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Genetics

Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy

(mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes)

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ABSTRACT **The segregation of mutant and wild-type mtDNA was investigated in transformants constructed by transferring human mitochondria from individuals belonging to four pedigrees with the MELAS encephalomyopathy-associated mtDNA mutation (MELAS is mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) into human mtDNA-less (ρ°) cells. Five of 13 clonal cell lines containing mixtures of wild-type and mutant mtDNAs were found to undergo a rapid shift of their genotype toward the pure mutant type. The other 8 cell lines, which included 6 exhibiting nearly homoplasmic mutant mtDNA, on the contrary, maintained a stable genotype. Subcloning experiments and growth rate measurements clearly indicated that an intracellular replicative advantage of mutant mtDNA was mainly responsible for the dramatic shift toward the mutant genotype observed in the unstable cell lines.**

The rules that govern selection, segregation, and complementation of different mtDNA molecules at the intracellular and cell population levels are poorly understood. In mammalian cells, until recently, the available evidence was limited to the behavior of drug-resistance-inducing mutations in cell hybrids and cybrids (cells fused to cytoplasts), and pointed to a random drift to the mutant or wild-type genotype in the absence of selection (1) and to a propagative advantage of the resistance-conferring mutant mtDNA in the presence of the drug (1, 2). The recent identification of human disorders associated with mtDNA mutations (3) has provided an opportunity for investigating the transmission of natural mutations in the patient's cells *in vivo* or in their *in vitro* derivatives. Thus, an accumulation of deleted mtDNA molecules relative to coexisting wild-type genomes has been reported in skeletal muscle fibers of patients with mitochondrial diseases (4, 5) and in cybrids derived from patient's cells (6).

In the present work, cell lines containing in heteroplasmic form the mitochondrial $tRNA^{Leu(UUR)}$ gene mutation associated with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) (7–9) that were constructed by introducing patient mitochondria into human mtDNA-less (ρ°) cells (10) were used to investigate the behavior of mutant and wild-type mtDNAs upon prolonged subculturing. The MELAS mutation has been shown (11, 12) to cause serious defects in mitochondrial protein synthesis and respiration in cell lines carrying it in homoplasmic form. The heteroplasmic transformants analyzed in the present work exhibited either an unstable or a stable genotype. Surprisingly, in all unstable heteroplasmic transformants, a dramatic shift toward the pure mutant genotype was observed. The rapidity of the shift and growth rate measurements clearly pointed to a marked replicative advantage of the mutant mtDNA as being mainly responsible for the phenomenon observed here.

MATERIALS AND METHODS

Myoblast Cultures and Mitochondria-Mediated ρ°206 Cell Transformation. Mass myoblast cultures were established from muscle biopsy specimens by using reported procedures (13–15). Conditions of growth of the ρ°206 cell line and ρ°206 cell transformation by cytoplast fusion were as described (16). Transformants were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) dialyzed fetal calf serum and bromodeoxyuridine at 100 μg/ml. The population doubling time of the transformants was determined from growth curves or by using the formula:

$$DT = (t - t_0)\log 2/(\log N - \log N_0),$$

where DT is doubling time, t and t_0 are the times at which cells were counted, N and N_0 are cell numbers at times t and t_0, respectively. Subcloning was carried out by mixing samples of ≈20 cells from each transformant with 10^5 ρ°206 cells in DMEM with 10% fetal calf serum and bromodeoxyuridine at 100 μg/ml and plating them in a 96-well plate at a final cell density of 0.2 transformant plus 10^3 ρ° cells per well. Plating efficiency varied between 16% and 100%.

DNA Analysis. The presence and proportion of mutated mtDNA in total DNA samples (prepared by using an Applied Biosystems 340A extractor or by proteinase K digestion of a 0.5% Tween 20 cell lysate) were determined by testing for the presence of the *Apa* I site created by the MELAS mutation at position 3243 (7–9) in a fragment amplified by a PCR using oligonucleotides corresponding to positions 3031–3050 and 3341–3360. The proportion of digested and undigested molecules was determined by laser densitometry after ethidium bromide staining of the agarose gel. To correct for possible resistance to digestion of heteroduplexes of wild-type and mutant mtDNA formed during the final stages of PCR amplification (17), a mixed-template standard curve was constructed by PCR amplification of a mtDNA fragment containing the mutation from total DNA samples of 43B cells, containing ≈99% mutated mtDNA, and transformant 94C, containing exclusively wild-type mtDNA (12). Different ratio mixtures of the mutant and wild-type PCR products were then used for a second cycle of PCR amplification. Fig. 1 compares the mixed template standard curve (curve T) thus constructed with the mixed product standard curve (curve P), obtained by mixing the PCR products independently amplified from 43B-derived and 94C-derived PCR products in different ratios and then subjecting the mixtures to *Apa* I digestion.

