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Very Rare Complementation between Mitochondria Carrying Different Mitochondrial DNA Mutations Points to Intrinsic Genetic Autonomy of the Organelles in Cultured Human Cells*

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In the present work, a large scale investigation was done regarding the capacity of cultured human cell lines (carrying in homoplasmic form either the mitochondrial $tRNA^{Lys}$ A8344G mutation associated with the myoclonic epilepsy and ragged red fiber (MERRF) encephalomyopathy or a frameshift mutation, isolated *in vitro*, in the gene for the ND4 subunit of NADH dehydrogenase) to undergo transcomplementation of their recessive mitochondrial DNA (mtDNA) mutations after cell fusion. The presence of appropriate nuclear drug resistance markers in the two cell lines allowed measurements of the frequency of cell fusion in glucose-containing medium, non-selective for respiratory capacity, whereas the frequency of transcomplementation of the two mtDNA mutations was determined by growing the same cell fusion mixture in galactose-containing medium, selective for respiratory competence. Transcomplementation of the two mutations was revealed by the re-establishment of normal mitochondrial protein synthesis and respiratory activity and by the relative rates synthesis of two isoforms of the ND3 subunit of NADH dehydrogenase. The results of several experiments showed a cell fusion frequency between 1.4 and 3.4% and an absolute transcomplementation frequency that varied between 1.2×10^{-5} and 5.5×10^{-4}. Thus, only 0.3–1.6% of the fusion products exhibited transcomplementation of the two mutations. These rare transcomplementing clones were very sluggish in developing, grew very slowly thereafter, and showed a substantial rate of cell death (22–28%). The present results strongly support the conclusion that the capacity of mitochondria to fuse and mix their contents is not a general intrinsic property of these organelles in mammalian cells, although it may become activated in some developmental or physiological situations.

The compartmentalization of mitochondrial DNA (mtDNA)[1] in distinct organelles and the changes in the distribution of the mitochondrial genome in a cell brought about by mitochondrial growth, division, and movement and, in some cells, by mitochondria fusion account for the fact that the organization of these organelles plays a critical role in determining the segregation and complementation behavior of wild-type and mutant mtDNA, when they co-exist in a cell.

In simpler eukaryotic cells, like *Saccharomyces cerevisiae* (1, 2), *Chlamydomonas* species (3) and slime mold (4), and in plants (5, 6), there is ample evidence of mtDNA complementation and recombination, which is made possible by the well established capacity of mitochondria to undergo fusion in these organisms. These fusion events are genetically regulated (2, 4, 7). In animal cells, studies using time-lapse bright-field, phase-contrast, interference and fluorescence microscopy and electron microscopy of serial sections have revealed in some cell types a mitochondrial organization in the form of a network of interconnected tubular organelles, with suggestion of fusion events (8–11). Recently, the occurrence of a developmentally regulated fusion of mitochondria in postmeiotic spermatids of *Drosophila melanogaster*, which is mediated by the transient synthesis of a novel GTPase encoded in the *fuzzy onions* (*fzo*) gene, has been described (12). Homologs of this gene have been found in mammals, nematodes, and *S. cerevisiae* (12–14). However, mitochondria of mammalian spermatids have been reported not to fuse but to form structurally distinct contacts (15). Similar contacts have also been described in rat cardiac tissue (16). A developmentally regulated formation of a reticulum of tightly joined mitochondria has been reported for rat diaphragm muscle (17).

Observations on cultured human cells have so far failed to provide an unambiguous picture of the extent of mtDNA interactions in these cells. In investigations carried out in this laboratory (18), two mtDNAs, each carrying a recessive mutation in a different mitochondrial tRNA gene, *i.e.* the $tRNA^{Lys}$ gene mutation associated with the myclonic epilepsy and ragged red fiber (MERRF) encephalomyopathy (19) and the $tRNA^{Leu(UUR)}$ gene mutation associated with the mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) encephalomyopathy (20, 21), were independently introduced into the same human mtDNA-less (ρ^0) cell (143B.$\rho^0$206) by enucleated cell (cytoplast) × ρ^0 cell fusion (22). After growing the fusion products under conditions non-selective for respiratory competence, no evidence of transcomplementation of the mutations was found in five clones carrying both types of parental mtDNA, even 3 months after cell fusion. Other investigators, analyzing by fluorescence microscopy the fusion products of enucleated wild-type human cells and human ρ^0 cells, obtained results that were interpreted to indicate the occurrence of a rapid and extensive fusion of host and exogenous mitochondria and subsequent rapid diffusion of

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[1] The abbreviations used are: mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; BrdUrd, bromodeoxyuridine; FCS, fetal calf serum; APRTase, adenine phosphoribosyltransferase; CAP, chloramphenicol; MERRF, myoclonic epilepsy and ragged red fiber.

mtDNA and its transcripts throughout the organelles (23). More recently, the same investigators (24, 25), after constructing cybrids carrying two types of mtDNA with appropriate markers within distinct organelles and culturing them under conditions either selective or non-selective for recovery of respiratory capacity, isolated a few clones that showed evidence of translational complementation.

In the present work, in order to obtain a more conclusive answer to the question of the genetic autonomy of mitochondria in cultured human cells, large scale fusion experiments were carried out between cells and cytoplasts (or cells) carrying different recessive mtDNA mutations, in particular either the $tRNA^{Lys}$ MERRF mutation (26) or a frameshift mutation in the NADH dehydrogenase ND4 subunit gene (27). The determination of both the efficiency of cell fusion and the frequency of transcomplementation between the two mutations showed unambiguously that events of intergenomic complementation did occur, but only very rarely and sluggishly in cells carrying both parental mtDNAs, with evidence of slow growth and significant cell death of the cell fusion products.

EXPERIMENTAL PROCEDURES

Cell Lines and Media—The pT1 human cell line, carrying in nearly homoplasmic form the A to G transition at position 8,344 in the mitochondrial $tRNA^{Lys}$ gene, which is associated with the MERRF syndrome (19), and the C4T cell line, carrying in homoplasmic form a cytidine insertion in a stretch of six Cs at positions 10,947 to 10,952 in the mitochondrial *ND4* gene (27), had been previously isolated by transfer into human mtDNA-less 143B.$\rho^0$206 cells of mitochondria from myoblasts of a MERRF patient (26) and, respectively, from a mutant of the VA_2B human cell line (27) by cytoplast × cell fusion. They were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% dialyzed fetal calf serum (FCS) and 100 μg of bromodeoxyuridine (BrdUrd) per ml. The parental line of $\rho^0$206, 143B.TK$^-$ (22) was grown in DMEM with 5% FCS and 100 μg of BrdUrd per ml. The $\rho^0$13.1 cell line, a mtDNA-less derivative of an adenine phosphoribosyltransferase (APRTase)-less mutant of 143B.TK$^-$ (26) was grown in DMEM supplemented with 5% FCS and containing 50 μg of uridine, 100 μg of BrdUrd, and 50 μg of 8-azadenine per ml. Transformants of the $\rho^0$13.1 cell line, obtained by cytoplast × cell fusion-mediated transfer of mitochondria from C4T cells (hereafter designated C4.13-E, -I, or -J), were grown in DMEM supplemented with 5% dialyzed FCS, 100 μg of BrdUrd, and 50 μg of 8-azadenine per ml (26). The population doubling times of the various cell lines were determined as described previously (29).

