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MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts

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ABSTRACT **The pathogenetic mechanism of the mitochondrial $tRNA^{Leu}_{UUR}$ gene mutation responsible for the MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) syndrome was investigated in transformants obtained by transfer of mitochondria from three genetically unrelated MELAS patients into human mitochondrial DNA (mtDNA)-less (ρ°) cells. Marked defects in mitochondrial protein synthesis and respiratory activity were observed in transformants containing virtually pure mutant mtDNA, as compared to the parent of the ρ° cells (the 143B cell line) or to transformants containing exclusively wild-type mtDNA, derived from one of the patients or a maternally related asymptomatic individual. A striking protective effect against the mutation was exerted in the transformants by levels of residual wild-type mtDNA above 6%. The MELAS mutation occurs within the mtDNA binding site for a protein factor (mTERF) that promotes termination of transcription at the 16S rRNA/$tRNA^{Leu}_{UUR}$ gene boundary. A marked decrease in affinity of purified mTERF for the mutant target sequence was observed in *in vitro* assays. By contrast, RNA transfer hybridization experiments failed to show any significant change in the steady-state amounts of the two rRNA species, encoded upstream of the termination site, and of the mRNAs encoded downstream, in the transformants carrying the MELAS mutation.**

In the present work, the approach of mitochondria-mediated transformation utilizing human mtDNA-less (ρ°) cells as recipients (1, 2) has been used to investigate the pathogenetic mechanism of the mtDNA mutation responsible for the MELAS syndrome (3–5). The mutation occurs in the binding site for the transcription termination factor (mTERF) (6) within the $tRNA^{Leu}_{UUR}$ gene. The results indicate that repopulation of ρ° cells with mitochondria carrying the MELAS mutation in their DNA produces transformants exhibiting severe protein-synthesis and respiration defects, but no significant change in the steady-state levels of the heavy (H)-strand-encoded rRNAs and mRNAs (7). Therefore, although the MELAS mutation strongly decreases the affinity *in vitro* of mTERF for the target sequence, its pathogenetic mechanism does not involve a change in the absolute and relative amounts of the RNA species encoded in the two mtDNA H-strand transcription units (7).

MATERIALS AND METHODS

Cell Lines, Myoblast Cultures, and Mitochondria-Mediated Transformation. Conditions of growth of the thymidine kinase-deficient 143B human osteosarcoma cell line (ATCC CRL 8303) and of the ρ°206 cell line, a mtDNA-less derivative of 143B, were as described (1). Mass myoblast cultures from the first patient ("43") and his asymptomatic maternally-related aunt ("94"), from the second patient ("2S"), and from the third patient ("59") were established by published procedures (8–10). Mitochondria-mediated transformation of ρ° cells was carried out by fusion with enucleated myoblasts (2).

Extraction, Electrophoresis, and Blot Hybridization Analysis of RNA. Extraction and oligo(dT)-cellulose chromatography of nucleic acids from the mitochondrial fraction were carried out essentially as described (11). Electrophoresis of nucleic acids in a 1.4% agarose/10 mM CH_3HgOH gel, electroblotting, and hybridization with a ^{32}P-labeled HeLa cell mtDNA probe were performed as previously detailed (12).

For tRNA blot hybridization analysis, samples of nucleic acids from the mitochondrial fraction were electrophoresed as described (13) and electroblotted onto a Nytran 0.2-μm membrane (Schleicher & Schuell). The tRNAs were then crosslinked to the membrane by irradiation with a short-wavelength transilluminator. The blots were hybridized with ^{32}P-labeled probes containing the $tRNA^{Leu}_{UUR}$ gene (clone A2, carrying the mtDNA fragment spanning positions 8287–8591, in pGEM-1) or the $tRNA^{Lys}$ gene (clone MTC2, carrying the fragment spanning positions 2953–4121, in pUC19) at 52°C overnight, as previously described (12), except for the omission of formamide and dextran sulfate.

