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Abstract In order to study the still poorly understood dynamics of mitochondrial gene segregation, we attempted to alter the percentage of deleted mtDNA (del-mtDNA) over wild-type mtDNA in cell-culture by manipulating respiratory chain capacity. For this purpose, we used a cell-line harbouring a 6-kb mtDNA-deletion which normally was present in 70% of the molecules. The results show that in the presence of low concentrations of doxycycline (DC), an inhibitor of mitochondrial protein synthesis, the average percentage of del-mtDNA in culture steadily declined. After short-term DC treatment most cells still contained del-mtDNA and removal of DC led to a rapid increase in the proportion of del-mtDNA. In contrast, long-term DC treatment rendered del-mtDNA undetectable by Southern analysis, reflecting the complete absence of del-mtDNA in most cells. In this case, del-mtDNA in culture remained at a constant low level after removal of the drug. The results clearly show the importance of phenotypic selection in the segregation of a deleterious mtDNA mutation.

Key words Human mtDNA · Deletion · Segregation · Phenotype selection

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Introduction

Mammalian mitochondrial DNA (mtDNA) contains a small, but essential, set of genes (Anderson et al. 1981; Bibb et al. 1981). Thirteen genes code for subunits of enzyme complexes involved in oxidative phosphorylation (OXPHOS). The other genes encode two rRNAs and 22 tRNAs that are essential for mitochondrial protein synthesis (Nelson 1987). The importance of the mitochondrial genome is indicated by the occurrence of a multitude of disorders resulting from specific mtDNA mutations (Wallace 1992). In most cases, mutant mtDNA molecules are found together with wild-type (wt) mtDNA within a tissue or cell type, a situation known as heteroplasmy. It is widely accepted that the distribution of different ratios of mutant to wt-mtDNA in different tissues is important in determining the clinical phenotype. Generally, heteroplasmy is known to be intracellular, and several reports suggest that heteroplasmy is intra-mitochondrial, in both humans and *Drosophila* (Lécher et al. 1994; Attardi et al. 1995; Matthews et al. 1995).

Single, heteroplasmic mtDNA deletions in humans are commonly linked with Chronic Progressive External Ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), Pearson syndrome, and other disorders (Holt et al. 1988; Rötig et al. 1989). These partial deletions, which generally remove several protein-coding and tRNA genes, are believed to arise spontaneously during oogenesis (Holt et al. 1988). Low levels of del-mtDNA (del-mtDNA) are detectable in the oocytes of healthy women (Chen et al. 1995), although the chance that these molecules will contribute to the developing foetus is very small (Hauswirth and Laipis 1985; Chen et al. 1995). This is consistent with the fact that the above disorders are rare. When a mutant mitochondrial genome is propagated in the foetus its eventual representation and tissue distribution are presumed to be strongly influenced by its replication and segregation behaviour. For example, due to their smaller size del-mtDNA molecules may possess an inherent replicative advantage over wt-mtDNA molecules (Wallace 1989; Shoubridge

et al. 1990), at least in some cell types, resulting in their accumulation over time, as has been observed in a patient with KSS (Larsson et al. 1990). The segregation behaviour of del-mtDNA can also depend on cell type (Bourgeron et al. 1993), and differentiation status (Collombet et al. 1996). It has been suggested that not only del-mtDNA but also mtDNA containing a point mutation may accumulate with time, in patients as well as in cell-culture (Yoneda et al. 1992; Attardi et al. 1995). Nuclear background was shown to influence this behaviour (Dunbar et al. 1995), which raises questions regarding the mechanism(s) of the replicative advantage of any mutant mtDNA, including mtDNA molecules of smaller than wild-type size, and its relationship with the phenotypic effects of the mutation.

For example, a lymphoblast cell-line containing a 6-kb mtDNA deletion maintained high levels of del-mtDNA (60–70%) and did not show severe mitochondrial dysfunction despite a 50% reduced synthesis of the proteins encoded by the deleted genes (Spelbrink et al. 1994). This could be explained on the basis of an over-capacity of mitochondrial protein synthesis in this cell type. Differences in mitochondrial protein synthesis between patient-derived and control cell-lines was demonstrated by showing that the patient cell-line was more sensitive to low concentrations of doxycycline (DC), an inhibitor of mitochondrial protein synthesis. This led to the idea that treatment with low DC concentrations could be used to select against cells with high percentages of del-mtDNA.

In the present paper, the results of DC treatments of a cell-line having an initially stable population of wt- and del-mtDNA molecules are reported. The del-mtDNA population was systematically manipulated by changing the DC regime and the culture conditions. The results indicate that del-mtDNA molecules are preferentially amplified resulting in their increase to, or maintenance at, a threshold level, above which their presence appears to confer a phenotypic disadvantage.

Materials and methods

Patient and cell-line. The lymphoblast cell-line (P) used in this study was created from cells of a patient with Pearson's syndrome. The clinical, biochemical and molecular genetic features of the patient and cell-line have been described previously (De Vries et al. 1992; Spelbrink et al. 1994). A 6-kb deletion from within the COX II gene to within the ND5 gene (bp 7778–13 794) was identified in this patient.

Cell-cultures. Patient-derived and control lymphoblastoid cells were cultured in RPMI 1640 (2 g/l glucose) or Dulbecco's Modified Eagle (DME) medium (4.5 g/l glucose) containing 1 mM pyruvate supplemented with 10% heat-inactivated foetal calf serum (FCS). Cells were collected by centrifugation and washed three times with sterile PBS at 4°C. Cell-pellets were used immediately or stored at –70°C prior to DNA isolation. Cell concentrations were determined using a counting chamber. Doxycycline (DC, Sigma) was added to the culture medium to different final concentrations, as indicated in the figure legends, from a stock solution in 70% ethanol. Doxycycline is believed to inhibit mitochondrial protein synthesis by binding to the small ribosomal subunit and preventing tRNA-binding.