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Abbreviation: MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

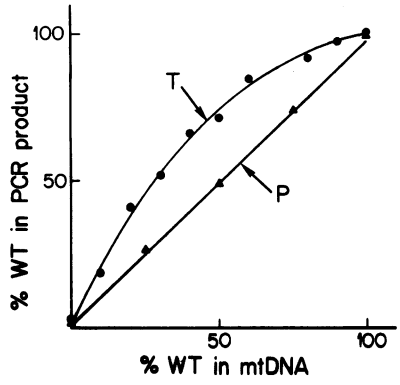

FIG. 1. Construction of standard curves for the quantitation of the MELAS mutation in mtDNA. Curves: T, mixed-template standard curve; P, mixed-product standard curve. WT, wild type.

Slot blot analysis of total DNA samples for quantitation of mtDNA was carried out as described (10) using the mtDNA clone pTZ18/K4 (containing a fragment from positions 41 to 2578), ^{32}P-labeled by random priming (18), as a probe. The data were normalized relative to hybridization to a nuclear 28S rRNA probe, constructed by PCR amplification from the 28S rRNA gene (19).

RESULTS

Transfer of Mitochondria from Myoblasts of MELAS Mutation-Carrying Individuals to ρ^0 Cells. Some of the mitochondrial transformants investigated here were derived from myoblast cultures of MELAS patient 43, his asymptomatic maternally related aunt (patient 94), and a second MELAS patient, 2S, as described (12). Other transformants were produced from myoblast cultures of three additional MELAS patients. Of these, two (patients 3E and 4H) were brothers of Afro-American lineage, 8 and 13 years old, respectively, and the third (patient 5X) was an isolated 11.5-year-old female Caucasian who suffered from MELAS syndrome and epilepsy. These three patients exhibited the MELAS 3243 mutation in heteroplasmic form in the skeletal muscle, urinary epithelial cells, and peripheral blood cells.

The majority of transformants obtained from the myoblast cultures of patients 3E and 4H (which contained 68% and 82% mutant mtDNA, respectively) exhibited strongly predominant mutant mtDNA, a few were homoplasmic or nearly homoplasmic for wild-type mtDNA, and one (3E7) exhibited an intermediate genotype. By contrast, 9 of 10 transformant clones produced from the myoblast culture of patient 5X (which contained 35% mutant mtDNA) exhibited pure or virtually pure wild-type mtDNA and one clone (5X1) had an intermediate genotype.

Segregation of Mutant and Wild-Type mtDNA in MELAS Transformants. In the course of continuous culturing, it was unexpectedly observed that the mitochondrial genotype in one of the relative 94-derived transformants, 94B, underwent a marked change. As shown in Fig. 2 *A Upper* and *D*, the original culture 94B exhibited a dramatic decrease in its wild-type mtDNA content, which dropped from 85% to 11% in 33 days, and then a slower decrease to 4.5% over the next 36 days. A culture derived from another vial of early 94B cells (94B′) exhibited the identical behavior (Fig. 2 *A Lower* and *D*). In contrast, another 94 transformant (94H) that initially contained 71% wild-type mtDNA maintained this level unchanged (71–74%) for 6 weeks (Fig. 2 *B* and *D*). Two other 94 transformants (94I and 94L) exhibited apparently pure wild-type mtDNA for 4–5 weeks (Fig. 2*D*). Fig. 2*D* also shows the behavior of the 43B transformant, which maintained a mutant mtDNA content of 97–99% for 7 weeks. A similar behavior was observed in another 43 transformant (43A) and a 94 transformant (94A), which contained strongly predominant mutant mtDNA (data not shown).