Southern Blot and PCR Analysis of DNA. Southern blot analysis of mtDNA in the total nucleic acids of the mitochondrial fraction, after digestion with *Eco*RV endonuclease, was carried out by standard procedures, with ^{32}P-labeled HeLa cell mtDNA as a probe. The presence and proportion of mutated mtDNA in total cell DNA, prepared by using an Applied Biosystems 340A DNA extractor or by lysis of cells with Tween 20 and proteinase K digestion, were determined by testing for the presence of the *Apa* I site created by the MELAS mutation (3–5) at position 3243 (14) in a PCR-amplified DNA fragment. The amplified fragment was digested with *Apa* I in the presence of pBluescript KS(+) (Stratagene) DNA linearized with *Xmn* I, as an internal marker for completion of digestion, and analyzed by agarose gel electrophoresis. The proportion of digested and undigested molecules was determined by laser densitometry after ethidium bromide staining and was corrected for the resistance to digestion of heteroduplexes of wild-type and mutant mtDNA on the basis of a mixed-template standard curve (M.Y., A.C., A.M., and G.A., unpublished data).

Gel Retardation Assays. Equal samples of affinity column-purified mTERF (6) were mixed with 2.5 ng of a double-

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stranded oligonucleotide containing the wild-type sequences protected by mTERF (6) or of the homologous double-stranded oligonucleotide carrying the MELAS mutation at position 3243 (labeled with [γ-^{32}P]ATP and polynucleotide kinase), and the mixtures were incubated on ice. After 45 min (time sufficient to give equilibrium binding) or longer, a 200-fold molar excess of the corresponding unlabeled probe was added to all samples but two controls, and the samples were incubated further and, finally, run in a nondenaturing polyacrylamide gel (15).

Analysis of Mitochondrial Protein Synthesis and Measurement of O_2 Consumption. Labeling with [^{35}S]methionine and electrophoresis of the mitochondrial translation products and O_2 consumption measurements were carried out as described (2).

RESULTS

Mitochondria Transfer from Three MELAS Patients and an Asymptomatic Relative's Myoblasts to ρ° Cells. The first patient ("43") was a 19-year-old Caucasian man who exhibited the MELAS mutation in heteroplasmic form in the mtDNA from muscle and blood (A.M., L. Bartolomei, R. Carrozzo, M. L. Mostacciuolo, C. Carbonin, V. Toso, E. Ciafaloni, S. Di Mauro, and C. Angelini, unpublished data).

The second patient ("2S") was an isolated 34-year-old Caucasian man who displayed a characteristic MELAS syndrome, as well as chronic progressive external ophthalmoplegia, sensorineural deafness, and pigmentary retinopathy and exhibited the MELAS mutation in heteroplasmic form in the muscle, peripheral blood, and urinary epithelial cells (16, 17).

The third patient ("59") was an isolated 28-year-old Japanese man who displayed typical MELAS symptoms and exhibited the MELAS mutation in heteroplasmic form in the muscle.

mtDNA analysis of muscle from patient 43 and his aunt ("94") showed a mutant mtDNA content of 93% and 75%, respectively (Fig. 1*a*). The patient 43-derived myoblast culture contained predominantly (92%) mutant mtDNA, while the relative-derived culture contained 55% mutant mtDNA. After fusion of enucleated myoblasts from the two sources with ρ°206 cells, 3 and 10 colonies, respectively, of presumptive mitochondrial transformants were isolated in selective medium (1).