Mitochondrial DNA and mRNA analysis. Total cellular DNA was isolated according to standard procedures (Sambrook et al. 1989), and DNA concentrations were measured spectrophotometrically. Approximately 3 µg of DNA was digested with *Eco*RI or with *Pst*I (as indicated), subjected to electrophoresis using 0.7% agarose TBE gels, and blotted overnight onto Hybond N^+ membranes (Amersham) using 20×SSC. To detect both normal mtDNA and mtDNA containing the 6-kb (np 7778–13 794) deletion, an M13 recombinant (mp18.XH5.2, King and Attardi 1993) containing most of the COX I gene was used to probe *Eco*RI-digested DNA. To probe DNA digested with *Pst*I, a second clone containing most of the cyt *b* gene (mp18.XK76) was used. The above site-specific probes, in contrast to the use of a total mtDNA probe, avoid having to correct for differences in the length of the tested mtDNA species. In some cases, a PCR-derived probe for 18S rRNA (Gonzalez and Schmickel 1986) was used to detect nuclear DNA. Total cytoplasmic RNA was isolated from NP-40-lysed cells by phenol extraction and analysed by Northern hybridisation after electrophoresis in formaldehyde-agarose gels (Sambrook et al. 1989; Spelbrink et al. 1994). A cDNA probe for 18S ribosomal RNA was used as a reference for the amount of total cytoplasmic RNA. Probes for the detection of COX I and COX II mRNAs were as described previously (Spelbrink et al. 1994). Generally, 10–20 ng of probe was labelled to an activity of 10–20 µCi, using random priming (Feinberg and Vogelstein 1983). Blots were hybridised as described (Church and Gilbert 1984) and washed several times at 65°C with 45 mM NaCl, 4.5 mM sodium citrate, 0.1% sodium dodecyl sulphate.

PCR and single-cell PCR. For the detection of small amounts of del-mtDNA, total cellular DNA was isolated and used in a 50-µl PCR reaction, containing 50 ng of each forward and reverse primer (as below), 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 5% DMSO, 200 µM of each dNTP and 0.8 U *Taq* polymerase (Gibco BRL). The following PCR program was used: 40 cycles; 30 s denaturation at 94°C, 1.5 min annealing at 53°C and 2 min extension at 72°C. For the detection of del-mtDNA the following primers were used: forward primer, 5′-CAC ACA TTC GAA-GAA CCC GT-3′ (mtDNA bp 7410–7429, numbering according to Anderson et al. 1981); reverse primer, 5′-TCT AGG GCT GTT AGA AGT CC-3′ (mtDNA bp 13 844–13 825), resulting in a product of 399 bp. These primers did not give a product with wt-mtDNA because the length of the region which has to be amplified is too great under the conditions employed. For the detection of normal (wt) mtDNA the following primers were used: forward primer, 5′-CCT CCC TGT ACG AAA GGA CA-3′ (mtDNA bp 3116–3135); reverse primer, 5′-GTG AAG-AGT TTT ATG GCG TC-3′ (mtDNA bp 3476–3457), resulting in a product of 361 bp. Single cells, which were used for PCR detection of del- or wt-mtDNA, were isolated as follows. Cultured cells were diluted to 1 cell/µl in sterile PBS at 4°C and 1-µl drops were pipetted on a sterile Petri dish. Each drop was subsequently examined microscopically (100–200 × magnification) for the presence of a single cell, and transferred to a reaction vessel containing 9 µl of sterile water. Samples were heated for 10 min at 95°C, centrifuged for 5 min at 12 000 g_{max}, and supernatants used for PCR amplification (Erickson and Castora 1993).

Miscellaneous. The activity of cytochrome *c* oxidase was measured spectrophotometrically (Van Kuilenburg et al. 1991) at 20°C in 30 mM phosphate buffer (pH 7.4) in cell lysates obtained by laurylmaltoside treatment as described previously (Spelbrink et al. 1994), using 20 µM of reduced bovine heart cytochrome *c* as the substrate. The activity was expressed as a first-order rate constant, *k*, per min. Citrate synthase activity was also measured in cell lysates obtained by lauryl maltoside treatment, essentially as described by Srere (1969). Hybridisation signals with [^{32}P]-labelled probes were measured using a PhosphorImager™ (Molecular Dynamics, Sunnyvale, Calif., USA).

Results

The effects of phenotypic selection on mitochondrial gene segregation remain poorly understood. In order to address

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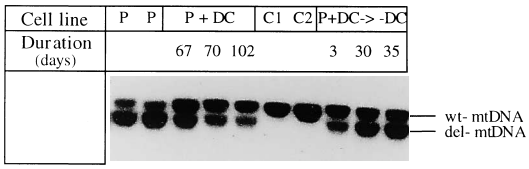

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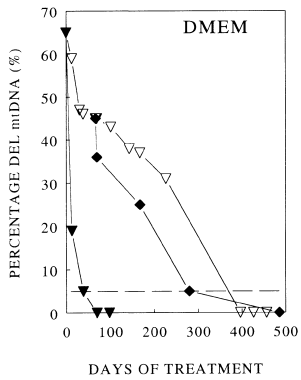

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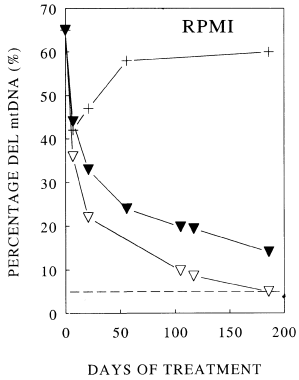