As shown in Fig. 2*D*, an O_2 consumption of 4.5 fmol per min per cell, which is within the normal range found for several 94 transformants containing 100% wild-type mtDNA (12), was measured when the wild-type mtDNA content of 94B was 11%. The O_2 consumption dropped to 2.1 fmol per min per cell when the wild-type mtDNA content had become

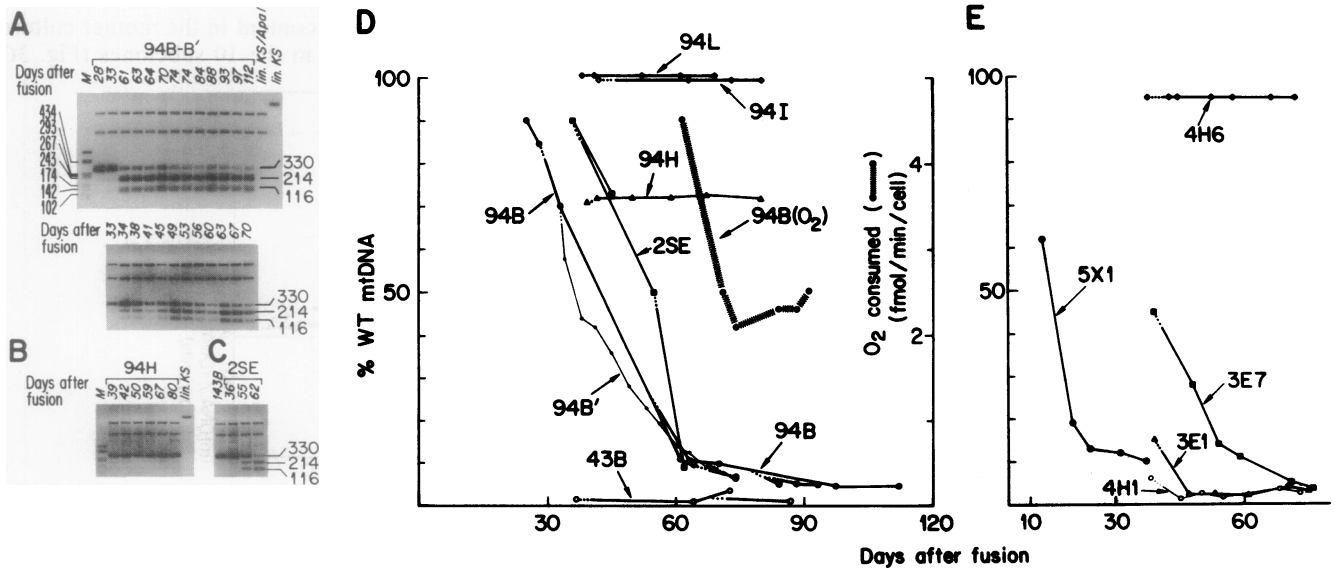

FIG. 2. mtDNA carrying the MELAS mutation rapidly replaces the predominant wild-type mtDNA or remains constant during growth of different heteroplasmic MELAS transformants. (*A–C*) Genotype analysis of transformants 94B (*A Upper*), 94B′, derived from another vial of early 94B cells (*A Lower*), 94H, isolated from the asymptomatic individual 94 (*B*), and 2SE, isolated from MELAS patient 2S (*C*), and of 143B.TK$^-$ cells (*C*). lin.KS, pBluescript KS+ DNA, linearized with *Xmn* I, added as internal marker for completion of digestion; M, *Hae* III-digested pBluescript KS+ DNA. (*D*) Quantitative behavior of wild-type mtDNA during growth of MELAS transformants 94B, 94H, 43B, and 2SE and O_2 consumption in transformant 94B. The dotted portions of the curves represent interruptions in growth due to freezing and thawing of cells. O_2 consumption was measured as described (10). (*E*) Quantitative behavior of wild-type mtDNA during growth of MELAS transformants 3E1, 3E7, 4H1, 4H6, and 5X1. WT, wild type.

