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Biochemical evidence for nuclear gene involvement in phenotype of non-syndromic deafness associated with mitochondrial 12S rRNA mutation

Min-Xin Guan, Nathan Fischel-Ghodsian[1] and Giuseppe Attardi*

Division of Biology, California Institute of Technology, Pasadena, California 91125, USA and [1]Ahmanson Department of Pediatrics, Steven Spielberg Pediatric Research Center, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA

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The phenotypic effects of the human mitochondrial 12S rRNA gene mutation at position 1555 associated with maternally inherited non-syndromic deafness and sensitivity to aminoglycoside-induced deafness have been analyzed in 25 lymphoblastoid cell lines derived from members of a large family carrying this mutation in homoplasmic form and from control individuals. A clear decrease in the rates of growth in galactose medium, mitochondrial protein synthesis, total oxygen consumption, and complex I-, complex III- and complex IV-dependent respiration was observed in two groups of nine and 10 mutant cell lines derived, respectively, from symptomatic and asymptomatic members of the family, as compared with six control cell lines. The severity of mitochondrial dysfunction in the mutant cell lines was correlated with the presence or absence of hearing loss in the donor individuals. These observations strongly suggest a role of a nuclear factor(s) in the phenotypic manifestation of the mutation. The approach used here provides a paradigm for the analysis of the nuclear background involvement in other mtDNA-linked disorders, including the putative ones associated with neurodegenerative diseases. Exposure of the cell lines derived from several symptomatic or asymptomatic individuals from the same family to high concentrations of neomycin or paromomycin decreased to a significant, nearly identical extent their rate of growth in glucose-containing medium, as contrasted with the unchanged growth rate of control cell lines or of mtDNA-less cells. These results support the hypothesis that the main target of the antibiotics is the mitochondrial 12S rRNA carrying the 1555 mutation, without any apparent role of the nuclear background.

INTRODUCTION

Susceptibility to aminoglycoside-induced deafness is maternally inherited in humans in a significant proportion of cases. Recently, a homoplasmic A to G transition at position 1555 of mtDNA in a highly conserved region of the mtDNA 12S rRNA gene has been found in families with aminoglycoside-induced deafness (1–3), as well as in an Arab-Israeli family with maternally inherited non-syndromic deafness (1). The homologous region of the bacterial small ribosomal subunit rRNA is known to bind aminoglycoside antibiotics (4–6), and mutations within this region conferring antibiotic resistance have been isolated in bacteria, yeast mitochondria and *Tetrahymena* (5,7–9). Furthermore, the corresponding region of 16S rRNA in *Escherichia coli* forms an essential part of the decoding site of the ribosome (10), and is crucial for subunit association either by RNA–protein or RNA–RNA interactions (11). Unlike most of the other mitochondrial DNA mutations that are associated with a variety of multisystem disorders, many of which include sensorineural deafness as a symptom (12), the 1555 12S rRNA mutation causes a tissue-specific disorder, with no general myopathy or neurological symptoms (13).

We report here the phenotypic characterization of 25 immortalized lymphoblastoid cell lines derived from members of the Arab-Israeli family (including 10 individuals exhibiting both the mutation and hearing loss, nine carrying the mutation but lacking a clinical phenotype, and three lacking the mutation) and from three other unrelated control individuals lacking the mutation. This investigation has shown a variable decrease in the rates of mitochondrial protein synthesis, oxygen consumption and growth in galactose medium in the cell lines carrying the mutation, as compared with control cell lines. In general, in the mutant cell lines, the severity of mitochondrial dysfunction was found to be correlated with the presence or absence of hearing loss in the donor individuals, pointing to the involvement of a nuclear factor(s) in the deafness phenotype. By contrast, all mutant cell lines, independently of the hearing state of the cell donor, exhibited, when compared with wild-type cells, an identically reduced growth rate in glucose medium in the presence of high concentrations of the aminoglycosides neomycin and paromomycin.

RESULTS

Description of the pedigree and selection of cell lines for analysis

A portion of the five generation pedigree of the Arab-Israeli family with maternally inherited non-syndromic deafness (13) is

