Introduction of heteroplasmic mitochondrial DNA (mtDNA) from a patient with NARP into two human ρ° cell lines is associated either with selection and maintenance of NARP mutant mtDNA or failure to maintain mtDNA

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Mitochondria from a patient heteroplasmic at nucleotide position 8993 of mitochondrial DNA (mtDNA) were introduced into two human tumour cell lines lacking mtDNA. The donor mitochondria contained between 85 and 95% 8993G:C mtDNA. All detectable mtDNA in the mitochondrially transformed cells contained the pathological 8993G:C mutation 3 months after transformation. These results suggest that 8993G:C mtDNA had a selective advantage over 8993T:A mtDNA in both lung carcinoma and osteosarcoma cell backgrounds. In contrast, two other presumed pathological mtDNA variants were lost in favour of ‘wild-type’ mtDNA molecules in the same lung carcinoma cell background. Taken together, these findings suggest that the transmission bias of mtDNA variants is dependent upon a combination of nuclear background and mtDNA genotype. A second phenomenon observed was a marked decrease in the growth rate of many putative transformed cell lines after 6 weeks of culturing in selective medium, and in these cell lines mtDNA was not readily detectable by Southern blotting. Restriction endonuclease analysis and sequencing of amplified mtDNA demonstrated that the slow growing cells contained little or no mtDNA. It is concluded that these cells represented transient mitochondrial transformants.

INTRODUCTION

Mutations in mitochondrial DNA (mtDNA) are now a well-recognized cause of disease (1). A common phenomenon in mitochondrial disease is heteroplasmy, i.e. the co-existence of mutant and wild-type mtDNA molecules. Of critical importance to these diseases is the mechanism(s) of transmission of different mtDNA genotypes. Several studies of pathological mtDNA mutations demonstrated biased transmission of human mtDNA variants in cultured cells (2–6). Biased segregation of 8993G:C mtDNA in vivo was implied by an analysis of seven oocytes, six of which contained predominantly 8993G:C mtDNA, whereas the seventh egg contained solely 8993T:A mtDNA (7).

The 8993G:C mtDNA mutation, located in the gene encoding ATP synthase subunit 6, was identified in a pedigree with a complex neurological disorder termed NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa) (8). The same mutation subsequently was found to be associated with maternally inherited Leigh’s syndrome (MILS) (9).

Human ρ° cell lines have been widely used in mitochondrial transformation studies to examine the phenotypic effects of presumed pathological mtDNA mutations in control nuclear backgrounds (2–6,10–12). Using this system, Trounce et al. (13) demonstrated that the 8993G:C mtDNA genotype was associated with a decrease in the rate of coupled respiration in isolated mitochondria.

In this study, mitochondria were transferred from enucleated fibroblasts carrying a mixture of 8993G:C and 8993T:A mtDNA to osteosarcoma and lung carcinoma ρ° cell lines. Mitochondrially transformed cells (cybrids) were selected on the basis of their ability to grow in medium without uridine, whereas untransformed ρ° cells died in this medium (14,15). DNA was harvested from cybrid clones at various times after fusion to assess the level of 8993G:C and 8993T:A mtDNA, and thereby determine whether or not mtDNA segregation occurred with time.

RESULTS

The detectable mtDNA in all the lung carcinoma and osteosarcoma cybrids screened carried the 8993G:C mutation associated with NARP 3 months after mitochondrial transformation. The mitochondrial transformant cybrid lines could be divided

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Figure 1. Fusion 1, lung carcinoma cybrids transformed with heteroplasmic NARP mitochondria were homoplasmic for mutant mtDNA at first screening, or else segregated to homoplasmic mutant over time. Amplified mtDNA (8278–9409) was labelled during the last cycle using [32P]dCTP (NEN), digested with SmaI that cleaves mtDNA containing the 8993G:C NARP mutation and fragments separated on a 6% polyacrylamide gel. Lane 1, nuclear-digested with PvuII and fragments separated on a 0.7% agarose gel. The filter was probed simultaneously with purified placental mtDNA and nuclear 18S rDNA. A second clone (data not shown) also contained a normal complement of mtDNA.