9% and remained around 2.3–2.5 fmol per min per cell over the next 17 days, as the wild-type mtDNA content declined to 6%. Previously, a striking protective effect of wild-type mtDNA against the phenotypic manifestation of the MELAS mutation, with a sharp transition around an average wild-type mtDNA proportion of 6% and with full protection by ≈10% wild-type mtDNA, has been observed (12). The present observations for 94B, therefore, fit in well with the above data. The finding that, in the 94B heteroplasmic transformant, the mtDNA shift to mutant type went beyond the threshold protective level of wild-type mtDNA, causing a defect in the respiratory capacity of the transformant to appear, indicates that this defect did not produce a significant growth disadvantage in the affected cells, probably due to an adaptation that allows the cells to rely on glycolysis for their energy requirements. The growth rate measurements discussed below support this conclusion.

To investigate whether the unusual behavior of the mtDNA in the 94 transformants was related to some property of its sequence, genotypes of heteroplasmic transformants derived from other genetically unrelated MELAS patients were analyzed. As shown in Fig. 2 *C*, *D*, and *E*, transformants 2SE, 3E7, and 5X1 (derived from patients 2S, 3E, and 5X, respectively) exhibited the same dramatic decrease of wild-type mtDNA observed in the 94B transformant. In particular, in transformant 2SE, the wild-type genomes decreased from 90% after thawing to 50% after 19 days in culture and to 9% after an additional 7 days (Fig. 2 *C* and *D*). In two other clones (3E1 and 4H1), the proportion of wild-type mtDNA was initially 15% and 6%, respectively, then declined to 2–3% in 7–8 days, and remained at this level over the next 4 weeks (Fig. 2*E*). It seems very likely that the 3E1 clone, at the time of its first analysis, was in the final stage of a rapid genotype shift toward pure mutant type. Finally, other transformant clones maintained their initial nearly homoplasmic (>95%) wild-type genotype [4H6 (Fig. 2*E*)] or nearly homoplasmic mutant genotype (2SC and 2SD) over 3–5 weeks (data not shown). In summary, in the present work, of 23 heteroplasmic transformants found among 48 clones derived from individuals belonging to four pedigrees with the MELAS mutation, the mtDNAs of 13 transformants were analyzed over a prolonged period and shown to undergo a rapid shift to the mutant genotype or to maintain a stable genotype. In no case was a shift toward the wild-type mtDNA genome observed. The remaining 10 heteroplasmic clones, containing nearly pure mutant mtDNA, were not analyzed further.

Subcloning of MELAS Transformants. To obtain some insight into the mechanism of the rapid genotype shift of the unstable heteroplasmic transformants described above and the basis for the behavior of the 94H and 4H6 transformants, cultures of 94B, 2SE, and 94H were subjected to one or two steps of subcloning at different times after their isolation. Among 17 subclones derived from the 94B transformant when its genotype exhibited >90% wild-type mtDNA, 9 subclones showed an apparently homoplasmic wild-type genotype, 1 (subclone 10) exhibited almost pure (≈99%) wild-type mtDNA, and 7 contained predominantly (>81%) mutant mtDNA presumably as a result of a shift in genotype (Fig. 3 *A Upper* and *B*). Second-step subcloning of clone 10 produced 10 clones, of which 8 maintained a nearly homoplasmic wild-type mtDNA for 18 days and the other 2 exhibited the beginning of a shift to mutant mtDNA (Fig. 3*B*). The poor growth of the last two clones prevented their further analysis.

In a second 94B subcloning experiment, carried out when the genotype showed 58% wild-type mtDNA, among 10 subclones, 5 subclones still exhibited apparently homoplasmic wild-type mtDNA and 5 contained heteroplasmic mtDNA, with a level of wild-type mtDNA between 8% and 22%. Upon further culturing, the 5 heteroplasmic subclones exhibited a progressive decrease of wild-type mtDNA, to 3.5–7.5% 3 weeks after subcloning (Fig. 3*C Upper*). All five apparently homoplasmic wild-type clones exhibited 100% wild-type mtDNA at the earliest analyzed time point (10 days after subcloning), without any evidence of transitional genotype, as opposed to the gradually changing genotype of the subclones shifting to the mutant type. This observation strongly argues that these apparently homoplasmic wild-type clones preexisted as such in the original 94B population. Furthermore, the progressive decline in the proportion of wild-type mtDNA in the 94B mother culture implies that a continuous recruiting of clones shifting to the mutant genotype must have occurred from the pool of apparently homoplasmic wild-type clones.

