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SEGREGATION OF MITOCHONDRIAL DNAs CARRYING A PATHOGENIC POINT MUTATION ($tRNA^{leu3243}$) IN CYBRID CELLS

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To investigate the mechanism of segregation of mitochondrial DNAs (mtDNAs) carrying a pathogenic point mutation in the $tRNA^{leu(UUR)}$ gene (A to G at position 3243) cytoplasts, derived from a heteroplasmic myoblast clone, were fused to rho^o cells to create cybrid cells carrying different proportions of mutant and wild-type mtDNAs. Although the individual myoblasts used as fusion partners contained predominantly mutant mtDNAs (mean proportion 0.77, range 0.46-0.94), the majority (56%) of the cybrid clones isolated after growth in selective medium were homoplasmic wild-type, indicating preferential replication of wild-type mtDNAs. Long-term culture of heteroplasmic cybrid clones in non-selective medium produced no change in the mean proportion of wild-type mtDNAs but an increase in population variance, consistent with a purely stochastic segregation mechanism. These results are at variance with a previous report suggesting a replicative advantage for mtDNAs carrying the $tRNA^{leu3243}$ point mutation.

Single base pair substitutions in mtDNA-encoded tRNA genes are the most common cause of a heterogeneous group of disorders referred to as the mitochondrial encephalomyopathies (1,2). These mutations are also rare causes of relatively common diseases such as diabetes (1-3). Patients with these diseases carry varying proportions of both wild-type and mutant mtDNAs, a condition referred to as mtDNA heteroplasmy. Because the copy number of mtDNA is on the order of 10^3-10^4 in most cells, the expression of a pathogenic biochemical or clinical phenotype depends on the relative proportion of mutant and wild-type mtDNAs within the cell. These expression thresholds have been determined for the $tRNA^{lys8344}$ mutation associated with MERRF (Myoclonus Epilepsy with Ragged Red Fibers) (4.5) and the $tRNA^{leu3243}$ mutation associated with MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like Episodes) (6). Severe mitochondrial translation defects were observed in cells homoplasmic for the tRNA

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mutations, whereas cells containing 85-90% mutant genomes appeared phenotypically normal (4-6). Thus these mutations are functionally recessive with steep thresholds for expression of the pathogenic phenotype.

The rules that govern segregation of pathogenic mtDNA mutations in the female germline and in somatic cells are not well understood, but are clearly important in understanding the pathogenesis of these disorders. Studies of autopsy tissues from MELAS patients carrying the $tRNA^{leu3243}$ mutation have suggested that the overall proportion of mutant mtDNAs in an individual is primarily determined by segregation during oogenesis, and that variation between tissues can be explained largely (though not entirely) by random replicative segregative (7). In contrast, it has recently been reported that mtDNAs carrying this mutation have a marked replicative advantage over wild-type mtDNAs in cultured cybrid cells in non-selective medium (8). While isolating cybrid cells to study the molecular mechanism of the translation defect produced by this mutation we obtained strikingly different results, showing that segregation of this mutant mtDNA is a completely stochastic process under non-selective conditions.

MATERIALS AND METHODS

Cells lacking mtDNA (rho^o cells) were made from an osteosarcoma cell line ($143B.TK^-$) and by long-term growth in ethidium bromide (40 ng/ml)(9). These cells are resistant to 5-bromo-2'-deoxyuridine (BrdU) and, because they lack a functional respiratory chain, auxotrophic for pyruvate and uridine (10). The rho^o cells were passaged in DMEM containing 5% fetal bovine serum, BrdU (100μg/ml), pyruvate (100μg/ml) and uridine (50μg/ml) (complete medium). Clonal myoblast cultures were established (4) from a patient carrying the $tRNA^{leu3243}$ mutation. The proportion of wild-type and mutant mtDNAs was determined in single myoblasts from a heteroplasmic clone using a PCR-based test in which the mutant allele is detected by the presence of a new HaeIII site in the PCR product (11). The PCR primers used were at the following positions in the standard (12) mtDNA sequence: forward 3130-3149 and reverse 3404-3423. PCR products were labelled with [^{32}P]dCTP in the last cycle of PCR, digested with HaeIII and run on 12% polyacrylamide gels. Results were quantified on a Molecular Dynamics Phosphorimager. Cybrid cells were made by fusing cytoplasts made from the clonal myoblast culture (13) with rho^o cells (14). Briefly, myoblasts were enucleated on plastic discs by centrifugation in the presence of cytochalasinB (10μg/ml). They were allowed to recover (2 hr) and rho^o cells were plated onto the disc to form a confluent monolayer. The following morning cells were fused in 45% polyethylene glycol containing 10% DMSO. The cells were cultured in medium which selects against rho^o cells (minus pyruvate and uridine) and against nucleated myoblasts (plus BrdU). Although cells with no functional respiratory chain are negatively selected in this medium, cybrid cells with severe respiratory chain defects can survive and grow. Colonies were picked after 15-20 days and genotyped. Heteroplasmic clones were passaged for 90 days in complete medium and genotyped again.