Isolation of Double Transformants—Unless other conditions are indicated, 1–5 × 10^5 cells of the chosen mitochondrial donor cell line were enucleated by centrifugation in the presence of cytochalasin B, and the predominantly enucleated cells were then fused, using polyethylene glycol 1500 (PEG), with 1–5 × 10^5 cells of the chosen recipient cell line, as described previously (30). After 1–6 days of incubation, as specified below, in non-selective medium, double transformants carrying both the MERRF $tRNA^{Lys}$ gene 8344 mutation and the *ND4* frameshift mutation were selected and thereafter grown in "special DMEM/galactose" medium. This medium contained 0.9 mg/ml galactose instead of glucose and 0.50 mg/ml pyruvate (28) and was supplemented with 10% dialyzed FCS, 100 μg of BrdUrd per ml, and, when C4.13-E, or -I, or -J were used as recipient cells, also with 50 μg of 8-azaadenine per ml. Colonies were recognized between 3 and 4 weeks after plating, and those that appeared healthy were picked up for analysis.

Karyotype Determination—Karyotype determination was done, as described previously (31), in cells arrested in metaphase by treatment with 0.5 μg of colchicine per ml for 3 h.

DNA Analysis—Total DNA was isolated from cells as described previously (32). Quantification of mtDNA mutations was carried out in most cases by allele-specific termination of primer extension (33, 34). For this purpose, a 346-base pair mtDNA segment between positions 10,762 and 11,108, containing the *ND4* mutation, and a 173-base pair segment between positions 8191 and 8364 of mtDNA, containing the MERRF $tRNA^{Lys}$ mutation, were amplified with *Taq* polymerase. The two amplified fragments were separated from free nucleotides on a Tris borate/EDTA, 1.5% agarose gel, and eluted from the gel by the QIAEX II gel extraction kit (Qiagen). Then each amplified product was used for allele-specific primer extension termination, using Sequenase (Amersham Pharmacia Biotech) and the corresponding ^{32}P-5′-end-labeled primer (33, 34). Nucleotide concentrations were 33 μM dGTP and 500 μM ddTTP for the quantification of the C insertion in the *ND4* gene, and 33 μM TTP, 33 μM dGTP, and 500 μM ddCTP for the quantification of the mutation in the $tRNA^{Lys}$ gene. The mixtures were heated to 95 °C for 3 min and annealed at 50 °C for 10 min. After addition of Sequenase, they were incubated for 6 min at 45 °C. Electrophoretic analysis of the products and quantification of the intensity of the bands were carried out as described previously (33, 34). In some experiments, quantification of the MERRF mutation was carried out by *Nae*I restriction enzyme digestion of a mtDNA segment that had been amplified by polymerase chain reaction, using a mismatched primer generating a new *Nae*I site (18).

Mitochondrial Protein Synthesis and O_2 Consumption Analysis—The cultures were labeled with [^{35}S]methionine (>800 Ci/mmol, 25 μCi/ml) for 2 h in the presence of 100 μg of emetine per ml, and translation products were electrophoresed as described previously (26). O_2 consumption measurements were made as detailed earlier (22).

RESULTS

Characterization of Parental Cell Lines—To verify the homoplasmic nature of the $tRNA^{Lys}$ gene mutation in the pT1 human cell line and of the *ND4* gene mutation in the C4T cell line (see "Experimental Procedures"), allele-specific primer extension experiments were carried out, using a previously described protocol for *ND4* (33) and a protocol illustrated schematically in Fig. 1*a* for the $tRNA^{Lys}$ gene (34). As shown in Fig. 1*b*, the two mutations appear to be homoplasmic. The MERRF mutation in pT1 has been shown to produce a defect in the aminoacylation of the mitochondrial $tRNA^{Lys}$, which causes a severe impairment of protein synthesis due to premature termination of translation (35). This results in an almost complete loss of synthesis of the largest polypeptides (ND5, ND4, and CYTb) (Fig. 1*c*) and the accumulation of premature translation termination products, prominent among which are pMERRF, a truncated *COI* product, and a truncated *ND2* product (indicated by *small arrows* in Fig. 1*c*). The frameshift mutation in C4T causes a complete loss of synthesis of ND4, with resulting lack of assembly of the membrane arm of the NADH dehydrogenase and loss of enzyme activity (27). As shown in Table I, in both the pT1 and C4T cell lines, as a result of their mtDNA mutations, O_2 consumption is dramatically decreased to a level close to that previously observed in 143B.$\rho^0$206 cells (22).

Selective Conditions for Full mtDNA Functionality—For a large scale screening of transcomplementing clones among mitochondrial cybrids and hybrids containing two types of mitochondria carrying either the $tRNA^{Lys}$ or the *ND4* gene mutation, a medium containing galactose instead of glucose, which was capable of selecting against the two parental defective cell lines, was used. Such type of medium has been previously shown to curtail severely the growth of cells deficient in oxidative metabolism (28, 36–40).

As shown in Table I, cells carrying wild-type mtDNA (143B.TK$^-$ (22)) and the mutant C4T and pT1 cell lines grew well in regular DMEM containing glucose and supplemented with 5% FCS. When glucose was substituted by galactose, the cells carrying wild-type mtDNA (143B.TK$^-$) continued to grow, although at a reduced rate, whereas both types of mutant cell lines not only stopped growing but showed extensive cell death. This death was indicated by the progressive cell detachment from the plates. To confirm that the normal expression of mtDNA was absolutely required for the survival and growth of the 143B.TK$^-$ cells in special DMEM/galactose medium, as suggested by the results described above, mitochondrial protein synthesis was specifically inhibited by adding 40 μg/ml chloramphenicol (CAP) to the incubation medium; furthermore, the medium was supplemented with 50 μg/ml uridine, since it is known that cells without full mitochondrial gene expression become auxotrophic for pyrimidines (22, 36, 41).