Quantitation of the mtDNA content in the mother culture at the time of subcloning and in the 10 subclones (Fig. 3*C*

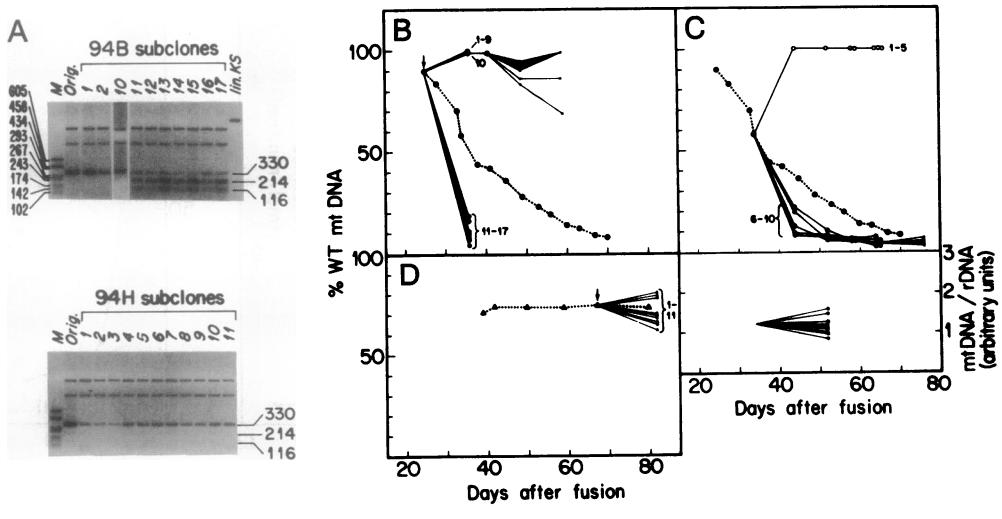

FIG. 3. Genotype behavior of subclones of mitochondrial transformants derived from asymptomatic individual 94. (*A*) Genotype analysis of some of the first-step subclones (1–17) of the 94B transformants and subclones (1–11) of 94H transformant. Arrows in *B* and *D* indicate times of subcloning. Orig., sample of mother culture. (*B* and *C*) Quantitative behavior of wild-type mtDNA in subclones deriving from 94B 25 days (*B*) and 34 days (*C*) after isolation of the transformant. The dotted line reproduces the curve of Fig. 2*D* illustrating the quantitative behavior of wild-type mtDNA in transformant 94B-94B'. Open symbols represent clones with apparently homoplasmic wild-type mtDNA. In *C Upper*, the first point in the curve for subclones 1–5 pertains to all subclones (see text for details). (*C Lower*) Total mtDNA content in the first-step subclones and the mother culture. (*D*) Quantitative behavior of wild-type mtDNA in subclones derived from 94H transformant. WT, wild type.

Lower) showed that no significant change in the average amount of mtDNA per cell had occurred between the isolation of the subclones and 2.5 weeks later, when the proportion of wild-type mtDNA in the heteroplasmic subclones had decreased to one-half (Fig. 3*C Upper*). This observation indicated that a decrease in the absolute average amount of wild-type mtDNA per cell had taken place in these subclones during the same period. Second-step subcloning of one of the heteroplasmic clones with 4% wild-type mtDNA produced 5 subclones that exhibited limited genotype variability around this value (Fig. 3*C Upper*).

Subcloning of the 2SE transformant ≈5 weeks and ≈9 weeks after its isolation gave results similar to those described above for 94B (Fig. 4). For both transformants, the subcloning experiments indicated that the cultures undergoing a shift of their genotype to mutant mtDNA consisted of cell populations that were very heterogeneous in their mutant to wild-type mtDNA ratio and exhibited a progressive general shift toward the mutant genotype, with the proportion of cells containing homoplasmic or near homoplasmic wild-type mtDNA decreasing progressively with the time in culture. These results indicated that the shift toward the mutant type started at different times and/or proceeded at different rates in the individual cells of the unstable populations.

Subcloning of the 94H transformant carried out ≈9 weeks after its isolation, when the genotype showed ≈73% wild-type mtDNA, gave 11 subclones that, in contrast to 94B and 2SE, exhibited only limited variability (between 62% and 80%) in wild-type mtDNA content (Fig. 3 *A Lower* and *D*).