Fig. 1A–C Treatment of Pearson lymphoblasts with low concentrations of doxycycline. Pearson lymphoblasts (*P*) were grown in the presence of 3 or 6 μg of DC/ml of DME medium, for a period of up to 1.5 years. Total cell DNA was isolated and digested with *Pst*I. Southern blots were hybridised with a single-stranded DNA probe against the cyt *b* gene, which detects both wt- and del-mtDNA. DNA digested with *Pst*I, results in a band from wt-mtDNA of 14 459 bp and a band of del-mtDNA of 10 552. **A** a sample autoradiogram showing the steady decline in the average percentage of del-mtDNA in culture, caused by proliferation of the cells in the presence of 3 μg of DC/ml (*P + DC*). Duration = the time cells were treated with DC starting from the first day of addition, or the time cells were grown in the absence of DC counting from the first day of omission. Part of the P + DC cell-culture was propagated without DC after 102 days of DC treatment (*P + DC → –DC*), which resulted in an increase in the percentage del-mtDNA. *P* Pearson cells cultured without DC. *C1*, *C2* control cell-lines. **B** graphical representation of the decline in the percentage of del-mtDNA in Pearson cells, cultured in the presence of 3 (▽) or 6 (▼) μg DC/ml. Cells treated for 10 weeks with 3 μg of DC/ml were propagated in medium containing 6 μg of DC/ml (◆) and further cultured in the presence of 6 μg/ml. The percentage of del-mtDNA was determined after the quantification of hybridisation signals. The combined results of several Southern blots are shown. The *dashed horizontal line* represents the detection limit by Southern analysis [a del-mtDNA level, barely detectable by eye, was quantified as 7% (see Fig. 4); below this level the signal for wt-mtDNA results in too-high background levels to distinguish del-mtDNA]. The results demonstrate that after long-term treatment with 3 μg of DC/ml, del-mtDNA can no longer be detected. Treatment with 6 μg of DC/ml resulted in undetectable del-mtDNA levels after 50 days of treatment. *Wt-mtDNA* = wild-type mtDNA; *del-mtDNA* = mtDNA harbouring a 6-kb mtDNA deletion. **C** Pearson lymphoblasts, which were cultured in DME medium, were transferred at 0 days to RPMI medium (+), RPMI + 3 μg DC/ml (▽) or RPMI + 6 μg DC/ml (▼). DNA was isolated and analysed at several time points following this change of culture medium. The results show that transition of Pearson cells to a poorer medium (RPMI) (Van den Bogert et al. 1992) results in a more rapid effect of treatment with 3 μg of DC/ml. Cells treated with 6 μg of DC/ml showed a slower decline in del-mtDNA, possibly caused by a severely reduced growth rate

this problem we attempted to systematically manipulate the segregation of a 6-kb mtDNA deletion in cultured lymphoblasts, by imposing stress on oxidative phosphorylation.

Selection against an apparently stable mtDNA deletion in Pearson lymphoblasts

The deletion in the Pearson cell-line used here has its breakpoints in the COXII and ND5 genes. No *Bgl*II restriction site is present in human mtDNA (Anderson et al. 1981). Total cellular DNA of the Pearson cell-line digested with *Bgl*II and hybridised with a COX I probe only showed full-length and del-mtDNA in relative concentrations of 30% and 70% respectively. No partially duplicated mtDNA was observed (data not shown).

Based on previous results (Spelbrink et al. 1994), we reasoned that the presence of del-mtDNA could be rendered phenotypically disadvantageous, and hence potentially selected against, in the presence of low concentrations of doxycycline (DC). Figure 1 a/b shows that the average percentage of del-mtDNA in Pearson (P) cells cultured in DMEM in the presence of 3 μg DC per ml rapidly decreased from 65% del-mtDNA to about 45% del-mtDNA. From this point on, it declined steadily to reach undetectable levels, based on Southern analysis, after a period of about 1 year. In the absence of DC the percentage of del-mtDNA in culture remained stable at 60–70% over a period of 3–4 years. This stability has also been observed by others (Bourgeron et al. 1993).

An aliquot of the cells that had been treated for 10 weeks with 3 μg/ml DC was subsequently cultured in 6 μg/ml of the drug. This initially accelerated the decline in del-mtDNA levels, after which a trend with a more gradual decline was resumed (Fig. 1b). Treatment with 6 μg of DC/ml, without pre-treatment at the lower dose, resulted in an immediate and rapid loss of del-mtDNA until it could no longer be detected (Fig. 1b).

Cell proliferation for 3–4 weeks in the presence of 15 μg/ml of DC, which fully inhibits mitochondrial protein synthesis (Van den Bogert et al. 1988; Spelbrink et al. 1994), did not result in selection against del-mtDNA. Thus, partial but not complete inhibition of mitochondrial protein synthesis results in a reduction in the average percentage of del-mtDNA in cell culture. This suggests that applying a stress that reduces the OXPHOS capacity affects cells with high del-mtDNA levels more than cells with low del-mtDNA levels, and that the observed effects are not the result of a property of DC other than the inhibition of mitochondrial protein synthesis.

As a control, to show that the original Pearson cell-culture was not contaminated with cells from other cultures that were subsequently selected during DC treatment, several of the subcultures and other cell-cultures that we normally use were subjected to a small-scale haplotype analysis using microsatellite markers. The original Pearson culture plus all subcultures had the same haplotype, which was different from the haplotypes of other cell-lines in use in the laboratory (data not shown).

Influence of culture conditions on the rate of loss of del-mtDNA

It has long been recognised that human cells cultured under different conditions are to a different extent dependent on mitochondrial function (Desjardins et al. 1985; King and Attardi 1989; Van den Bogert et al. 1992). Various cell types cultured in RPMI are highly dependent on mitochondrial function when compared with cells cultured in DMEM; a 50% decline in OXPHOS capacity in RPMI results in cellular dysfunction and a block to cellular proliferation (Van den Bogert et al. 1992). Thus, we expected that cells cultured in RPMI should be more sensitive to DC treatment, which was indeed observed at 3 μg of DC/ml (Fig. 1c). However, cells cultured in RPMI in the presence of 6 μg of DC/ml showed a slower decline in the level of del-mtDNA, most likely due to a significantly reduced growth rate under these conditions (as previously observed by Spelbrink et al. 1994). Cells transferred from DMEM to RPMI without DC treatment also showed an initial decline in the amount of del-mtDNA, although it gradually returned to the 60–70% level.