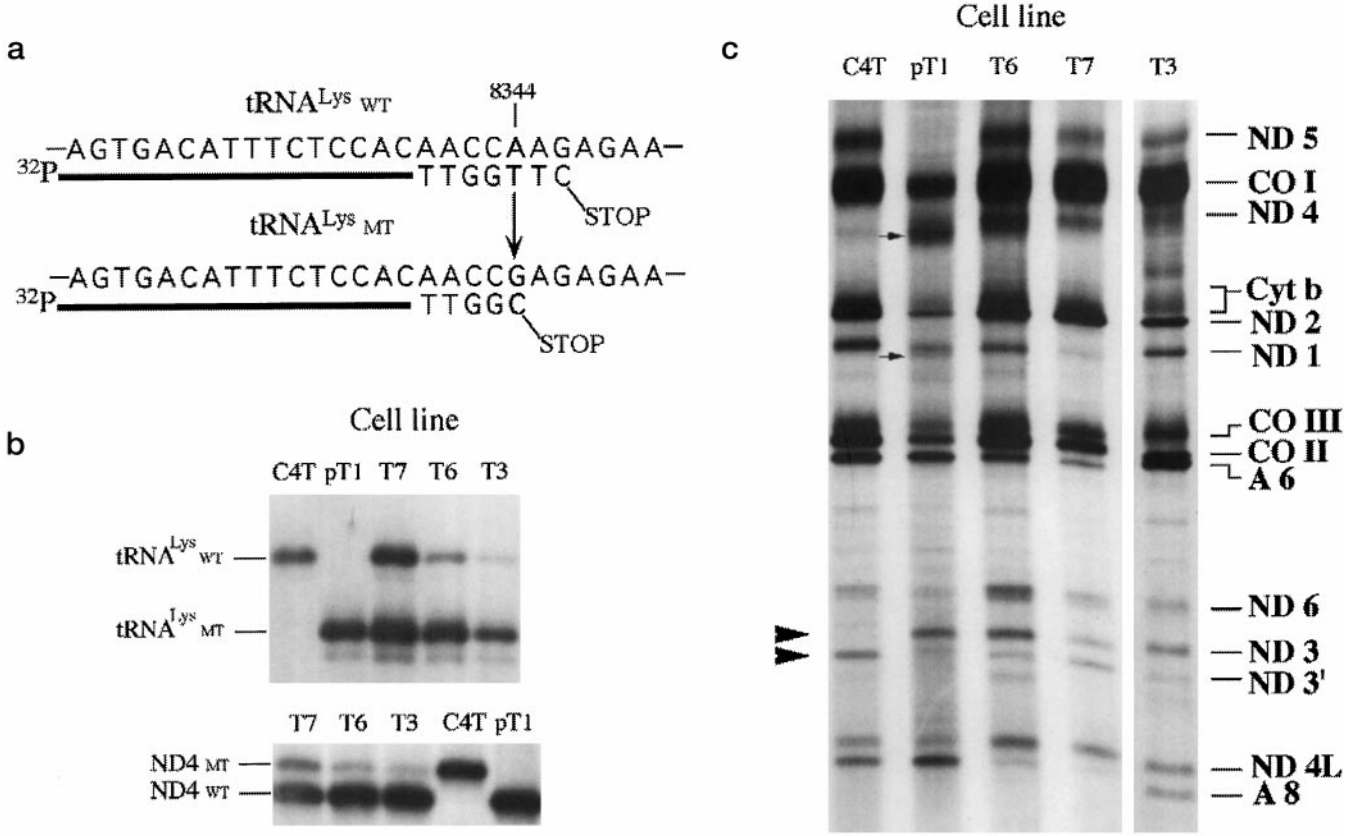

FIG. 1. **Amounts of wild-type (*WT*) and mutant (*MT*) tRNA^{Lys} and *ND4* genes and patterns of mitochondrial translation products in the pT1 and C4T cell lines and in three transcomplementing clones derived from the enpT1xrecC4T cell fusion experiments.** *a,* scheme showing the principle of the sequence-dependent termination of primer extension to quantify the A8344G transition in the mitochondrial tRNA^{Lys} gene. The same approach was used to quantify the *ND4* gene mutation (33). *b,* fluorogram, after electrophoresis through a sequencing gel, of the ^{32}P-5′-end-labeled primer extension products obtained from samples of total cellular DNA purified from the indicated cell lines. The separation of the mutant and the wild-type versions of the tRNA^{Lys} gene, as well as of the *ND4* gene, is clearly illustrated. The relative proportions of the two mutant mtDNAs are shown in Table I. *c,* fluorogram, after electrophoresis through an SDS-polyacrylamide gradient gel, of the mitochondrial translation products of pT1 and C4T cells and of three transcomplementing clones, labeled with [^{35}S]methionine for 2 h in the presence of emetine. *COI, COII,* and *COIII,* subunits I, II, and III of cytochrome *c* oxidase; *ND1 ND2, ND3, ND4, ND4L, ND5,* and *ND6,* subunits 1, 2, 3, 4, 4L, 5, and 6 of the respiratory chain NADH dehydrogenase; *A6* and *A8,* subunits 6 and 8 of H^+-ATPase; *Cyt b*, apocytochrome *b*. The *small arrows* indicate premature translation termination products characteristic of the pT1 cells, and the *large arrowheads* indicate the two polymorphic variants (*ND3* and *ND3′*) of the ND3 polypeptide.

143B.TK$^-$ cells grew well in the presence of CAP in glucose-containing medium supplemented with uridine but stopped growing and became detached from the plate in special DMEM/galactose supplemented with CAP and uridine (data not shown).

Evidence of Reciprocal Complementation of tRNA^{Lys} and ND4 Mutations in Double Transformants—Advantage was taken of the selective pressure provided by growth in special DMEM/galactose medium in order to isolate clones exhibiting transcomplementation of the tRNA^{Lys} and *ND4* gene mutations. For this purpose, 10^5 pT1 cells were subjected to enucleation (22), and the predominantly enucleated cells were then fused with 5×10^5 C4T cells. After a 2-day incubation in non-selective medium, a portion of the fusion mixture, corresponding to 2.5×10^5 recipient cells, was transferred to special DMEM/galactose medium in a Petri dish. After 3–4 weeks, eight clones were detected. Two of them subsequently died, and the other six were picked up. However, of them, only three (T3, T6, and T7) kept growing and were utilized for further analysis (Table I and Fig. 1). An equivalent portion of the fusion mixture was transferred to special DMEM/galactose medium after a 6-day incubation in non-selective medium and yielded initially six clones which, however, were not picked up for further growth.