Figure 2. Fusion 2, many lung carcinoma cybrids transformed with heteroplasmic NARP mitochondria contain little or no detectable mtDNA on Southern blotting. Whole-cell DNA was digested with PvuII and fragments separated on a 0.7% agarose gel. The filter was probed simultaneously with purified placental mtDNA and nuclear 18S rDNA. A third clone (data not shown) also contained a normal complement of mtDNA.

Figure 3. Quantification of NARP mutant mtDNA in lung carcinoma cybrids. NARP cybrids with a normal complement of mtDNA (lanes 1 and 8, Fig. 2) are homoplasmic for the NARP mutant (8993G:C) mtDNA (lanes 1 and 8 in this figure). NARP cybrids with little or no mtDNA on Southern blotting (lanes 2–7) have little or no NARP mutant mtDNA but a substantial amount of 8993T:A DNA, that subsequently was shown to be nuclear in origin (see text and Fig. 2 for details). Nested PCR products of the region 8539–9211 were digested with SmaI and separated on a 6% polyacrylamide gel.

The proportion of 8993G:C (NARP mutant) mtDNA was quantified in the patient-derived fibroblasts before and after the fusion experiments and found to be 85 and 95%, respectively (data not shown). Thus, it appears that segregation to NARP mutant mtDNA was occurring in the patient’s cultured fibroblasts during the period when the fusion experiments were performed. Eight clonal cell lines were expanded from the first fusion of enucleated heteroplasmic 8993 cells and lung carcinoma ρ° cells. DNA was amplified directly from sonicated cells ~21 days after fusion. At later time points, ~50 and 80 days after fusion, DNA was extracted from 5 × 10^6 cells prior to amplification. Six clones were homoplasmic for 8993G:C mtDNA at first screening, and were unchanged at subsequent time points (two examples are shown in Fig. 1, lanes 8–13). Two clones were heteroplasmic at first screening, but were homoplasmic for 8993G:C mtDNA at later time points (Fig. 1, lanes 2–7). Thus segregation to NARP mutant mtDNA occurred in these two cell lines and may well have occurred in some or all of the other cybrids with 100% 8993G:C mtDNA prior to first screening.

A second fusion of the same type was performed and a further 15 cell lines analysed. Southern blotting and hybridization suggested that only three of the 15 clones contained a normal amount of mtDNA; the remainder had little or no detectable mtDNA (Fig. 2). PCR and restriction enzyme analysis indicated that the three cell lines with normal mtDNA copy number were homoplasmic for the NARP mutation. Moreover, the analysis confirmed that eight cell lines contained no mtDNA, as distinct from nuclear mtDNA sequence (pseudogene). A description of how mtDNA was distinguished from nuclear mtDNA sequence appears at the end of the results. The remaining four clones yielded a mixture of NARP mutant mtDNA and pseudogene after nested PCR (Fig. 3). The ratio of mitochondrial to nuclear DNA was <1:1 in all four clones, and <1:9 in three clones, suggesting that many of the cells in these cell lines had fewer than one copy of mtDNA per cell. Although all the transformant cell lines grew normally in the weeks immediately after transformation, by 2 months post-fusion there was a marked decrease in growth rate of the clonal cell lines with little or no mtDNA compared with cells containing a normal mtDNA copy number.

To test the behaviour of NARP mutant mtDNA in a second control nuclear background, the same patient-derived cells were enucleated and fused to osteosarcoma ρ° cells. Of eight putative transformants analysed, four were homoplasmic for NARP mutant mtDNA at first screening, and had normal mtDNA copy number, whereas four contained only nuclear mtDNA pseudogene (data not shown).