The Genotype Shift Does Not Result from Intercellular Selection Due to Differential Growth Rates. The rapidity of the shift toward mutant mtDNA observed in the unstable cell lines argued strongly against its being caused by cells with more mutant mtDNA growing faster than those with less mutant mtDNA. Evidence against intercellular selection was provided by a comparison of the doubling times of many MELAS transformants exhibiting widely different proportions of mutant mtDNA (Table 1). Small differences in doubling times were observed among different clones, probably reflecting variations in nuclear or mtDNA background, or in clone age. However, for a given transformant clone, no significant consistent difference in growth rate was found between stages or subclones homoplasmic or nearly homoplasmic for wild-type mtDNA or stages with 50–70% wild-type mtDNA and stages or subclones exhibiting nearly homoplasmic or strongly predominant mutant mtDNA. This is dramatically illustrated by

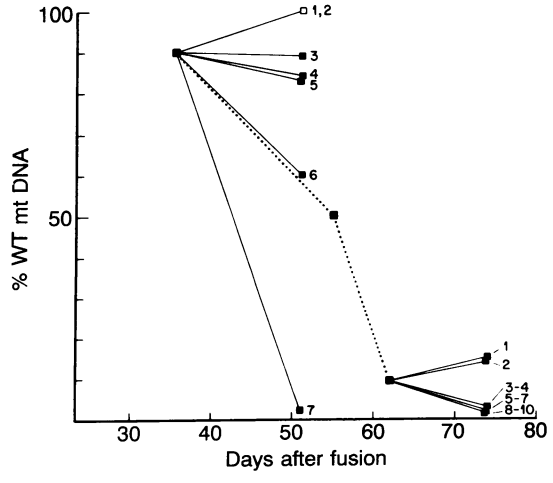

FIG. 4. Genotype behavior of subclones (as indicated) of mitochondrial transformant 2SE, derived from MELAS patient 2S. The dotted line reproduces the curve of Fig. 2*D* illustrating the quantitative behavior of wild-type mtDNA in transformant 2SE. WT, wild type.

Table 1. Growth rates of MELAS transformants

Transformant	% WT mtDNA	Doubling time, hr
94B early*	>90	31
94B early†	70	26
94B late‡	10 → 4.5	25
94B′	58 → 19	24
94B′	19 → 8	24
94B-subclones 1–5§	100	27–34
94B-subclones 6–10§	4.5–6	25–31
94H	72	30
94I	100	34
94L	100	35
43B	2–3	29
2SA	100	25
2SB	100	24
2SD	6	23
2SE	50	23

WT, wild type.
*At 24–28 days after fusion.
†At 33 days after fusion, culture derived from a frozen stock.
‡At 63–97 days after fusion.
§Derived from subcloning of 94B carried out 34 days after fusion (Fig. 3*C*).

the experiment shown in Fig. 5, where 94B′ cells, kept in continuous culture for 41 days, exhibited a decrease in the proportion of wild-type mtDNA from 58% to 8%, while maintaining an absolutely constant 24-hr doubling time. Furthermore, even in the cases where a slightly longer doubling time was estimated for cells with mostly wild-type mtDNA, as compared to cells with mostly mutant mtDNA, the increase in percent mutant mtDNA expected as a result of differential growth rates would have been much less than the increase actually observed. Thus, for 2SE cells grown continuously for 26 days, with an observed decrease of wild-type mtDNA from 90% to 9% (Fig. 2*D*), the decrease in proportion of wild-type mtDNA expected from intercellular selection would have been much less [i.e., from 90% to 67%; as calculated conservatively by using the values 25 hr (obtained for clone 2SA) and 23 hr (obtained for clone 2SD) (Table 1) as doubling times for cells containing a high percentage and a low percentage of wild-type mtDNA, respectively]. These quantitative considerations and the direct observations shown in Fig. 5 are not compatible with a significant role of intercellular selection in the phenomenon investigated here and rather point to an intracellular process involving a replicative advantage of the mutant mtDNA molecules.