Primer extension experiments revealed that all three clones contained both the mtDNA carrying the A8344G MERRF mutation and the mtDNA carrying the *ND4* mutation (Fig. 1*b*), with the former one being strongly predominant. A quantification by PhosphorImage analysis showed that the proportion of the tRNA^{Lys} mutation-carrying mtDNA varied between 71 and ~88% and that of the *ND4* mutation-carrying mtDNA between 10.5 and ~22% of the total mtDNA, and that the two mutant types of mtDNA accounted for all mtDNA, within experimental error (Table I). The latter observation indicated that these clones did not contain any significant amount of residual wild-type mtDNA from either parental cell line. Furthermore, the proportion of wild-type tRNA^{Lys} genes present in *ND4* gene mutation-carrying mtDNA exceeded the minimum previously shown to be able to complement the MERRF mutation (~10%) (18). Therefore, one expected that, if there was complete mixing of the gene products in the three clones, there would be full complementation of the MERRF mutation. There are no data on the quantitative effects of a null mutation in the *ND4* genes on their expression. However, if the same pattern of control applies to these genes as recently observed for the human *ND5* genes (42), one would expect in the three clones between 70 and 90% of the normal rate of synthesis of the ND4 protein.

The analysis of mitochondrial protein synthesis in the three isolated clones fully confirmed the predictions mentioned above. In fact, as shown in Fig. 1*c*, the three clones exhibited a

TABLE I
Mitochondrial genotype, chromosome number, oxygen consumption rate, and doubling time of different cell lines

Cell line	Mitochondrial genotype		Chromosome number (mean ± S.E.)	O_2 consumption rate	Doubling time	
	% $tRNA^{Lys}$ MT	% ND4 MT			Glucose	Galactose
				fmol/min/cell	*h*	
143B.TK$^-$	0	0	54	5–6[b]	21	46
C4T	0	100	56.3 ± 1.3	0.56	29	NG[d]
C4.13-J	0	100	57.7 ± 1.2	0.45	32	NG
C4.13-I	0	100		0.32		NG
pT1	100	0	55.0 ± 2.2	0.36	26	NG
pT1.C3	100	0	54.7 ± 1.1	0.44	24	NG
T3	87.7 ± 1.8[a]	10.5 ± 0.2[a]	100 to 110	6.4	51	78 (22%)[c]
T6	85.9 ± 3.0[a]	11.2 ± 1.6[a]	100 to 110	4.8	76	197 (28%)[c]
T7	71.0 ± 2.6[a]	22.3 ± 3.1[a]	61.2 ± 2.0	7.8	48	79 (23%)[c]
F31	61	40	60.0 ± 2.8	4.8		54
F34	79	24	106.3 ± 9.4	7.1		63
F35	61	40	109.5 ± 3.5	7.4		90
F39	76	24	106.0 ± 12.3	7.8		61
F310	81.0	19	81.0 ± 5.8	6.2		65
FJ1	71	30	56.3 ± 2.2	6.5		130
FJ2	60	41	64.2 ± 5.8	4.8		47
FJ3	82	18	54.7 ± 2.7	4.6		135
FI1	67	34	62.8 ± 9.8	8.0		

[a] Mean ± S.E.
[b] From Refs. 18 and 44.
[c] Numbers in parentheses represent proportion of dead (floating) cells.
[d] NG, no growth.

near to normal rate of ND5, ND4, and CYTb polypeptide synthesis. Furthermore, there was no clear evidence of premature translation termination, with the exception of a small amount of the main premature translation termination product, pMERRF (35), migrating just ahead of ND4 in T6. The occurrence in C4T mtDNA, as in the VA_2B parental cell mtDNA, of an ND3 polymorphism previously described in HeLa cells, which causes a faster migration of this subunit in a polyacrylamide gel (43), offered an additional useful marker of transcomplementation. Thus, in the protein synthesis patterns of Fig. 1*c*, the relative labeling of the ND3 and ND3′ bands, characteristic of pT1 and C4T mtDNA, respectively, showed a strong prevalence of ND3 in clones T3 and T6 and nearly equal labeling of the two bands in T7. Considering the large excess of MERRF mutation-carrying mtDNA in the three transformants and the expected low rate of ND3 synthesis in homoplasmic MERRF mutant cells relative to the wild-type cells (~35% (35)), the observed high ratio of ND3 to ND3′ labeling in T3 and T6 is consistent with a substantial level of transcomplementation in these clones of the $tRNA^{Lys}$ mutation by *ND4* mutation-carrying mtDNA.

Chromosome count in the three clones showed that T7 had an average chromosome number very similar to those of the parental cell lines, as expected for cybrids, whereas T3 and T6 had a chromosome number nearly double, strongly suggesting that these two clones represented cell hybrids (Table I). Oxygen consumption measurements (Table I) showed a recovery of the normal rate in all three clones, when compared with the rate expected in 143B cells (5–6 fmol/min/cell (18, 44)), in agreement with the conclusion that a reciprocal complementation of the two mutations had occurred in them. The calculated absolute frequency of transcomplementation in this experiment was thus $\sim 1.2 \times 10^{-5}$ (Table II). However, a surprising finding was that the three clones grew in regular DMEM considerably more slowly than 143B cells (Table I). Also in special DMEM/galactose medium, they grew more slowly than 143B cells (Table I). Furthermore, in the latter medium, they showed evidence of a significant rate of cell death, as revealed by the detachment of cells from the plate (Table I). It was particularly intriguing that these clones kept growing at a low rate and exhibited an abnormally high rate of cell death even after 18 weeks of continuous culturing since their isolation.

Residual Wild-type mtDNA in the Parental pT1 Cell Line—The very low frequency of transcomplementing clones in the experiments described above raised the possibility that the pT1 cell line and/or the C4T cell line contained a very minor residue of wild-type mtDNA, not detectable by the primer extension assays, and distributed in a non-uniform way among the cells. Rare cells in one or the other parental cell line might conceivably produce, by intramitochondrial complementation of the mutation, clones mimicking true transcomplementing clones. To exclude this possibility, 10^7 C4T cells were maintained for 6 weeks in special DMEM/galactose medium. As expected, massive cell death was observed, but not a single C4T clone able to grow in galactose medium was detected. A similar experiment carried out with pT1 cells also revealed a massive cell death. Surprisingly, however, surviving clones were detected, at a very low frequency, *i.e.* of ~2 per 10^5 original cells. In order to investigate further this phenomenon, eight of the surviving clones were isolated, and their mtDNA was analyzed. In all cases, these clones were found to carry the MERRF mutation in heteroplasmic form (Fig. 2*a*), exhibiting a minimum of 9% wild-type mtDNA, which was sufficient to restore mtDNA functionality in MERRF mutation-carrying cells (18).