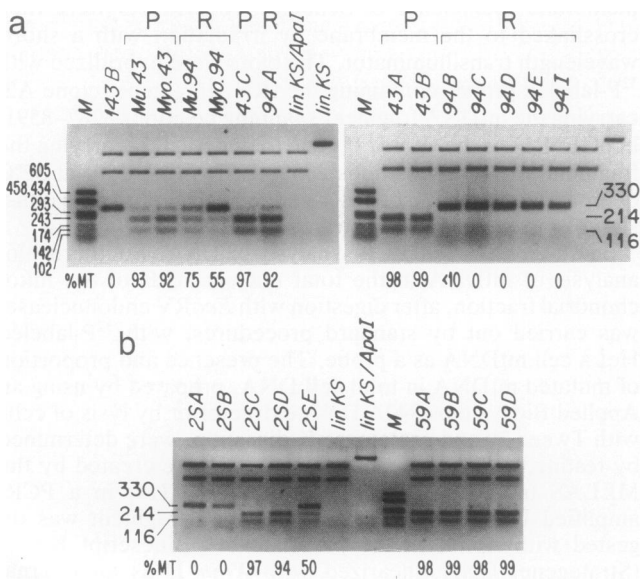

FIG. 1. Detection of the MELAS mutation in the muscle (Mu.) biopsy specimens, myoblast (Myo.) cultures, and mitochondrial transformants from patient 43 and his aunt ("94") (*a*) and from patients 2S and 59 (*b*). Indicated are the expected sizes of the PCR product (330 base pairs) and of the fragments produced by *Apa* I digestion of the MELAS mutation-containing PCR product (214 and 116 base pairs). For the quantitation, see *Materials and Methods*. P, patient; R, relative; M, *Hae* III-digested pBluescript KS(+) DNA; lin.KS, *Xmn* I-linearized pBluescript KS(+) DNA.

Three to 6 weeks after transformation, the three patient 43-derived transformants (43A, 43B, and 43C) contained almost exclusively (97–99%) mutant mtDNA (Fig. 1*a*). By contrast, five of the six relative-derived transformants chosen for analysis (94B to 94E and 94I) exhibited pure or virtually pure wild-type mtDNA. One relative-derived transformant (94A) contained 92% mutated mtDNA.

Among 14 transformant clones obtained from patient 2S, 10 clones were found, 5 weeks after transformation, to be apparently homoplasmic for wild-type mtDNA, and 4 contained strongly predominant mutant mtDNA. Five of the above clones were chosen for further study (Fig. 1*b*). Four transformant clones were obtained from patient 59, and, when tested 8 weeks after transformation, they all showed strongly predominant mutant mtDNA (Fig. 1*b*).

Mitochondrial Protein Synthesis in Transformants. Four to 6 weeks after transformation, transformants 94A, 94B, and 94C exhibited a pattern and rate of labeling with [^{35}S]methionine of the mitochondrial translation products comparable to those of 143B cells (Fig. 2*a*). This result was unexpected for transformant 94A, which contained 92% mutated mtDNA (Fig. 1). By contrast, the overall rate of labeling of the mitochondrial translation products in transformants 43A, 43B, and 43C appeared to be significantly decreased (64–77% and 67–78%, respectively) when compared with the labeling of 94B and 143B cells. Moreover, the labeling of cytochrome *c* oxidase subunits COII and COIII and of ND2 was more severely affected than that of the other polypeptides. No relationship was detected between the extent of decrease in labeling of the individual polypeptides and their absolute content in UUR-encoded leucine residues (data not shown).

The pattern of mitochondrial protein synthesis in the transformants derived from patient 2S, 8 weeks after transformation, also showed a clear decrease (50–70%) in the overall rate of labeling in the transformants with strongly predominant mutant mtDNA (2SC and 2SD) (Fig. 3*a*), as compared to 2SA, 2SB, and 2SE. A repeat of genotype analysis at this time revealed, unexpectedly, in 2SE an increase in proportion of mutant mtDNA from apparently none to 50% (Fig. 1b). In 2SC and 2SD, the COII, COIII, and ND2 polypeptides were more severely affected in their labeling than the other polypeptides. The four transformants with strongly predominant mutant mtDNA derived from patient 59 showed, 8 weeks after transformation, a marked overall decrease in the rate of protein labeling relative to 143B cells (85–95%), the reduction in labeling being again more pronounced for COII, COIII, and ND2 (Fig. 3*b*).