Effects of removal of DC on the del-mtDNA population

Figure 2 shows the effects when DC was removed from the medium after the cells had been cultured in DMEM in the presence of 6 μg of DC/ml for 5–6 weeks. Starting from a level of del-mtDNA that was just detectable, the percentage of del-mtDNA rapidly increased to levels of 50–60%. These results clearly demonstrated the preferential accumulation of del-mtDNA over wt-mtDNA. A preference for the amplification of del-mtDNA could be the result of several mechanisms, such as a replicative advantage due to its smaller size (Wallace 1989) or preferential proliferation of defective mitochondria containing high levels of del-mtDNA (Attardi et al. 1995 and see Discussion).

Removal of DC from cells that had been subjected to long-term treatment with 3 μg/ml of the drug in DMEM, after which del-mtDNA was undetectable by Southern analysis, gave a different result. Del-mtDNA was not re-established at high levels, even after prolonged culture of the cells without DC. This could have several interpretations: (1) that cells had lost del-mtDNA entirely; (2) that only a minority of cells still had del-mtDNA, in which case the maximal percentage del-mtDNA that could be present would be less than the percentage of cells that still had del-mtDNA; (3) that del-mtDNA was still present at low concentrations in most cells but that del-mtDNA no longer had an advantage because it was present in fully functional mitochondria. This last option seemed unlikely, given that cells harbouring as much as 60–70% del-mtDNA do not show a clear biochemical phenotype (see above). The first of these three options was excluded by demonstrating, using PCR, that del-mtDNA was still present in the culture (data not shown). To confirm that most cells no longer contained del-mtDNA, a PCR analysis on single cells was performed (Fig. 3). The results show that del-mtDNA could be detected in all single cells of the untreated Pearson cell-line. In most single cells isolated from subcultures in which we could no longer detect del-mtDNA by Southern analysis del-mtDNA was not detected by single-cell PCR. Combined with the observation that del-mtDNA could still be detected with PCR on DNA isolated from a large number of cells, this strongly suggests that only a small proportion of cells in culture still harboured del-mtDNA. The reliability of the method (see Materials and methods) is shown by PCR-analysis of single cells containing intermediate to high levels of del-mtDNA. In these cases, all single cells were shown to contain del-mtDNA and although we cannot fully exclude that more than one cell was present on some occasions it does show that in each single-cell isolate at least one cell was present. To further demonstrate that each sample contained a cell we only used half the sample volume for amplification of the del-mtDNA. The other half was used for amplification of wt-mtDNA. The sensitivity of PCR detection after 40 amplification cycles was demonstrated by the detection of a single-copy nuclear gene in single-cell isolates (data not shown). For this method we used a CCD camera that was about ten times more sensitive than the human eye. Although we could identify a single-copy gene in only two out of eight single cells, it proves that this method is capable of detecting only one or a few copies of a DNA molecule. Finally, several samples in which we could not detect any signal after the first 40 cycles were re-amplified for another 40 cycles. No del-mtDNA-specific band was observed after this second amplification round (data not shown).

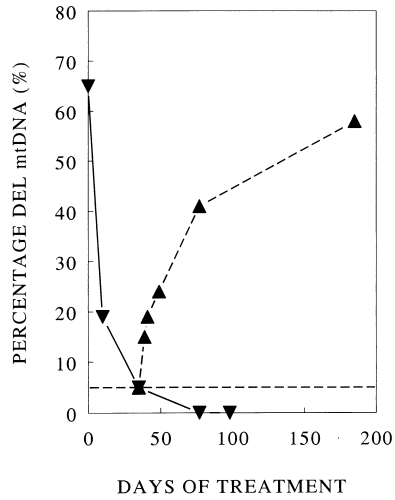

Fig. 2 The effect of transient DC treatment on the percentage del-mtDNA. Pearson lymphoblasts, cultured in DME medium, were treated with 6 μg of DC/ml (▼—▼) for 6 weeks after which DC was omitted (▲----▲). The percentage of del-mtDNA was determined at several time points. The graph shows the combined results of two 2 independent experiments. The results show that the percentage of del-mtDNA rapidly increased after stopping DC treatment, suggesting that del-mtDNA has a replicative advantage over wt-mtDNA

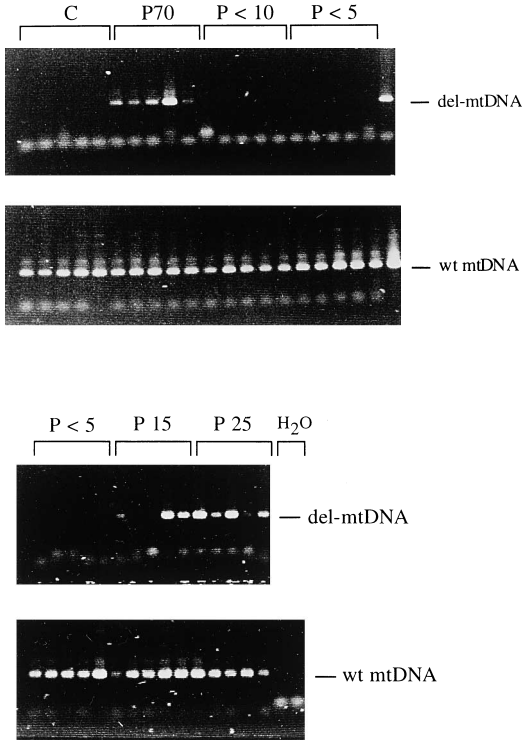

Fig. 3 PCR detection of del-mtDNA, using DNA isolated from single cells of various P subcultures. Five microliters of a 10-µl single-cell isolate was used for the detection of del-mtDNA by PCR (see Materials and methods). The other 5 µl was used for the detection of wt-mtDNA, as indicated. Shown are the amplification reactions from five single cells of various Pearson subcultures. The percentage of del-mtDNA, as determined by Southern analysis, is given for each P subculture. The *last lane* of the upper figure shows a positive control for del-mtDNA and wt-mtDNA. *C*=control lymphoblast line. The results demonstrate that, in cultures in which del-mtDNA was no longer detected by Southern analysis, the number of cells that still contain del-mtDNA must be low