In order to analyze the distribution of the residual wild-type mtDNA in the MERRF mutant cell population, 10 clones were isolated from this population in regular DMEM medium, and 10^6 cells of each clone were plated in special DMEM/galactose medium. As shown in Fig. 2*b*, the frequency of the subclones growing in this medium varied greatly among the original 10 pT1 clones, with the cumulative frequency (2.4×10^{-5}) being very similar to that observed in the original cell population (2×10^{-5}). The experiment described above allowed the isolation of pT1 clones carrying the MERRF mutation in reliably homoplasmic form, as judged from the fact that 10^6 of their cells did not generate any surviving subclones under strictly selective conditions. This conclusion was confirmed for clone 3 by an experiment in which 10^7 cells were replated in special DMEM/galactose, and no surviving cells were detected after 5 weeks of culture. As expected, this clone had maintained the respiratory-deficient phenotype of the pT1 cell line (Table I). Clone 3 (hereafter designated pT1.C3) was therefore chosen for all subsequent experiments.

Role of the Nuclear Background in Transcomplementa-

TABLE II
Summary of the transcomplementation experiments

Expt.	Enucleated cells	Recipient cells	Glucose medium			Galactose medium				Relative complementation frequency[e]
			Clones analyzed	Clones with both genomes	Fusion frequency[a]	Recipient cells plated	Maximum clones expected[b]	Surviving clones[c]	Absolute complementation frequency[d]	
1	pT1 (10^5)	C4T (5×10^5)				2.5×10^5		3 (8)	1.2×10^{-5}	
2	C4T (5×10^5)	pT1.C3 (5×10^5)				1.67×10^5		10 (31)	6.0×10^{-5}	
3	pT1.C3 (5×10^5)	C4.13-E (5×10^5)	144	4	0.028	1.67×10^5	4639	14 (35)	8.4×10^{-5}	3.0×10^{-3}
4	pT1.C3 (5×10^5)	C4.13-J (5×10^5)	212	3	0.014	1.67×10^5	2363	9	5.4×10^{-5}	3.8×10^{-3}
5	pT1.C3 (5×10^5)	C4.13-J (5×10^5)	140	0		1.67×10^5		3	1.8×10^{-5}	
6	pT1.C3 (5×10^5)	C4.13-I (5×10^5)	140	0		1.67×10^5		2	1.2×10^{-5}	
7	pT1.C3 (5×10^5)	C4.13-J (10^5)	160	0		3.33×10^4		1	3.0×10^{-5}	
8	pT1.C3 (5×10^5)	C4.13-J (2×10^4)	88	3	0.34	2.00×10^4	682	11	5.5×10^{-4}	16.1×10^{-3}

[a] Determined from the ratio of the number of clones with both genomes to the number of clones analyzed.
[b] Values calculated assuming that transcomplementation would occur in all fusion products.
[c] The values in parentheses indicate the total number of clones originally detected, some of which subsequently died.
[d] Determined from the ratio of surviving clones to the number of recipient cells plated.
[e] Determined from the ratio of absolute complementation frequency to fusion frequency.

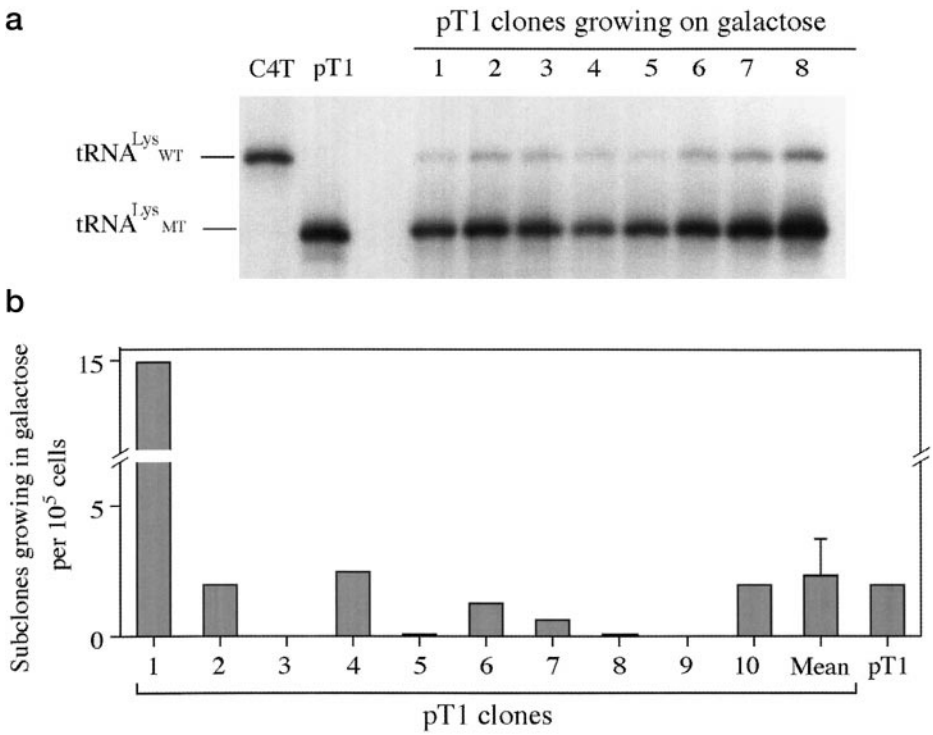

FIG. 2. **Evidence of the presence of a residual minor amount of wild-type (*WT*) mtDNA in pT1 cells.** *a,* shows a fluorogram, after electrophoresis through a sequencing gel, of the ^{32}P-5′-end-labeled primer extension products obtained from samples of total cellular DNA extracted from the parental pT1 and C4T cell lines and eight pT1 clones able to grow in galactose-containing medium. *b,* the yield of subclones growing in galactose-containing medium from 10^6 cell samples of 10 independent clones isolated in glucose-containing medium from the original pT1 cell line is compared with the yield obtained from the original pT1 cell population. Mean indicates the average yield of subclones from the 10 clones ± S.E.

tion—In the transcomplementation experiment described above, a surprising finding was that in the transcomplementing clones, including the cybrid T7, the MERRF mutation-containing mtDNA was in large excess over the mtDNA carrying the *ND4* gene frameshift mutation. In order to test the possible role of the nuclear background in this phenomenon, as well as explaining the low frequency of transcomplementation, another cell fusion experiment was carried out, this time using 5×10^5 predominantly enucleated C4T cells as mitochondria donors and 5×10^5 pT1.C3 cells as recipients. The use of the pT1.C3 cells was also expected to provide a test of the possible influence of the small amount of endogenous wild-type tRNA^{Lys} genes in the previous experiment. A portion of the fusion mixture, corresponding to $\sim 1.7 \times 10^5$ recipient pT1.C3 cells, after 24 h incubation in non-selective medium, was transferred to special DMEM/galactose in two 10-cm dishes. Of 31 detected clones, only 10 continued to grow, and among these, five clones (F31, F34, F35, F39, and F310) were randomly selected for further analysis.