Respiration Capacity of Transformants. Eight to 10 weeks after transformation, transformants 43A, 43B, and 43C exhibited a marked decrease in O_2 consumption as compared to 143B cells (Fig. 2*b*). By contrast, the five analyzed transformants derived from the unaffected relative, including, most remarkably, the 94A transformant, which had 92% mutant mtDNA, all exhibited an O_2 consumption rate comparable to that of 143B cells.

An analysis of the 43B and 94B mtDNA at the time of O_2 consumption revealed that, while the 43B genotype was unchanged, in 94B there had been, unexpectedly, a dramatic increase in the proportion of mutant mtDNA, from <10%, as measured 5 weeks earlier, to 89%. This phenomenon has been investigated further (M.Y., A.C., A.M., and G.A., unpublished work). In contrast to the change in genotype, O_2 consumption in 94B still was in the normal range, therefore reproducing the situation observed for 94A.

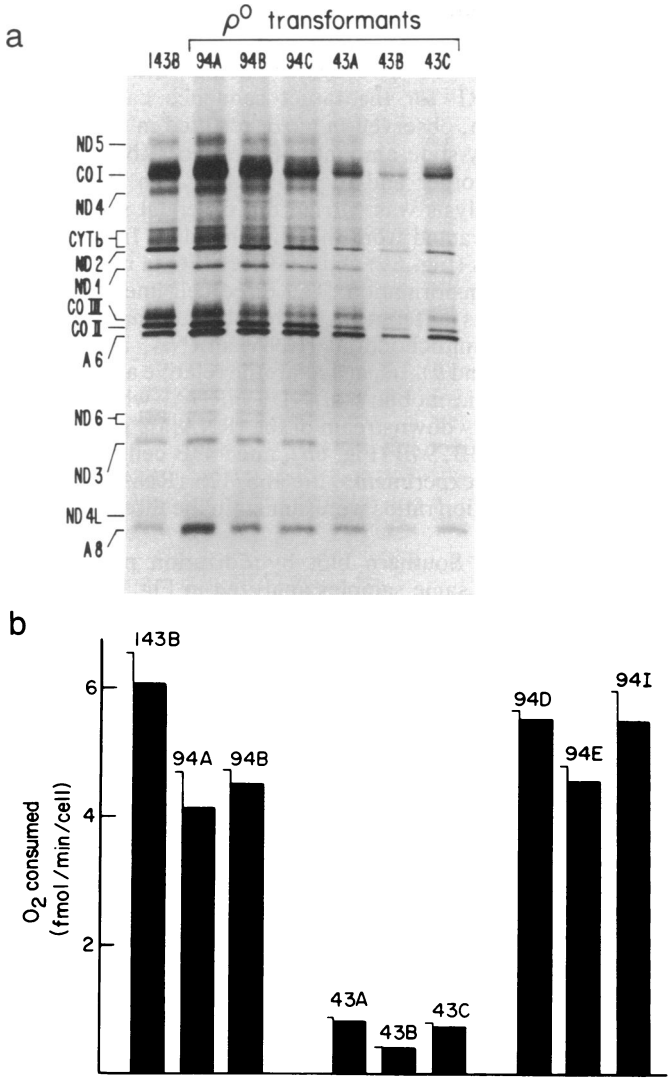

FIG. 2. Mitochondrial transformants derived from patient 43 myoblasts are defective in mitochondrial protein synthesis and respiratory capacity, but those derived from the myoblasts of asymptomatic relative 94 have a normal phenotype. (*a*) Mitochondrial translation products (30-min [^{35}S]methionine pulse; equal protein samples after correction for decay, except the 94A sample, which contained 50% more protein) run in an SDS/polyacrylamide gradient gel. 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 the H^+-ATPase; CYTb, apocytochrome *b*. (*b*) Rate of O_2 consumption (error bars represent 2 SE) in transformants 43A, 43B, 43C, 94A, 94B, 94D, 94E, and 94I and in 143B cells.

