernier Instruments data logger software. For each experiment, ~0.5 mg/ml of mitochondrial protein was incubated with a respiration buffer (100 mM KCl, 20 mM HEPES pH 7.4, 5 mM KH₂PO₄, 1 mg/ml BSA) in a water-jacketed chamber at 37°C to measure the following state 3 reactions: oxidation of glutamate (5 mM) plus malate (5 mM) for Complex 1; succinate (2 mM) for Complex 3; and ascorbate (10 mM) plus N,N,N′,N′-tetramethyl-p-phenylenediamine (0.2 mM) for Complex 4. The following specific inhibitors were used to assess the rates of the individual complexes in the respiratory chain: rotenone (2 µM) for Complex I; antimycin A (5 µg/ml) for Complex III; and KCN (2 mM) for complex IV.

In vivo labelling of mtDNA with BrdU

Mice were given an interperitoneal injection of BrdU, 1 mg/g body weight. Mitochondria were isolated from the liver by differential centrifugation and mtDNA extracted by standard procedures (44). To distinguish BrdU incorporation into NZB and BALB, mtDNA was digested with NheI, which cuts NZB once, producing a 16.3 kb fragment and BALB twice, producing 4.4 and 11.8 kb fragments. Digested mtDNA was separated on a 0.7% agarose gel, transferred to a Zeta-probe and UV cross-linked. Membranes were blocked in TBST (10 mM Tris–Cl, pH 8.0; 150 mM NaCl; 0.1% Tween 20)–5% milk and incubated with a 1:667 dilution of an anti-BrdU antibody (Becton Dickinson) overnight at 4°C. Detection and stripping of the anti-BrdU antibody was done according to the manufacturer’s protocol for the ECF western blotting kit (Amersham Pharmacia Biotech). Membranes that were stripped of antibodies were then hybridized to a full-length mtDNA probe. A standard curve was generated by mixing together known proportions of BALB and NZB mtDNA.

Immunofluorescence

Cells were grown on collagen-covered glass cover slips and washed thoroughly with phosphate-buffered saline (PBS) before fixing in chilled acetone. Albumin was detected by incubating with rabbit antiserum against mouse albumin (Cappel) (1:1000 in PBS) for 1 h at room temperature. Cover-slips were washed with three rinses of PBS and incubated with a biotinylated goat anti-rabbit IgG antibody (1:200) (Cedarlane) for 45 min at room temperature. After washing with PBS they were then incubated with Cy3-conjugated streptavidin (1:1000) (Jackson ImmunoResearch) for 30 min at room temperature and examined under a fluorescence microscope.

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REFERENCES