Primer extension analysis revealed that all five clones contained both the MERRF mutation-carrying mtDNA and the *ND4* mutation-carrying mtDNA (Fig. 3), with the former being again predominant, although somewhat less than in the first experiment (Table I). The *ND4* mutation-carrying mtDNA, with the MERRF-mutant mtDNA, again accounted, within $\pm<3\%$, for the total mtDNA (Table I). Karyotype analysis indicated the likely cybrid nature of the F31 and F310 clones, with an increased parental chromosome number, and the probable hybrid nature of the F34, F35, and F39 clones (Table I).

As observed for the clones isolated in the first experiment, the new five selected clones grew very slowly in special DMEM/galactose medium (Table I), again with evidence of a significant rate of cell death as revealed by cell detachment from the plates (data not shown). The recovery of mtDNA functionality in these clones was indicated by the re-establishment of full respiratory capacity (Table I) and of a nearly normal mitochondrial protein synthesis pattern, as well as by the substantial reduction of the premature translation termination products pMERRF and truncated ND2 polypeptide and by the relatively high rate of labeling of the ND3 subunit. These results indicated that transcomplementation of the two mutations had occurred in these clones, although the evidence suggested a less effective

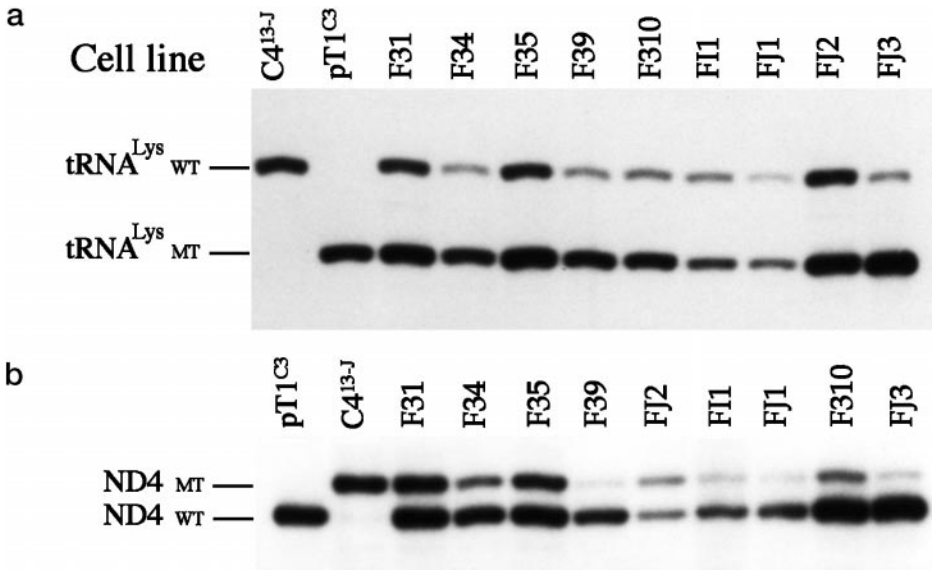

FIG. 3. **Amounts of tRNA^{Lys} gene or *ND4* gene mutation-carrying mtDNAs in the transcomplementing clones derived from enC4T × recpT1.C3 and enpT1.C3 × recC4.13 cell fusions.** *a* and *b*, fluorograms, after electrophoresis through a sequencing gel, of the ^{32}P-5′-end-labeled primer extension products obtained from samples of total cellular DNA extracted from the indicated cell lines. The relative proportions of the two mutant mtDNAs in each cell line, estimated from the fluorograms, are shown in Table I. *WT*, wild type; *MT*, mutant.

expression of the C4T mtDNA than in the previous experiment. The calculated absolute complementation frequency was ~6×10^{-5}, *i.e.* considerably higher than that measured in the first experiment (Table II), presumably reflecting the higher ratio of mitochondrial donor to recipient cells used in the cell fusion. In conclusion, the results obtained in this experiment, which utilized pT1.C3 cells as recipients, showed that the small residue of wild-type mtDNA in pT1 cells was not responsible for the transcomplementation observed in the previous experiment. Furthermore, they indicated that the nuclear background of the recipient cells did not play any significant role in the predominance of the MERRF mutation-carrying mtDNA in the transcomplementing clones.

Frequency of Transcomplementation Relative to Cell Fusion Frequency—A critical factor in evaluating the significance of the low absolute frequency of transcomplementation in the experiments described in the previous sections was the efficiency of cytoplast or cell × cell fusion. In order to estimate the frequency of the fusion events, growth of the products under conditions non-selective for respiration competence, *i.e.* in regular glucose-containing DMEM, was required. The quantification in this type of experiment, in turn, was expected to be greatly facilitated by the elimination from the mitochondrial donors, in this case pT1.C3 cells, of non-enucleated cells. These, in fact, could grow in medium non-selective for respiration competence and thus interfere with the identification of the A8344G mutation-carrying mtDNA in the cybrids or hybrids, to be carried out by *Nae*I digestion of total cell DNA (see "Experimental Procedures"). In order to eliminate the non-enucleated mitochondrial donor cells, a C4T derivative carrying a second drug resistance marker, besides BrdUrd resistance, was isolated. For this purpose, the *ND4* mutation-carrying mitochondria were transferred, as detailed under "Experimental Procedures," into a cell line different from 143B.$\rho^0$206, *i.e.* 143B.$\rho^0$13.1. This cell line had been previously isolated by long term exposure to a low concentration of ethidium bromide of an APRTase-less mutant, which was, therefore, resistant to 8-azaadenine, of 143B.TK$^-$ cells (26). Several clones resistant to BrdUrd and 8-azaadenine were thus isolated, and three of these (C4.13E, C4.13J, and C4.13I) were utilized as recipients for fusion with enucleated pT1.C3 cells in subsequent experiments. It was verified that they contained pure mutant *ND4* gene and had a respiratory-deficient phenotype, as shown for clones C4.13J and C4.13I in Fig. 3 and Table I.