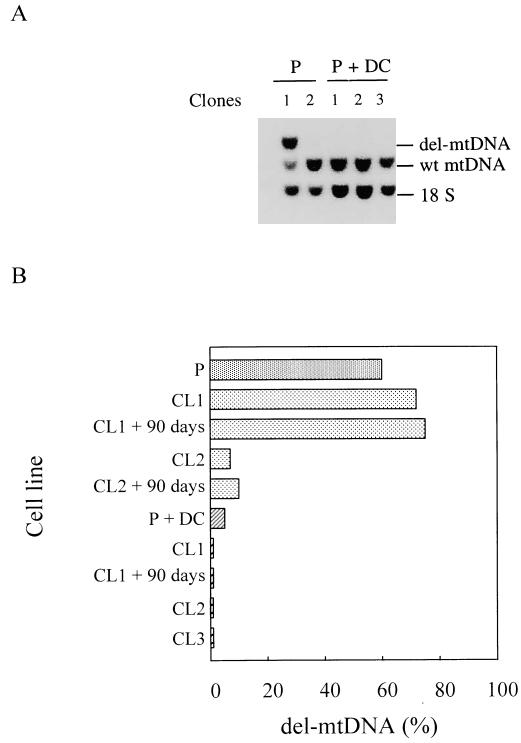

Fig. 4 A, B Percentage of del-mtDNA in several derivatives obtained from Pearson lymphoblasts. Derivatives (*CL*) from normal Pearson lymphoblasts (*P*) and P lymphoblasts that were treated for a 40-day period with 6 µg of DC/ml (*P+DC*) were obtained with the limiting-dilution technique. DNA was isolated and digested with *Eco*RI, and subjected to Southern analysis. Blots were hybridised with a probe against the mitochondrial COX I gene and the 18*s* rRNA multicopy nuclear gene. DNA digested with *Eco*RI results in a COX I-detectable band from wt-mtDNA of 7366 bp and a band from del-mtDNA of 9399 bp. **A** autoradiogram showing the percentages of del-mtDNA in a few Pearson lymphoblast derivatives. The results show that in both P-cell derivatives del-mtDNA was still detectable (*P-CL2*, very faint). The derivatives from the 'P+DC' cell culture did not have detectable del-mtDNA levels, although del-mtDNA could be detected with PCR. It is also evident that total mtDNA content in P-CL1 is elevated compared with the total mtDNA content in the other lines (see Fig. 5). **B** graphical presentation of the percentages of del-mtDNA of the parental cultures at the start of the cloning experiment (P, P+DC) and the derivative lines shown under **A** (P and derivatives are indicated by *stippled bars* and 'P+DC' and derivatives are indicated by *hatched bars*). The results are combined with Southern analysis using DNA isolated from some of the same proliferating cultures, 90 days after the first DNA isolation

In an attempt to obtain Pearson cells without any del-mtDNA we tried to derive clonal cells by the limiting-dilution technique. Both untreated Pearson cells (P) as well as Pearson cells (PDC) in which del-mtDNA was no longer detectable by Southern analysis were used. Compared with control lymphoblasts, the cloning efficiency for both of the above cell-lines was extremely low compared to that using control lymphoblasts (< 0.3% with Pearson lymphoblasts, 50% or more with control lymphoblasts). Two of a potential four-hundred derivatives were isolated from P cells and three from PDC cells. Both P derivatives still contained detectable amounts of del-mtDNA by Southern analysis; one contained 70–75% del-mtDNA while the other contained only about 10% del-mtDNA. The three PDC-derived subcultures did not contain amounts of del-mtDNA detectable by Southern analysis (Fig. 4), even after prolonged periods in culture. However, PCR analysis showed that all three PDC derivatives still contained cells with del-mtDNA (data not shown). The low cloning efficiency suggests that no true single-cell clones had been isolated, but instead that proliferating cultures which were derived from several cells had been obtained. Although we were unable to obtain a (clonal) culture without any detectable del-mtDNA, the above results suggest that, in principle, it should be possible to obtain such cultures by maintaining selective pressure on proliferating cells, and possibly by serial cloning.

Characterisation of the various Pearson subcultures

Since several subcultures with very low, stable levels of del-mtDNA were obtained it was possible to compare them

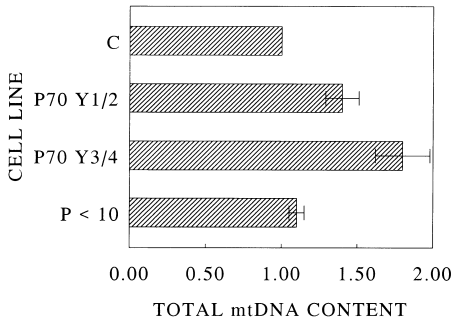

Fig. 5 Total mtDNA content in Pearson subcultures. Total mtDNA content was determined by Southern analysis, as described in Fig. 4 and elsewhere (Spelbrink et al. 1994). The graph shows the combined results of several analyses performed during 4 years of culturing this cell-line and several of its subcultures. *P70 Y1/2* shows the total mtDNA content determined for P cells with high percentages of del-mtDNA (60–70%) during the first 2 years of P-cell culturing. *P70 Y3/4* shows the same for years 3 and 4 and includes the values that were obtained with the clonal P-cell culture with a high del-mtDNA level (P-CL1, Fig. 4). *P < 10* shows the average value obtained with all P subcultures, including the derivatives obtained with limiting dilution (Fig. 4) and cultures receiving long-term treatment with DC, which contained no, or almost no, detectable levels of del-mtDNA, and which were stable in this respect. *Error bars* show the standard error of the mean (SE). All values are expressed relative to the value (set to 1), obtained in the same experiment, using control lymphoblasts. The results show that, irrespective of the cell-culture history, total mtDNA content is higher than normal in cultures having an average high percentage of del-mtDNA, but normal in cultures having an average low percentage of del-mtDNA