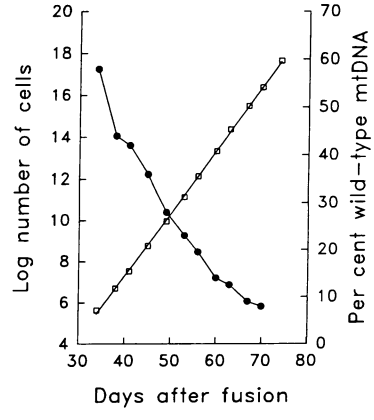

FIG. 5. Rapid genotype shift toward mutant mtDNA of the 94B′ transformant does not involve any change in growth rate. The figure shows the growth curve (open symbols) and the genotype change (solid symbols) during continuous subculturing of the 94B′ clone.

DISCUSSION

The central observation reported here (i.e., the preferential replication of mtDNA molecules carrying the MELAS mutation over wild-type molecules) extends the phenomenon of "suppressiveness" to a higher eukaryote mtDNA with a point mutation. Suppressiveness was originally described in yeast for a subclass of "petite" mtDNA mutations (20) and, subsequently, in *Neurospora* and other filamentous fungi for senescence processes leading to the replacement of normal mtDNA with grossly abnormal mtDNA (21–24). In the present work, the evidence obtained excludes a significant role of intercellular selection of faster growing mutant mtDNA-enriched cells in the genotype shift observed in unstable heteroplasmic MELAS transformants. The alternative possibility of an unequal segregation of mutant and wild-type mtDNA into daughter cells at cell division, with the daughter cells receiving more mutant mtDNA undergoing a compensatory nonselective mtDNA amplification, seems very unlikely in the present mammalian cell system. In fact, it would require a unidirectionality of unequal segregation events in successive division cycles, for which no known mechanism exists. Therefore, the most reasonable interpretation of the present data is that a replicative advantage of mutant mtDNA molecules over wild-type molecules is the main factor responsible for the observed genotype shift. This replicative advantage may explain the observations made here and in previous work (11, 12), indicating that the great majority of the transformants obtained by transfer of mitochondria from MELAS patient into ρ° cells contained either homoplasmic or near-homoplasmic wild-type mtDNA or strongly predominant mutant mtDNA, with very few transformants exhibiting an intermediate genotype.

As to the mechanism of the phenomenon investigated here, neither a smaller size of the mutant mtDNA molecules (25) nor, presumably, the loss of cis-acting control elements (5), factors that have been previously considered to explain the accumulation of mtDNA molecules with large deletions in mitochondrial diseases, could play a role. A plausible mechanism is one involving a feedback phenomenon that induces the selective replication of mtDNA in organelles functionally compromised by the presence of mutated mtDNA. This possibility has been raised before to account for the preferential accumulation of deleted or otherwise grossly altered mtDNA (5, 24). However, any model involving selection at the level of individual organelles would have to explain the present findings of two transformants (94H and 4H6) that did not exhibit an amplification of the mutant DNA over 5–6 weeks. The observation reported here and elsewhere (12) that the phenotypic manifestation of the MELAS mutation can be complemented by a small minority (>6%) of wild-type genomes suggests that segregation of mutant molecules in pure form in separate organelles (i.e., intermitochondrial heteroplasmy) may be the critical factor controlling the replicative advantage of the defective mtDNA molecules. Transformants 94H and 4H6 may represent examples of a situation where stable intermixing of mutant and wild-type mtDNA molecules (intramitochondrial heteroplasmy) would allow a complementation of the defect(s) caused by the mutation and thus prevent the replicative advantage of defective mtDNA molecules from being manifested. Another important question is whether the replicative advantage of mutant mtDNA molecules observed here is somehow linked to the particular nature of the MELAS mutation or whether it reflects a more general behavior of defective mtDNA.

It seems possible that the "crippled mitochondrion" model for the replicative advantage of mutant mtDNA is also applicable to the clonal expansion of the single deletion events early in development that produces the accumulation of deleted mtDNA molecules (26). Furthermore, it is reasonable that the shift in genotype toward higher proportions of mutant mtDNA, with increasing signs of mitochondrial dysfunction, observed in successive generations of Leber hereditary optic neuropathy pedigrees (27, 28) and of a MELAS pedigree (29), could reflect the operation in the oocytes of the same mechanism for a replicative advantage of the mutant mtDNA molecules.

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