Several fusions between C4.13 clones and enucleated pT1.C3 cells were carried out, as detailed in Table II. In two experiments (experiments 7 and 8), a 5-fold and, respectively, 25-fold higher ratio of cytoplasts to recipient cells was used, as compared with the other experiments. In each case, after 24 h incubation in non-selective medium, a portion of the fusion mixture was plated in an appropriate number of 96-microwell plates, at a concentration of ~0.2 cell/microwell, in regular DMEM containing 100 μg of BrdUrd and 50 μg of 8-azaadenine per ml. Another portion of the fusion mixture (30 to ~100%, as specified in Table II) was plated, either in one Petri dish or in 96-microwell plates (in general three), in special DMEM/galactose medium containing BrdUrd and 8-azaadenine, and the remainder was frozen.

Total cell DNA from 88 to 212 clones, isolated in different experiments from a portion of the cell fusion mixture grown under conditions non-selective for respiration competence (in regular DMEM), was tested by restriction enzyme *Nae*I digestion for the presence and proportion of the A8344G tRNA^{Lys} gene mutation in mtDNA. In three of the fusion mixtures analyzed (experiments 3, 4, and 8 in Table II), a small fraction of the clones tested showed the presence of the tRNA^{Lys} gene A8344G mutation in a proportion varying between 26 and 90% of total mtDNA. Because of the apparent complete absence of wild-type tRNA^{Lys} genes in pT1.C3 cells and of wild-type *ND4* genes in C4.13 cells (Fig. 3), in the clones which carried the tRNA^{Lys} gene mutation in a portion of their mtDNA, the remainder of mtDNA was considered to represent mtDNA with mutant *ND4* genes. Accordingly, the percentage of clones with mutant tRNA^{Lys} genes provided an estimate of the frequency of fusion of the two parental lines. This varied between 1.4 and 3.4% (Table II). In three of the fusion experiments, no clone containing the tRNA^{Lys} gene A8344G mutation was found, due presumably to the very low frequency of cell fusion.

A few clones derived from the pT1.C3xC4.13 clone fusions, which grew in galactose medium containing 8-azaadenine, were analyzed for genotype (Fig. 3), chromosome number, oxygen consumption rate (Table I), protein synthesis (Fig. 4), and growth rate. All the clones tested contained both types of mutant mtDNA, with the proportion of MERRF mutation-carrying mtDNA varying between 60 and 82%, and with the two mutant forms accounting, within 1–3%, for the totality of mtDNA (Fig. 3). These clones exhibited a chromosome number indicating their cybrid nature, as expected from the recessive nature of the APRTase null mutation, which would have killed any hybrid cell. Furthermore, they were fully respiratory-competent,

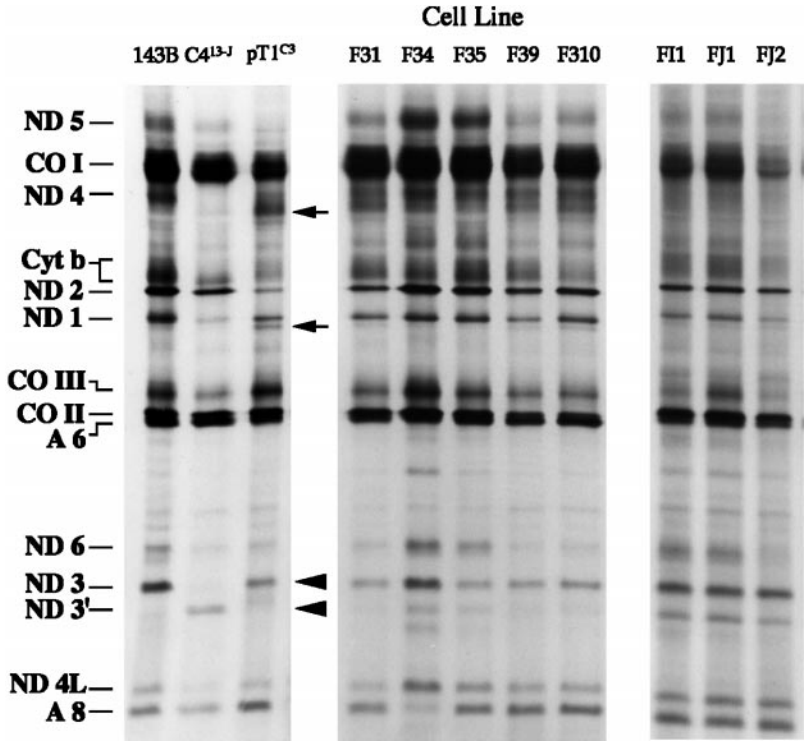

FIG. 4. **Fluorogram, after electrophoresis through an SDS-polyacrylamide gradient gel, of the mitochondrial translation products of the indicated cell lines labeled with [^{35}S]methionine for 2 h in the presence of emetine.** Symbols are as described in the legend of Fig. 1.

as expected from the selection medium used (Table I). Their protein synthesis patterns (Fig. 4) revealed the reacquisition of the capacity to synthesize the ND5, ND4, and CYTb polypeptides and the strong reduction or absence of the abnormal premature translation termination products pMERRF and truncated ND2 polypeptide. In addition, they exhibited again a preponderant labeling of the ND3 polypeptide characteristic of pT1.C3 mtDNA (Fig. 4). As observed in the previous experiments, the clones analyzed grew in general very slowly in galactose medium (Table I) and exhibited a significant rate of cell death (data not shown).

The evidence presented above indicated clearly that the clones growing in galactose medium were true transcomplementing clones. As shown in Table II, the absolute transcomplementation frequency among the clones isolated in the experiments with high cell fusion frequency (experiments 3, 4, and 8) was similar to that previously observed for the enC4T × pT1.C3 fusion (experiment 2), *i.e.* 5.4 and 8.4×10^{-5} (experiments 3 and 4), or much higher (5.5×10^{-4}), in the case of the fusion utilizing the largest excess of cytoplasts (experiment 8). It was, on the contrary, considerably lower (1.2 to 3×10^{-5}) in the experiments with low cell fusion frequency (experiments 5–7), as expected. The absolute frequency of the transcomplementing clones, divided by the frequency of cell fusion, provided an estimate of the relative efficiency of transcomplementation. As shown in Table II, this varied between 3.0×10^{-3} and 3.8×10^{-3} in experiments 3 and 4, and 1.6×10^{-2} in experiment 8. These results indicated clearly the intrinsically very low frequency of the transcomplementation events.