The transformant with equivalent proportions of mutant and wild-type mtDNA and the two transformants homoplasmic for wild-type mtDNA derived from patient 2S showed, 8 weeks after transformation, an O_2 consumption comparable to that of 143B cells (Fig. 3*a*). By contrast, the two patient 2S-derived (Fig. 3*a*) and four patient 59-derived (Fig. 3*b*) transformants with strongly predominant mutant mtDNA showed a marked decrease in O_2 consumption.

A Small Minority of Wild-Type mtDNA Protects the Transformants Against the Effects of the MELAS Mutation. A diagram illustrating the relationship between proportion of mtDNA carrying the MELAS mutation and degree of respiratory activity in various mitochondrial transformants analyzed in the present work is shown in Fig. 4. There is a sharp threshold, around 6%, in wild-type mtDNA proportion,

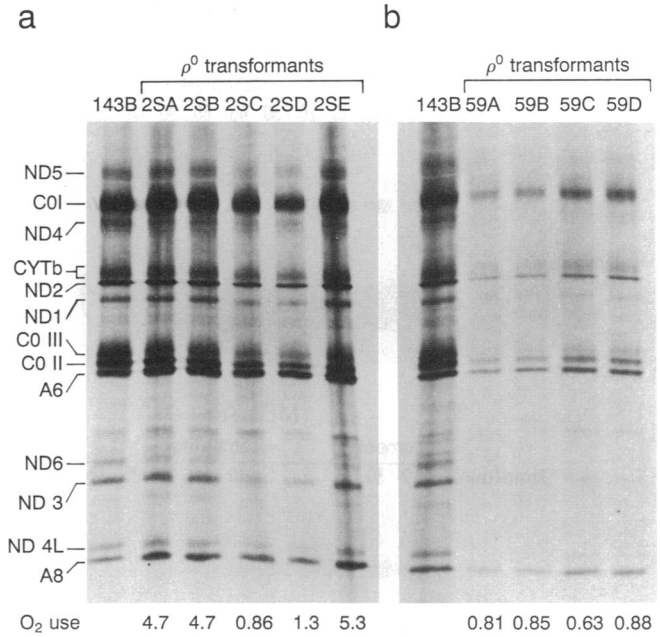

FIG. 3. Mitochondrial protein synthesis pattern and respiratory capacity of transformants derived from patients 2S (*a*) and 59 (*b*). Shown are the labeling patterns after a 30-min [^{35}S]methionine pulse (25 μg of protein per sample) in several transformants and in 143B cells. Symbols are as in Fig. 2. The O_2 consumption data for the transformants are expressed in fmol/min per cell.

above which this DNA protects against the phenotypic effects of the mutation, with full protection by ≈10% wild-type mtDNA.

The MELAS Mutation Strongly Reduces the Affinity of mTERF for the Termination Site. To test whether the MELAS mutation affected the affinity of mTERF for the termination site, dissociation rates were measured in gel retardation assays (15). The half-life of the complex involving the mutated sequence was between 5 and 6 min, whereas that of the complex involving the wild-type sequence was between 40 and 50 min (Fig. 5).

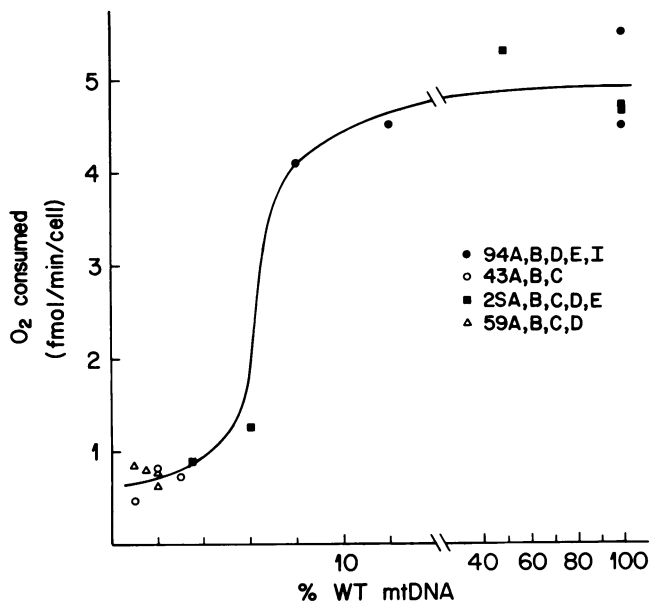

FIG. 4. A small minority of wild-type (WT) mtDNA can protect the mitochondrial transformants against the respiratory defect caused by the MELAS mutation.