Those clones devoid of mtDNA, as judged by nested PCR and restriction fragment length polymorphism (RFLP) analysis, are by definition ρ° cells. However, it is highly unlikely that they were untransformed ρ° cells that somehow proliferated under selective conditions. First, in all other cytoplast–ρ° cell fusion experiments performed in our laboratory, putative
mitochondrial transformants capable of growing without uridine for 2 weeks or more have always contained mtDNA, although two osteosarcoma cybrids with predominantly partially duplicated mtDNA were observed to lose their mtDNA over time (6). Second, four of the putative mitochondrial transformed cell lines contained a low level of mtDNA that harboured the NARP mutation (as described above and documented in Figure 3). In contrast, Smal did not cleave nuclear mtDNA pseudogenes amplified from osteosarcoma and lung carcinoma p* cells (data not shown).

We infer that all the clonal cell lines grown in the absence of uridine for 4 weeks or more after fusion were true mitochondrial transformants, but many of these cybrids proved incapable of propagating the mtDNA they received at transformation. Nevertheless, the mtDNA received from the donor cytoplasts was presumably functionally expressed in all cybrids otherwise those cells unable to propagate mtDNA would have been phenotypically indistinguishable from p* cells, i.e. they would not have grown at a normal rate in the absence of uridine for >4 weeks. Thus, in the absence of propagation, donor mtDNA was diluted with each cell division, eventually leading to respiratory insufficiency and cell death. Cells that received mtDNA, but were unable to propagate it, are henceforth described as transient NARP cybrids.

The osteosarcoma and lung carcinoma p* cell lines used in this study have been repopulated previously with mitochondria from a normal control subject. The resultant cybrids were found to have a normal mtDNA copy number and to be respiratory competent (16; I.J. Holt and D.R. Dunbar, unpublished data).

Previously we have found comparison of lactate with pyruvate production in cultured cells to be a sensitive measure of respiratory function. Therefore, the ratio of lactate to pyruvate (L/P) production was measured for two lung carcinoma NARP cybrids that appeared to be heteroplasmonic at first screening. The amplified product was digested with Smal to remove most, if not all, of the 8993G:C-containing DNA. Uncut PCR product was used as template for a second round of PCR with primers C and D (8539–9211). Some of the amplified product contained the additional Sma I site compared with cells with 8993G:C mtDNA (Fig. 4A).

The clones must therefore have received both 8993G:C and 8993T:A mtDNA in this region once at 8883 and additionally at 8718 in the corresponding nuclear mtDNA sequence. Less than 1% of the amplified product contained the additional Dde I site (Fig. 4A, lane 2). It is concluded that the 8993T:A DNA of these two NARP cybrids was mtDNA, not nuclear mtDNA pseudogene. The clones must therefore have received both 8993G:C and 8993T:A mtDNA from the mitochondrial donor fibroblasts used in the fusion experiment. Figure 4B shows in cartoon form how Smal and Dde I differentiate between NARP mutant mtDNA, wild-type mtDNA and nuclear mtDNA pseudogene.

Distinguishing mitochondrial genome DNA from nuclear mtDNA sequence

Estimating mtDNA copy number and the proportion of mutant mtDNA in NARP cybrids was complicated by the fact that nested PCR was necessary for some samples. Nested PCR generated product from the two recipient p* cell lines that lacked mtDNA, implying that nuclear mtDNA pseudogene had been amplified. Nuclear mtDNA sequences are believed to have arisen during evolution, via transfer from mitochondrial to nuclear DNA. This phenomenon has been described in a number of organisms including human (17), and its importance when quantifying mtDNA mutations has been discussed recently (18). To test this assumption, three nested PCR products were sequenced. The samples derived from: the mitochondrial donor fibroblasts of the heteroplasmic NARP patient (A); a NARP cybrid with normal mtDNA copy number (B); and a transient NARP cybrid with no 8993G:C DNA (e.g. lanes 18–21, Fig. 3) (C). Prior to the second round of amplification, sample A was digested with Smal that cleaves NARP mutant mtDNA at nucleotide position 8993 to ensure that amplified product from the second round of amplification was substantially 8993T:A DNA. Nested PCR products from samples A–C were sequenced directly. Sample A was identical to the published sequence (19) and sample B differed from the published sequence only at nucleotide position 8993 (i.e. carried the NARP mutation). Sample C had four differences from the Cambridge sequence (19) in a short stretch of DNA (nucleotides 8700–9000), but lacked the NARP mutation at 8993. Sample C was therefore adjudged to be nuclear mtDNA pseudogene. One of the four differences, an A to G transition at nucleotide pair 8718, predicted a Dde I site gain. Dde I was therefore used to screen nested PCR products from p* cells and a panel of transient and permanent NARP cybrids. The 8539–9211 PCR products of transient NARP cybrids and p* cells contained an additional Dde I site compared with cells with normal mtDNA copy number (Fig. 4A).