DISCUSSION

In the present work, large scale fusion experiments between cultured human cell lines carrying two non-allelic recessive mtDNA mutations have allowed the detection of stable transcomplementation between the two mutations under growth conditions selective for recovery of respiratory capacity. However, the phenomenon occurred only very rarely among the double transformants, with a 0.3 to 1.6% frequency, accounting for the failure to detect it in earlier experiments not involving selection for respiratory competence (18). The transcomplementing clones became established very sluggishly and exhibited in general slow growth and a substantial rate of cell death. The present results confirm the previous observations of transcomplementation of mtDNA mutations in human cell cultures (24, 25). However, they are in striking contrast to the general model that has been proposed of human mitochondria functioning as a single dynamic unit in a living cell, with rapid diffusion of mtDNA and/or its products throughout the organelles (23). They support the conclusion that the capacity of mitochondria to fuse and mix their genetic contents is not an intrinsic general property of these organelles in mammalian cells, although it may be susceptible to developmental and physiological regulation.

Previously, the occurrence of apparent mitochondrial fusion in mammalian cultured cells, independent of cell fusion, has been reported by others (9, 10, 11) on the basis of studies using optical or electron microscopy. Although fusion is necessary for the occurrence of genetic complementation, any physical evidence of fusion would not necessarily imply a mixing of the mtDNAs and/or their products from different organelles. It is in fact quite possible that only the external mitochondrial membranes fuse or that the mtDNAs and their transcripts remain compartmentalized in fully fused mitochondria.

Mechanism Underlying the Transcomplementation of the Two Mutations—In the present work, one must assume that some form of mitochondrial fusion event produced the observed transcomplementation of the two mtDNA mutations analyzed. However, nothing is known about the mechanism involved. The very low frequency of reciprocal complementation of the two mutations detected in the cytoplast or cell × cell fusion products argues strongly against a programmed phenomenon. One cannot exclude that, in a small fraction of the transformed recipient cells, a gene homologous to the *fzo* gene transiently activated during spermatid development in *D. melanogaster* (12) and to its equivalent found in *S. cerevisiae* (13, 14) and the genes encoding other components of the mitochondrial fusion

machinery (13) are expressed at a low level. However, a more likely possibility, which has not been excluded in any of the experiments reported so far in which transcomplementation has been observed (23–25), is that the transient exposure of the cells to polyethylene glycol (PEG) used to induce cell fusion may be responsible for the fusion of mitochondria. The low frequency of stable transcomplementing clones observed in the present and previous work would imply that these mitochondrial fusion events, which would produce heteroplasmic organelles, would be followed by a reversal of transcomplementation, due to division of the organelles and re-segregation of the two mtDNAs. According to this hypothesis, only very infrequently would transformants with stable intramitochondrial heteroplasmy be selected, accounting for the great rarity of stable transcomplementing clones observed. A similar hypothesis had been previously proposed, which associated mitochondrial fusion with a transient response to a cell fusion stimulus, like during myotube formation (45). One may further elaborate this alternative model by assuming that the transient response to a cell fusion stimulus would involve an activation of the *fzo* gene and related genes. If an extensive *reversible* mitochondrial fusion event during cell fusion does indeed occur, the real *in vivo* frequency of transcomplementation events may be much lower than observed here. This possibility is open to experimental test through the introduction of donor mitochondria into the recipient cell by microinjection (46).

Factors Affecting the Efficiency of Transcomplementation—In the scenario discussed above of mitochondrial fusion being transiently activated during cell fusion, the very rare, slowly established transcomplementing clones detected in this work and previously by others (24, 25) would represent those few clones that underwent no segregation or only partial intermitochondrial segregation of the two mutant mtDNAs. The considerable proportion of dead cells that was observed in the present work during growth of the surviving clones after cell fusion may well be the result of an equilibrium situation between selection of cells having reached the threshold of respiratory competence associated with the minimum necessary number of heteroplasmic organelles and continuous loss of this competence due to segregation of the two defective mtDNAs by mitochondrial division.

It has been suggested that the very low frequency of transcomplementation between homoplasmic recessive mtDNA mutations in cell fusion products grown under conditions selective for respiration may result from lack of production of sufficient energy to overcome the energy barrier of membrane fusion (25). However, this suggestion is not supported by the results of previously mentioned work from this laboratory (18), which had shown that the non-transcomplementing clones isolated under conditions non-selective for respiration grew at a high rate (doubling times between 22 and 28 h) and were presumably able to produce adequate ATP from glycolysis to carry out mitochondrial fusions. In the present work, the possibility that the observed very low frequency of transcomplementation was due to the fact that the incubation of the cell fusion products under non-selective conditions was too short to allow adequate mitochondrial fusion, and genetic complementation is not supported by the available evidence. In fact, in the first experiment, a 2-day and a 6-day incubation of the fusion products under non-selective conditions yielded about the same number of clones surviving under selective pressure. Furthermore, an argument against the idea of a quick death of respiratory-deficient cells in selective medium is the observation that the proportion of dead cells after a 2-day incubation in such medium of the two parental cell lines was only 12–40%. Finally, the observation that, in *ND4* gene frameshift mutation-carrying mitochondria, all proteins other than ND4 exhibited a normal rate of synthesis and the expectation that these organelles carried an ~2-fold excess of aminoacylated $tRNA^{Lys}$ over that required to support normal translation (35) strongly suggests that, if they underwent a fusion with $tRNA^{Lys}$ mutation-carrying mitochondria, there would be a rapid complementation of the latter mutation.

The finding in the present work that, independently of whether $tRNA^{Lys}$ gene mutation- or *ND4* gene mutation-carrying cells were used as recipients for cell fusion, the MERRF mutation-carrying mtDNA was always predominant (60–90% in the various complementing clones) presumably reflected the need for the cell to have an adequate rate of NADH dehydrogenase activity. It is known, in fact, that Complex I-dependent O_2 consumption is usually the rate-limiting step in respiration (47, 48).

Implications for Disease-causing or Aging-related mtDNA Mutations—The present observations have important implications for understanding the segregation and complementation behavior of disease-causing mtDNA mutations in man and the transmission of such diseases (49), as well as for potential therapeutic approaches involving mitochondria-mediated gene transfer. Another area where the observed tendency of mammalian mitochondria to remain genetically autonomous has significant implications is that of aging-dependent mtDNA damage. There is substantial evidence of an aging-related occurrence in mtDNA of oxidative derivatives of nucleotides (50), of small deletions and insertions, and of large deletions (51–54). Most significantly, very recently an aging-dependent large accumulation of specific mutations in a critical control region for mtDNA replication has been demonstrated in human fibroblasts (55). The functional effects of these mutations depend not only on their frequency but also on whether or not mitochondria can fuse and mix their mtDNA products, so as to allow wild-type genes to complement mutant genes. Additivity of the aging-dependent dysfunctions of the individual organelles in a cell, as opposed to transcomplementation between mitochondria, would magnify considerably the overall damage to the cell.

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