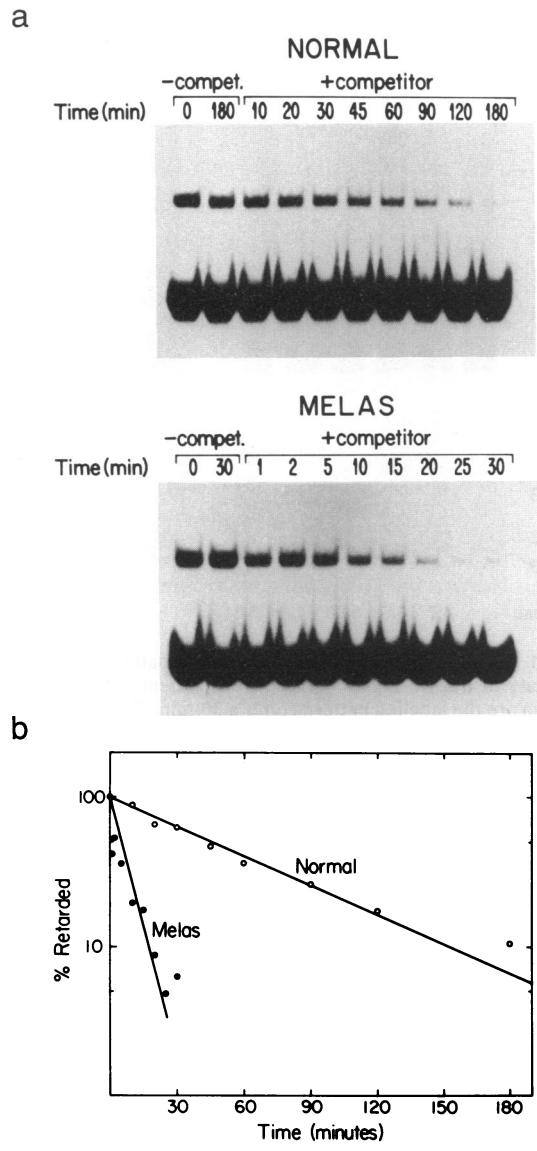

FIG. 5. Kinetics of dissociation of complexes of mTERF with the wild-type DNA probe or the MELAS mutation-carrying probe. (*a*) Gel retardation assays. The slower band in each lane represents the mTERF-DNA complex, the faster band, uncomplexed probe. The times of incubation of the samples (after the initial 45 min) in the absence (controls) or presence of competitor are indicated. (*b*) Densitometric quantitation of the autoradiograms in *a*.

The Steady-State Amounts of Mitochondrial rRNAs, mRNAs, and tRNA$^{Leu}_{UUR}$ Are Not Significantly Affected by the MELAS Mutation. To investigate whether the decreased affinity of mTERF for the target sequence carrying the MELAS mutation, observed *in vitro*, resulted in changes *in vivo* in the steady-state amounts of the two rRNA species relative to each other and to the mRNAs, an RNA blot hybridization analysis was carried out on equal samples of nucleic acids extracted from the mitochondrial fractions of 43B and 94B cells (Fig. 6*b*) and 143B cells (data not shown) 9 weeks after transformation. The three cell lines failed to show any obvious differences in the absolute and relative amounts of the mitochondrial rRNA, mRNA, and tRNA species (Fig. 6 *a* and *b*). In particular, the relative amount and the electrophoretic mobility of ND1 mRNA, which is encoded immediately downstream of the tRNA$^{Leu}_{UUR}$ gene, were not different in 43B, 94B (Fig. 6*b*), and 143B cells (data not shown). In these experiments, the 16S/12S rRNA and 16S/mRNA hybridization ratios were similar in the three cell lines (Table 1).