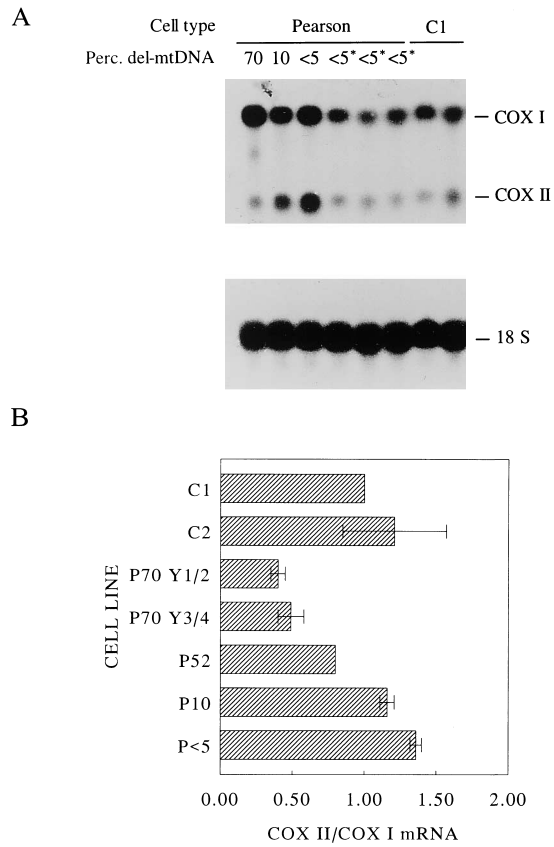

Fig. 6 A, B Mitochondrial mRNA levels in Pearson lymphoblast cell-cultures with varying percentages of del-mtDNA. Total cytoplasmic RNA was isolated from Pearson cell-cultures with varying average del-mtDNA levels, and used for Northern analysis of mitochondrial transcript levels. Blots were hybridised simultaneously with a probe for the COX I mRNA and a probe for the COX II mRNA. A probe against 18S rRNA was used as a measure of the amount of RNA loaded in each lane. **A** sample autoradiogram showing the COX I and COX II mitochondrial mRNA levels in Pearson cultures with high and low percentages of del-mtDNA, and in control lymphoblast cultures. The results show that cells with low levels of del-mtDNA have increased COX II mRNA levels when compared with P cells with high del-mtDNA levels. P cells with < 5% del-mtDNA that are still under DC treatment are indicated with an *asterisk*. **B** COX II/COX I mRNA ratios of several experiments as under **A** were calculated after quantification of hybridisation signals. All values are expressed relative to the value (set to 1) obtained in the same experiment with a control lymphoblast line (*C1*). *Error bars* show the standard error of the mean. *C2* = a second independent control lymphoblast line. The percentage of del-mtDNA at the time of the RNA isolation is indicated behind each P subculture (see also previous figures for an explanation of P subcultures). The results show that the COX II/COX I mRNA ratio steadily increases with decreasing percentages of del-mtDNA, which is the result of increasing COX II mRNA levels

with untreated cells having high del-mtDNA levels. Pearson cells with high del-mtDNA levels (P70) show an increased total (wt+del) mtDNA content (Spelbrink et al. 1994). During the past 4 years we have frequently analysed total mtDNA content in these Pearson cells. Surprisingly, during the last 2 years total mtDNA content had further increased (from 1.4 to 1.8–2.0 times the mtDNA content in control cells, Fig. 5), resulting in levels of wt-mtDNA of about 70% compared to the mtDNA content in control cell-lines. This further increase was clearly not the result of increased del-mtDNA levels alone, because the ratio of del-mtDNA to wt-mtDNA remained constant over the 4-year period. Subcultures that no longer contained high levels of del-mtDNA were found to have returned to control levels of total mtDNA (Figs. 4, 5). This observation included one subculture with low del-mtDNA levels that had not been obtained by DC treatment but by the cloning experiment. These results suggested that an increase in the level of del-mtDNA might be accompanied by an increase in the total mtDNA content.

The mtDNA deletion in the Pearson cell-line that was used in this study starts within the COX II gene (bp 7778), encompasses the COX III gene, and ends within the ND5 gene (bp 13 794, see De Vries et al. 1992). In order to better evaluate the effects of different levels of del-mtDNA on mitochondrial gene expression we directly compared COX II mRNA levels (gene contained within the deletion) with those of COX I mRNA (gene outside the deletion) by means of Northern analysis (Fig. 6a). Use of the COX II/COX I mRNA ratio removed from the analysis the effects of variation in total mtDNA content in the Pearson subcultures since, in all subcultures tested, the COX I mRNA content reflected the total mtDNA content (data not shown). The results show that in cells with high levels of del-mtDNA the COX II/COX I mRNA ratio was low compared to that in controls (Fig. 6b). The COXII/COX I

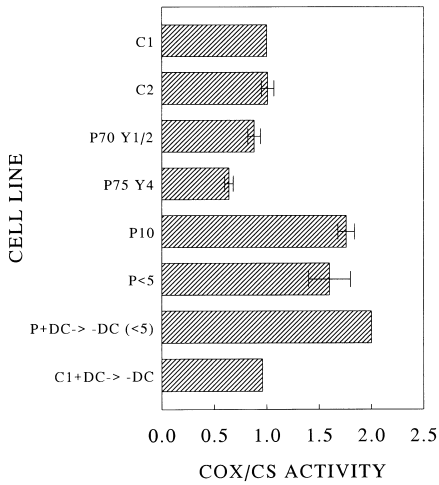

Fig. 7 Cytochrome *c* oxidase activity in Pearson lymphoblast cell-cultures with varying percentages of del-mtDNA. Cytochrome *c* oxidase (COX) and citrate synthase (CS) activity were measured using the same sample. The ratio of COX activity over CS activity is used as a measure of COX activity. CS activity corrects for mitochondrial volume/mass (Spelbrink et al. 1994). With few exceptions, the several Pearson subcultures are essentially the same as the cultures described in the previous figures. *P75 Y4* = P-CL1 with 75% del-mtDNA (see Fig. 4); *P + DC → –DC* = a Pearson subculture subjected to long-term treatment with DC, followed by prolonged proliferation in the absence of DC; *C1 + DC → –DC* = control cell-line 1 treated the same as *P + DC → –DC*. The results show that Pearson cells with low del-mtDNA levels have a high COX activity compared to controls, which can not be attributed to DC treatment