Nuclear mtDNA sequence presented a further complication in that it might account for the 8993T:A PCR product of the presumed heteroplasmic lung carcinoma NARP cybrids (Fig. 1, lanes 1 and 4). Therefore, the mtDNA region 8278–9409 was amplified from the cybrids that appeared to be heteroplasmic at first screening. The amplified product was digested with Smal to remove most, if not all, of the 8993G:C-containing DNA. Uncut PCR product was used as template for a second round of PCR with primers C and D (8539–9211). Some of the nested PCR product was digested with Smal; as expected, the enzyme did not cleave the vast majority of the DNA, indicating that 8993T:A DNA had been enriched. A separate portion of the second round product was digested with Dde I that cuts mtDNA in this region once at 8883 and additionally at 8718 in the corresponding nuclear mtDNA sequence. Less than 1% of the amplified product contained the additional Dde I site (Fig. 4A, lane 2). It is concluded that the 8993T:A DNA of these two NARP cybrids was mtDNA, not nuclear mtDNA pseudogene. The clones must therefore have received both 8993G:C and 8993T:A mtDNA from the mitochondrial donor fibroblasts used in the fusion experiment. Figure 4B shows in cartoon form how Smal and Dde I differentiate between NARP mutant mtDNA, wild-type mtDNA and nuclear mtDNA pseudogene.

DISCUSSION

NARP (or MILS) is associated with a thymine to guanine transversion at nucleotide position 8993 of human mtDNA (8,9). Here we report that the mutant 8993G:C genotype can be selected and stably maintained in a proportion of osteosarcoma and lung carcinoma cybrids. The NARP patient-derived 8993T:A mtDNA genotype was not propagated in either nuclear background. The biased transmission of 8993G:C mtDNA over 8993T:A mtDNA in cell cybrids was paralleled in the oocytes of a mother with an affected child, where six of seven oocytes had predominately 8993G:C mtDNA (7). Taken together, these studies strongly suggest that 8993G:C mtDNA can be fixed via selection, not merely genetic drift.
The molecular basis of the selective advantage of 8993G:C mtDNA is not known. It has been proposed that mitochondria with impaired respiratory function sequester limiting proliferation factors, so that mitochondria with high levels of mutant DNA and impaired respiratory capacity proliferate at the expense of organelles with wild-type mtDNA. In this model, the more deleterious a particular mutation is, the greater is the expected selective advantage. Our findings are difficult to reconcile with this model as to date we have found no marked respiratory phenotype in cybrids with NARP mutant mtDNA.

Mitochondrial DNA depletion is a well-recognized cause of disease (20) and it is known from the genetics of the syndrome that nuclear factors are involved. It is possible that mtDNA depletion is the result of nuclear–mitochondrial incompatibility and requires a minimum of two mutations, one in mtDNA and one in nuclear DNA. This model assumes that individually each mutation is benign. All forms of mtDNA depletion represent a failure of copy number control. Numerous factors, involved in mtDNA replication, resolution of daughter molecules and mitochondrial (DNA) partitioning, are required for transmission and maintenance of mtDNA. However, to date, only one factor has been demonstrated to be essential for maintenance of mammalian mtDNA, TFAM (21). Defective mtDNA partitioning could explain both the phenomena reported here, if it is assumed that a partial defect was associated with failure to partition 8993T:A-containing mtDNA normally, whereas a severe defect led to complete failure of mtDNA partitioning, and thereby depletion. This in turn would imply that there was at least one additional unrecognized mutation in the molecules with 8993T:A mtDNA. Conceivably, the primary mutation in NARP may be elsewhere than nucleotide 8993 and cause loss of mtDNA at high frequency. 8993G:C mtDNA would have a selective advantage if it ameliorated this tendency to lose mtDNA.