Fig. 6*c* shows Southern blot hybridization patterns of mtDNA from the same samples analyzed in Fig. 6 *a* and *b*. The ratios of amounts of mtRNA to mtDNA and of mRNA to mtDNA are comparable in the three cell lines (Table 1). The mtDNAs from 94B and 43B exhibited a fragment length polymorphism relative to the mtDNA of 143B.

In other experiments, equal samples of the same nucleic acid preparations analyzed in Fig. 6 *a* and *b* were subjected to RNA transfer hybridization with a tRNA$^{Leu}_{UUR}$ probe alone, or with a mixture of the tRNA$^{Leu}_{UUR}$ probe and a tRNA^{Lys} probe. In both blots, the content of tRNA$^{Leu}_{UUR}$ per unit of mtDNA was slightly lower (≈20%) in 43B cells than in 94B or 143B cells (Fig. 6*d* and Table 1). No difference in electrophoretic mobility of the tRNA$^{Leu}_{UUR}$ in 43B cells, as compared to 94B or 143B cells, could be detected. The tRNA^{Lys} level appeared to be 40–50% higher in 94B and 43B cells than in 143B cells (Table 1).

DISCUSSION

It seems very likely that the MELAS mutation caused the disease phenotype in the affected individuals by inhibiting mitochondrial protein synthesis in muscle and nerve cells, and probably other cells, and thus by affecting the assembly of respiratory complexes. Cytochemical evidence that cytochrome *c* oxidase deficiency is associated with the MELAS mutation has been recently reported (18). The observation that the presence in heteroplasmic transformants of levels of wild-type mtDNA above 6% can protect the cell against the phenotypic effects of the MELAS mutation has extended to this mutation the *in vitro* observations (M.Y., T. Miyatake,

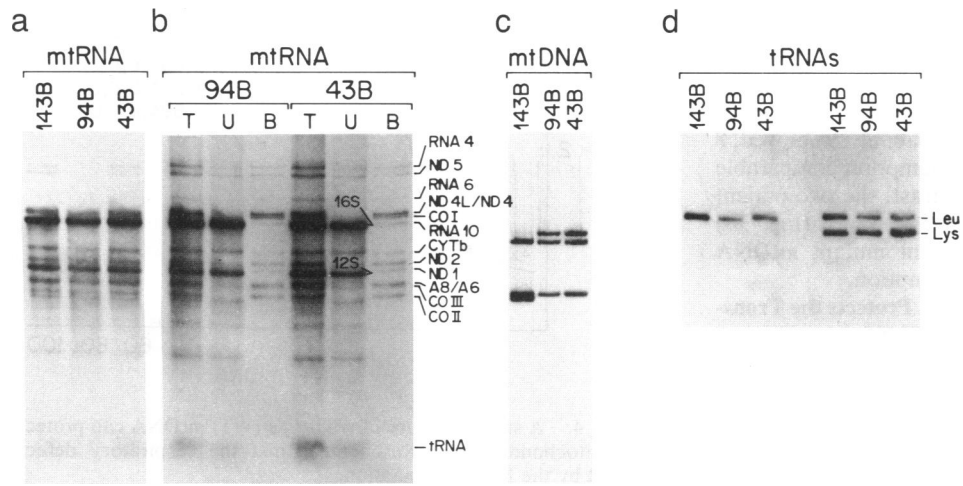

FIG. 6. Blot hybridization analysis of mitochondrial nucleic acids from 143B, 94B, and 43B cells. (*a* and *b*) Equal amounts of nucleic acids were analyzed either directly after DNase I digestion (*a*) or after oligo(dT)-cellulose chromatography (*b*) (T, total; U, unbound fraction; B, bound fraction), by RNA gel blot hybridization. (*c* and *d*) Equivalent samples of nucleic acids of the three preparations were used for Southern blot analysis, after *Eco*RV digestion (*c*) or for quantitation of tRNA$^{Leu}_{UUR}$ and tRNA^{Lys} (*d*).