RESULTS

All myoblasts in the clonal population used to make the cytoplasts were heteroplasmic for the $tRNA^{leu3243}$ mutation. The proportion of wild-type mtDNAs ranged from 0.06-0.54 with a mean of 0.23 (Figure 1a). Thus one can assume that all successful cybrid fusions initially

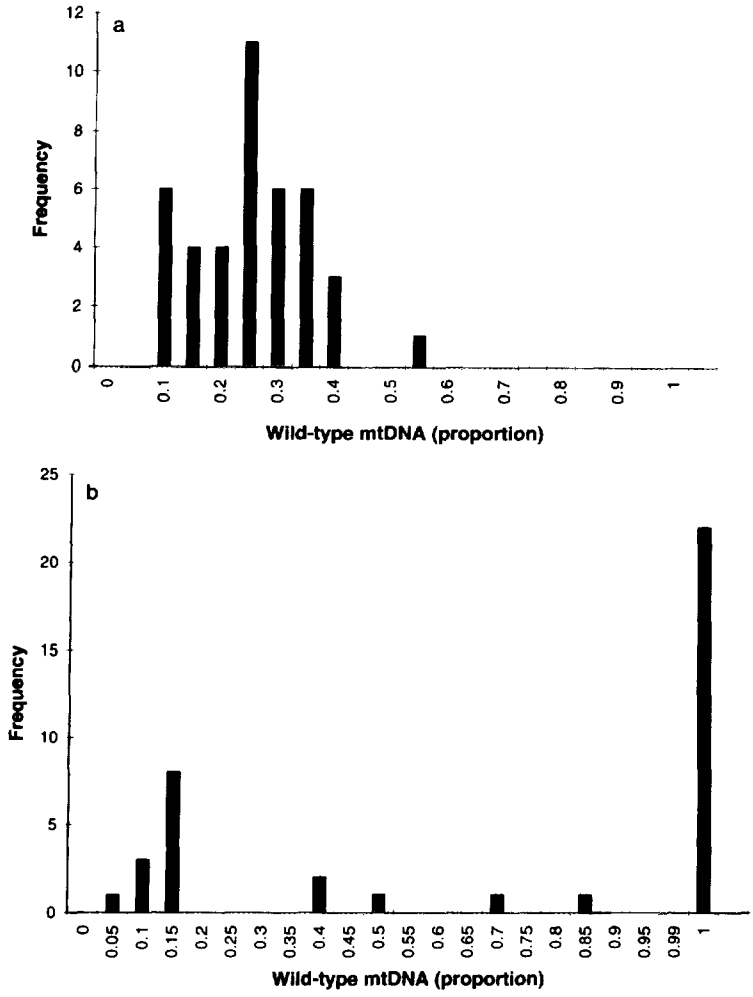

Figure 1. Frequency analysis of the proportion of mutant and wild-type mtDNAs in (a) the primary myoblast clone used to prepare cytoplasts and (b) cybrid clones picked after 15-20 days growth in selective medium. The values on the x axis correspond to the upper boundary of each bin used in the analysis. The 22 clones in the bin labelled 1 in (b) were homoplasmic for wild-type mtDNA.