Mitochondrial DNA from one patient with mtDNA depletion was propagated successfully on transfer to the lung carcinoma p° cells used in this study (22), suggesting that a different problem underlies the failure to maintain mtDNA reported here. Fusion experiments with other cytoplast donors have been performed in our laboratory subsequent to those reported here, and all cybrids screened contained a normal complement of mtDNA. Thus, the inability of many clones to propagate mtDNA was related to NARP mtDNA of the patient-derived cytoplasts and did not reflect a general decline in the p° cells.

Non-random segregation of pathological and wild-type mtDNA molecules in osteosarcoma and lung carcinoma cells has been observed previously. The 3243G:C mtDNA mutation associated with MELAS was selected in osteosarcoma cybrids (3), whereas 3243A:T mtDNA was selected in lung carcinoma cybrids (5). Partially duplicated mtDNAs can be maintained in osteosarcoma cells, whereas they are lost in favour of mtDNA molecules of normal size in lung carcinoma cybrids (6). In the present study, however, NARP mutant mtDNA was propagated in both lung carcinoma and osteosarcoma cybrids. Thus, the lung carcinoma cell background does not select against all pathological mtDNA variants, indicating that mitochondrial genotype as well as nuclear genetic background plays a role in determining transmission bias. The different segregation bias of mixtures of mtDNA molecules in human cultured cells (2–6; this study) suggest that mtDNA transmission biases in vivo are likely to be complex, and on this basis genetic counselling should proceed with caution.

**MATERIALS AND METHODS**

The fibroblasts derived from a member of the original NARP pedigree, 3 (8), The standard growth medium for all the cells used in the study was Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose and 110 mg/l sodium pyruvate, with 10% fetal bovine serum. p° cells were, in addition, supplemented with 200 µM uridine, and osteosarcoma p° and p° cells were grown in the presence of 100 µM bromodeoxyuridine. Patient-derived fibroblasts were enucleated by centrifugation at 7000 g in DMEM containing 10 µg/ml cytochalasin B (Sigma, Poole, UK), and the resultant cytoplasts fused to p° cells using polyethylene glycol 1500 (Merck, Poole, UK) as described previously (15).
DNA was extracted from cells lysed in 0.5% SDS, 0.075 M NaCl, 0.05 M EDTA, pH 8, and incubated with 0.5 μg/ml proteinase K for 16 h at 37°C, by precipitation with an equal volume of isopropanol (23). Primers A and B were used for the first round of amplification, and primers C and D were used for nested PCR: A, 8278–8298 5′–3′ CTA CCC CCT CTA GAG CCC ACT; B, 9409–9390 5′–3′ TGG TAT GTG CCT TCT CTT GT; C, 8539–8558 5′–3′ CTT TTC GCT TTA TTC ATT GC; D, 9211–9192 5′–3′ GTG ATT ATG TGT TGT CGT GC.

Amplification conditions for oligonucleotide primers A and B were 30 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 100 s. DNA amplification with oligonucleotides C and D was achieved after 20–25 cycles of 94°C for 60 s, 60°C for 50 s and 72°C for 55 s.

Southern blotting and hybridization were carried out as described in Sambrook et al. (24). Purified placental mtDNA and an 18S rDNA clone were used as mitochondrial and nuclear probes, respectively. The proportion of 8993G:C and an 18S rDNA clone were used as mitochondrial and nuclear probes, respectively. The proportion of 8993G:C and an 18S rDNA clone were used as mitochondrial and nuclear probes, respectively.

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