Table 1. Quantitation of mitochondrial RNAs

Comparison		Ratio* 143B	94B	43B
RNA/mtDNA	Exp.1	0.74	1.0	0.82
	Exp.2		1.0	0.86
mRNA/mtDNA	Exp.1	1.1	1.0	0.97
	Exp.2		1.0	1.1
$tRNA^{Leu}_{UUR}$/mtDNA	Blot 1	1.1	1.0	0.78
	Blot 2	1.0	1.0	0.79
$tRNA^{Lys}$/mtDNA	Blot 2	0.73	1.0	1.1
16S/12S†	Exp.1	3.4	3.4	3.2
	Exp.2	2.7	3.6	3.1
16S/mRNA‡	Exp.1	1.7	1.7	1.9
	Exp.2		1.2	0.96

*Derived from densitometric analysis of appropriate exposures of the autoradiograms shown in Fig. 6*a* (Exp. 1), Fig. 6*b* (Exp. 2), Fig. 6*c* and Fig. 6*d*. Data from comparison of two different autoradiograms are normalized to the 94B value.

†Calculated from densitometric analysis of the autoradiogram for total RNA shown in Fig. 6*a* (Exp. 1) or for RNA that did not bind to oligo(dT)-cellulose, two samples of which are shown in Fig. 6*b* (Exp. 2).

‡Calculated from densitometric analysis of autoradiograms for total RNA. The mRNAs analyzed were those for ND5, ND4 and ND4L, COI, cytochrome *b*, COII, ATPase 6 and ATPase 8, and COIII.

and G.A., unpublished data) and the *in vivo* observations (19) previously made for the MERRF (myoclonic epilepsy and ragged-red fiber) mutation, making this phenomenon amenable to analysis in an *in vitro* cell culture system.

As to the mechanism(s) whereby the mutation produces the protein synthesis defect, the observation that the reduction in labeling of the various mitochondrial translation products in the defective transformants was not correlated with their UUR-encoded leucine content would seem to argue against an effect of the MELAS mutation on the $tRNA^{Leu}_{UUR}$ function or stability. On the other hand, one cannot exclude that the MELAS mutation affects the $tRNA^{Leu}$ synthetase charging specificity (20), thus producing an instability of the aberrant polypeptides not necessarily correlated with their UUR-encoded leucine content.

A defect of transcription termination *in vitro* at the 16S rRNA/$tRNA^{Leu}_{UUR}$ boundary in a template carrying the MELAS mutation was recently reported (21). However, the present work has provided no evidence to support the occurrence of such a phenomenon *in vivo*. The significance of the slight (≈20%) decrease observed in the steady-state level of $tRNA^{Leu}_{UUR}$ in 43B cells relative to that in 143B cells is uncertain. There were no size alterations of the $tRNA^{Leu}_{UUR}$ or of the immediately downstream-encoded ND1 mRNA or of the 16S rRNA, as detectable by changes in their electrophoretic mobility.

It is possible that the reduction in affinity of mTERF for the mutated target sequence detected in the *in vitro* assays is compensated *in vivo* by hyperexpression of the protein. On the other hand, the present results do not exclude the possibility that alterations in the rates of synthesis of the various RNA species caused by the MELAS mutation could have been compensated by changes in their stability, nor do they provide information as to possible mistakes in the precise processing of the H-strand polycistronic transcripts or fine alterations in the 3′-terminal sequence of 16S rRNA which could affect its incorporation into the large ribosomal subunit or its function. However, the present data at least argue against the simplest possibility, namely, that the defect in protein synthesis in the transformants was caused by a change in the absolute or relative amounts of the different RNA components, due to defects in the transcription termination step or to misprocessing of the total H-strand polycistronic transcripts at the level of the $tRNA^{Leu}_{UUR}$ sequence.

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