contained a heteroplasmic population of mtDNAs which was predominantly mutant. Analysis of the cybrid clones that survived selection in medium containing BrdU, but lacking pyruvate and uridine, showed that the majority (56%) were homoplasmic wild-type (Figure 1b). The rest contained various proportions of mutant mtDNAs, however, none was completely homoplasmic for the mutant mtDNA species. To test whether segregation of mtDNAs was directional in the heteroplasmic clones, 15 of these clones were passaged in complete medium for 90 days then genotyped again (Figure 2). The mean frequency of wild-type mtDNA in all 15 clones was 0.25 at the start of this experiment (variance=0.058) and the mean frequency at the end of the

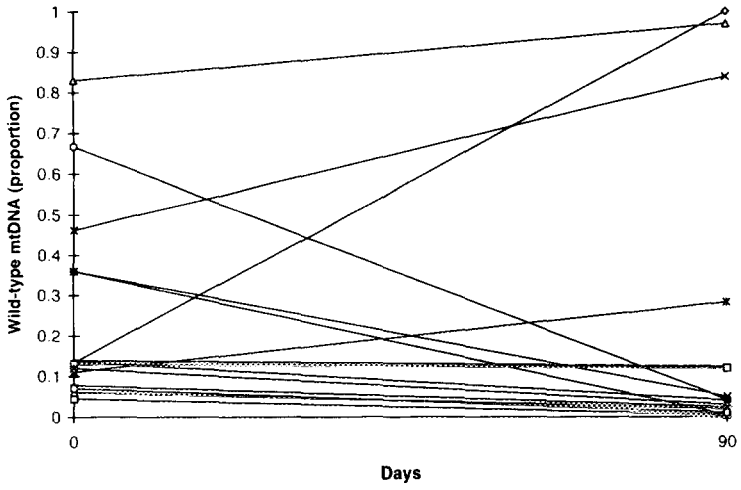

Figure 2. Change in mtDNA genotype in 15 heteroplasmic cybrid clones after 90 days of culture in non-selective medium. Each symbol represents an individual clone.

experiment was 0.24 (variance=0.136). Thus the mean was unchanged while the variance increased, characteristic of a completely stochastic process. Assuming that the doubling time of each clone was on the order of 24 hr (8) one can calculate the effective population size of the mtDNA pool (N) using a model borrowed from population genetics in which the variance (V) in the n^{th} generation is related to the initial gene frequency of one mtDNA genotype (p_o) by the equation $V_n=p_o(1-p_o)[1-(1-1/N)^n]$ (15). Calculation of this variable N gives a value of 5.5×10^3 which is close to the estimated mtDNA copy number of 9.1×10^3 in 143B cells (9).

DISCUSSION

Two principal conclusions can be drawn from this study. First, extremely rapid and selective amplification of wild-type mtDNAs occurs in successful cybrid fusions in selective medium. Second, segregation of the $tRNA^{3243}$ mutation in heteroplasmic cybrid clones is a stochastic process under non-selective conditions. Mitotic segregation of mtDNAs results from relaxed control of mtDNA replication during the cell cycle and the random partitioning of mtDNAs to daughter cells during cytokinesis (16,17). Although mtDNA copy number appears to be tightly regulated (by unknown mechanisms), individual mtDNA molecules may replicate more than once during the cell cycle (or not at all) and their progeny assort to the daughter cells without regard to ancestry. These factors lead to more rapid segregation than would be predicted from a truly random sampling of mtDNAs at each generation.