mRNA ratio was observed to increase steadily with decreasing del-mtDNA levels (Fig. 6b). COX II/COX I mRNA ratios, which were measured in cells during long-term DC treatment or shortly after, did not give clear-cut results. In part this may be attributed to compensation mechanisms caused by treatment with a mitochondrial protein-synthesis inhibitor, acting on the stability of mitochondrial mRNAs (as observed by Chrzanowska-Lightowlers et al. 1994).

Despite high del-mtDNA levels, cytochrome *c* oxidase activity remained almost unchanged over 2 years (Spelbrink et al. 1994). Having derived cells with very low levels of del-mtDNA, we compared COX activity in the various cell-lines (Fig. 7). All values were corrected for citrate synthase (CS) activity, as described previously (Spelbrink et al. 1994). Correction for protein content gave essentially the same results. A derivative with the highest del-mtDNA levels (75%, see Fig. 4) had the lowest COX activity, which was still 65% of control activity. Subcultures with very low del-mtDNA levels had higher than normal COX/CS activity ratios. Apparently this was not a consequence of long-term DC treatment alone, since control lymphoblastoid cells, which were also treated for a similar period of time prior to measurement, showed normal COX activity.

Discussion

It is commonly believed that the tissue distribution of mutated and wt-mtDNA is an important determinant of a mitochondrial disease phenotype. Understanding the mechanisms that lead to this distribution may provide important insight into the possibilities of manipulating these events and thereby altering the course of the disease. In the present study we looked at the dynamics of deleted and wt-mtDNA in a lymphoblastoid cell-line obtained from a patient with the Pearson syndrome. Most importantly, the results show that in this cell-line mitochondrial genome segregation can be manipulated, favouring full-length mtDNA, by the use of a mitochondrial protein-synthesis inhibitor, doxycycline. The behaviour of del-mtDNA before, during, and after DC treatment suggests that the percentage of mutant mtDNA is a balance between its preferential amplification and its phenotypic disadvantage.

Selection against del-mtDNA by chronic DC treatment

During 4 years of continuous cell proliferation, a stable heteroplasmy of 60–70% del-mtDNA and 30–40% wt-mtDNA was observed in the cell-line used in this study. Although no severe biochemical deficiency could be demonstrated, the cell-line appeared to be more sensitive to doxycycline than control cell-lines (Spelbrink et al. 1994). Since different cells in a single culture generally contain different percentages of mutant mtDNA (Mita et al. 1989; Matthews et al. 1995), this meant that treatment with low concentrations of DC could, in principle, be used to select against those cells in culture harbouring the highest percentages of del-mtDNA, and thereby reduce the average percentage of del-mtDNA in culture. Previous findings had already indicated that the addition of low concentrations of DC resulted in a stronger inhibition of growth in cells with a high level of del-mtDNA compared to control lymphoblasts (Spelbrink et al. 1994). The findings presented here show that selection against high levels of del-mtDNA was indeed achieved by the use of low concentrations of DC (3–6 μg/ml). In contrast, complete inhibition of mitochondrial protein synthesis in cells with a high proportion of del-mtDNA, using 15 μg of DC/ml (Van den Bogert et al. 1988; Spelbrink et al. 1994), did not result in any reduction of del-mtDNA levels over a period of weeks. The results strongly suggest that the effects on mutant mtDNA levels are brought about by artificially reducing respiratory capacity, thus lowering the threshold level at which mutant mtDNA molecules become deleterious.

Intra-cellular and intra-mitochondrial heteroplasmy

The length of the treatment necessary to obtain a cell population in which del-mtDNA could no longer be detected by Southern analysis and single-cell PCR suggests that, in this Pearson cell-line, mtDNA heteroplasmy is basically

intracellular, i.e. deleted and wt-mtDNA co-exist in the same cell. The present findings do not support the idea of two major cell populations, one containing mainly wt-mtDNA and the other containing only high levels of del-mtDNA, since this would most likely result in very rapid selection against del-mtDNA. The single-cell PCR experiments also argue against this possibility. Likewise, the segregating unit during the division of cells with intermediate to high levels of del-mtDNA must also be heteroplasmic, because mtDNA genotype fixation should otherwise occur rapidly, eventually resulting in a shift towards mainly wt-mtDNA (Solignac et al. 1984; Matthews et al. 1995). This implies that, if mitochondria are the segregating units, mtDNA heteroplasmy must be mainly intra-mitochondrial, meaning that both deleted and wt-mtDNA are present in the same organelle.

Preferential accumulation of del-mtDNA

The results presented here clearly show that del-mtDNA can be preferentially amplified. Based on the PCR experiments, an increase of del-mtDNA following DC treatment was always observed when most cells in culture still contained del-mtDNA, but not when most cells had only wt-mtDNA . Because it can be expected that cells with low del-mtDNA levels can still give rise to cells with high del-mtDNA levels by random partitioning of mitochondria to the daughter cells (as demonstrated for a point mutation, Shoubridge 1995), the results seem to exclude a growth advantage of cells containing high del-mtDNA levels. If this was the case, the overall percentage of del-mtDNA would always increase no matter how few cells still contained del-mtDNA. However, no del-mtDNA was detectable by Southern analysis, even after about 100 cell divisions, in cultures initially containing few cells with del-mtDNA. Also, except for a single derivative, the cultures with low del-mtDNA levels had similar growth rates as cultures with high del-mtDNA levels.