The rapid segregation to wild-type homoplasmy which we observed in the cybrid clones cannot be explained by random processes. The largest proportion of wild-type mtDNAs in the

initial cybrid population would have been about 0.50 and the cybrid clones in the present experiments were sampled and genotyped after the equivalent of 15-20 population doublings. The mtDNA copy number in 143B cells is ~10^4/cell and mtDNA in transmitochondrial cell lines constructed from 143B rho° clones repopulates to similar levels (9). The inescapable conclusion from these considerations is that replication of wild-type mtDNA occurred preferentially during mtDNA repopulation and clonal growth of the initial cybrid cell population. Whether this occurred at the cellular or organellar level (or both) is unknown; it is, however, difficult to imagine how intercellular selection alone could produce such a large shift in mtDNA genotype in such a small number of generations. A similar rapid and selective amplification of exogenous mtDNA has been reported in mtDNA-depleted 143B cells following micro-injection of a single mitochondrion carrying chloramphenicol-resistant mtDNA and growth on selective medium (18). This was attributed to an intracellular selective advantage of the mtDNA conferring drug resistance and a rapid restoration of mtDNA copy number in the injected cells following removal of ethidium bromide. In contrast, studies of segregation of chloramphenicol-resistant mtDNAs in cybrid or hybrid cells show relatively slow segregation, even in selective medium (19), suggesting that the rapid restoration of mtDNA copy number following fusion with a rho^0 (or mtDNA-depleted) partner might be the most significant determinant of rapid mtDNA segregation in cybrid cells.

Non-random replication of mtDNA in the initial cybrid cell could result from competition at the organelle level for a limiting essential factor, such that only a subset of all possible templates participate in mtDNA replication. Replication of mtDNA is dependent on tRNA primers generated from transcription initiation at the L-strand promoter (16). The only known transcriptional activator of mtDNA, mtTFA, is undetectable in cells lacking mtDNA although steady-state mRNA levels for this factor are unchanged, suggesting that mtDNA is necessary to stabilize the protein (20). One might predict that mtTFA would be relatively scarce in the initial cybrid cell, leading to competition for this factor and an effective genetic bottleneck. Such a model would, however, require a competitive advantage for mitochondria containing wild-type genomes to explain the bias in favour of homoplasmic wild-type clones observed in the present experiments. Whatever the mechanism, any replicative advantage for wild-type mtDNA in selective medium cannot be absolute since some heteroplasmic cybrid clones contained very high proportions of mutant mtDNAs.

The stochastic nature of the segregation process observed for the $tRNA^{leu3243}$ mutant in the heteroplasmic clones in non-selective medium contrasts with results of Yoneda et.al. (8) who reported a marked replicative advantage for the mutant mtDNA species. Although it is not clear why our results differ from those reported by Yoneda et.al. (and it may simply reflect the limited number of clones they analyzed), there are other differences which could have contributed. First,

their cybrids were derived from mixed fibroblast/myoblast cultures. The relative proportion of myoblasts or fibroblasts was unknown as was the degree of heteroplasmy in each cell type. It is possible that some of the cybrids they investigated were derived from fibroblasts and that mitochondria derived from fibroblasts and myoblasts behave differently. Second, the outcome of the segregation experiment may depend on the nuclear background of the rho^0 cells. Although the rho^0 cells we used were derived from the same parental osteosarcoma cell line (143B.TK$^-$), the line is likely highly aneuploid and individual clones could have different nuclear DNA backgrounds. In the present study marked differences were observed in clonal growth and survival in low concentrations of ethidium bromide; some clones did not survive this treatment and others grew very poorly. Since treatment with ethidium bromide selectively depletes mtDNA, this heterogeneity suggests that different clones have different abilities to survive respiratory chain dysfunction, likely reflecting different nuclear backgrounds. All of the cybrids analyzed by Yoneda et.al. (8) were constructed with the same rho^0 cell clone.

A stochastic model of segregation for this mutation in somatic cells is consistent with the observation of a nearly random distribution of mutant tRNAs in different tissues of individual MELAS patients (7). If mtDNAs carrying the $tRNA^{leu3243}$ mutation had a replicative advantage one would predict that mutant mtDNAs would accumulate to levels near the threshold for expression of a biochemical phenotype. Such an effect ought to be most apparent in cells with a rapid turnover, such as peripheral blood cells. In fact, the proportion of mtDNAs carry this mutation is always lower in peripheral blood than in skeletal muscle of MELAS patients (19).

Our results show that the dynamics of mtDNA segregation in cybrid cells can be complex depending on mtDNA copy number, selection and nuclear background and suggest caution in interpreting selection for different mtDNA genotypes.

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