In cybrids, an increase in the level of del-mtDNA relative to wt-mtDNA has also been observed (Hayashi et al. 1991). In hybrids, however, del-mtDNA accumulated compared to its own parental wt-mtDNA but not compared to wt-mtDNA of the recipient cell-line (Sancho et al. 1992). Thus, in hybrids and cybrids preferential accumulation might be a property not only of del-mtDNA. A cybrid's nuclear genome may be involved in mtDNA segregation as well, as was clearly demonstrated in relation to the MELAS 3243 point mutation (Dunbar et al. 1995). Although it is impossible from the present study to determine to what extent the accumulation of del-mtDNA was influenced by its original parental nucleus, the ease with which del-mtDNA was selected against suggests a passive role of the nuclear genome.

Accumulation of del-mtDNA can also show cell-type and tissue-specific differences. In patient myoblasts, a deletion or point mutation may be barely detectable, while muscle from the same patient shows high levels of these mutations (Collombet et al. 1996; Fu et al. 1996). Following the fusion of myoblasts to myotubes, del-mtDNA was observed to accumulate rapidly with time (Collombet et al. 1996). Del-mtDNA accumulated to high levels in fibroblasts from a Pearson syndrome patient in the presence of uridine and pyruvate, but was lost rapidly in the absence of these chemicals (Bourgeron et al. 1993). A lymphoblast-line from the same patient showed stable heteroplasmy at a phenotypic threshold level, irrespective of the presence or absence of uridine/pyruvate. Thus, cell-type-specific differences clearly play a role in the preferential accumulation of del-mtDNA. We have now shown that mtDNA segregation can be straightforward, as the result of a dynamic equilibrium that is mainly determined by preferential accumulation of del-mtDNA on the one hand and phenotypic selection in favour of wt-mtDNA on the other. One might even hypothesise that differences in the eventual outcome of mtDNA segregation in different cell types or tissues are mainly the result of differences in the phenotypic expression of the mutant mtDNA versus a tendency to accumulate this mtDNA species preferentially.

The mechanisms by which deleterious mtDNA mutations may accumulate are the subject of much debate. A del-mtDNA could have a replicative advantage as a consequence of its smaller size (Wallace 1989; Shoubridge et al. 1990). If this is the case, elongation would be a rate-determining step in mtDNA replication. Although elongation is slow in vitro as well as in vivo (e.g. see Clayton 1982), it remains unproven that it is actually rate-determining in vivo. Unless there is a mechanism that tightly regulates the number of mtDNA molecules per cell, a direct replicative advantage of del-mtDNA also implies that the number of mtDNA molecules should always increase with increasing percentages of del-mtDNA. Therefore, if no restraints are present on the total mtDNA content per cell, in Pearson cells with 60–70% del-mtDNA one might expect a total mtDNA content of about 2.5–3.5 times the content of control cells, hence a normal wt-mtDNA content. The wt-mtDNA content was, however, consistently reduced in cells with high levels of del-mtDNA, whereas total mtDNA content was only 1.4–1.8 times the control mtDNA content. Although we favour a mechanism with an inherent replicative advantage of del-mtDNA, it is unlikely that this is solely the consequence of the smaller size of del-mtDNA combined with a rate-determining elongation rate. Additional mutations might contribute to the replicative advantage of del-mtDNA (by analogy with the observations of Marchington et al. 1996). Likewise, additional mutations could have a negative effect on the replication of 'wt'-mtDNA when present in this molecule, but not in del-mtDNA.

A second possible mechanism that could explain the increase in del-mtDNA after short-term DC treatment could be preferential or stimulated proliferation of defective mitochondria (Shoubridge et al. 1990; Attardi et al. 1995). This is believed to apply mainly to those situations were mutant mtDNA levels are already high, or heteroplasmy is not intra-mitochondrial but inter-mitochondrial, meaning that del- and wt-mtDNA are present in separate organelles (Attardi et al. 1995). In the patient-derived cell-line used

in this study a rapid increase in the proportion of del-mtDNA was observed, even when starting from a low level. Furthermore, because we did not observe a generalized protein-synthesis defect or severe OXPHOS deficiency at high del-mtDNA levels, heteroplasmy is very likely to be intra-mitochondrial (Spelbrink et al. 1994). Taken together these results make the second mechanism unlikely. Although an increased total mtDNA content could be an indication of stimulated mitochondrial proliferation, citrate synthase levels in all Pearson-cell derivatives were normal after correction for total cellular protein content, also arguing against this possibility.

Possible applications

The approach used here to study mitochondrial genome segregation may be valuable for several lines of research. For example, it may help to distinguish deleterious heteroplasmic mtDNA mutations from heteroplasmic polymorphisms in cell culture, without the use of cell-fusion techniques such as described, for example, by Chomyn et al. (1991). Also, partial inhibition of mitochondrial protein synthesis in cell-lines may be useful in establishing possible OXPHOS defects that were not evident from a comparison of patient and control cells without the treatment. The applicability of using partial inhibition of protein synthesis in order to manipulate the segregation of heteroplasmic mtDNA mutations in mitochondrial disorders, as suggested before (Clarke 1990), must await further studies. Nevertheless, the results of the present paper suggest that administration of doxycycline could be used as a therapy to reduce levels of mutant mtDNA (del-mtDNA or otherwise) in patients. However, one has to use such an approach with care as it might have the adverse effect of further impairing respiratory chain function. Furthermore, although DC treatment may result in selection against rapidly dividing cells with high mutant mtDNA levels, it is difficult to envisage the outcome of such a therapy in post-mitotic tissues. Animal models could be used to address the issue of, for example, the possible toxicity of chronic DC treatment. It is worth noting that doxycycline is a commonly used antibiotic that may even, in the past, have been given to some patients with heteroplasmic mtDNA mutations.

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