*To whom correspondence should be addressed

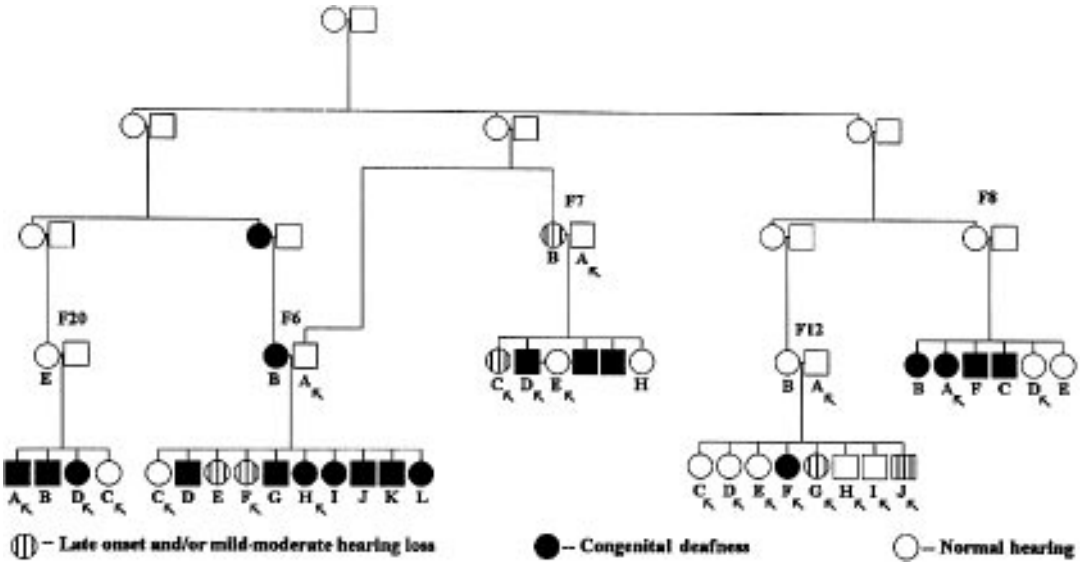

Figure 1. A portion of the Arab-Israeli pedigree with maternally transmitted deafness analyzed in this work is shown. Probands used for the investigation are indicated by arrows.

shown in Figure 1. In this family, most hearing-impaired members exhibited congenital, severe to profound hearing loss, while a few showed late onset and/or mild-to-moderate hearing impairment, without sex predilection; finally, other members had normal hearing. All maternally related individuals in this pedigree exhibited the 1555 12S rRNA mutation (13). Nine mutant cell lines derived from maternally related asymptomatic individuals, aged between 20 and 60 years, 10 mutant cell lines derived from maternally related symptomatic individuals, aged between 20 and 40 years, and six control cell lines derived from unrelated individuals (three 'married in', F7A, F7E and F12A), aged between 25 and 70 years, were selected for analysis.

Genotype of cell lines

An analysis of the mitochondrial genotype of the 25 immortalized lymphoblastoid cell lines confirmed the previously observed (1) presence of the A1555G mutation in homoplasmic form in all the 19 maternally related members of the Arab-Israeli family, and its absence in the six unrelated controls (data not shown). An analysis was carried out of the mtDNA content of the individual cell lines by slot blot hybridization using a ^{32}P-labeled mtDNA probe, and by normalizing the data for quantitative differences among DNA samples on the basis of hybridization with a nuclear 28S rRNA gene fragment, as detailed in the Materials and Methods section. Although there were some variations among the different cell lines in their mtDNA level, which ranged in general between 50 and 100% of that found in 143B.TK$^-$ cells (9100 molecules per cell) (14), no significant differences in the average mtDNA/rDNA ratio were detected among the three groups of control, asymptomatic and symptomatic cell lines. One cell line, derived from the asymptomatic individual F6C, exhibited an abnormally high mtDNA content, corresponding to 140% of the 143B.TK$^-$ mtDNA level (data not shown).

Growth properties of cell lines in glucose- or galactose-containing medium

Figure 2 shows the analysis of the growth properties of the different cell lines in glucose- or galactose-containing medium. Measurements of the population doubling times (DTs) (carried out as described in Materials and Methods) revealed considerable fluctuations among the different cell lines within each group. Despite this variation, a pattern clearly emerged. Thus, in glucose-containing medium, all 10 mutant cell lines derived from symptomatic individuals (S), except one (F7C), exhibited a decrease in their growth rate relative to the average value observed for the wild-type cell lines, which, however, reached only the 91% confidence level (Fig. 2a). The nine mutant cell lines derived from asymptomatic individuals (AS) exhibited DT values slightly shorter in the average than those of the cell lines from symptomatic individuals, although still clearly longer than those of the wild-type cell lines.

It has been previously shown that cell lines with defective oxidative metabolism have reduced growth capacity in media containing galactose instead of glucose (15,16). Therefore, the growth properties of the lymphoblastoid cell lines was investigated in medium containing galactose and an increased concentration of pyruvate relative to Dulbecco's modified Eagle's medium (DMEM). It is clear from Figure 2b that all the cell lines from the symptomatic individuals, except F7C, were markedly affected in their growth rate in galactose medium relative to the control cell lines, the difference between the DTs of the two groups being statistically significant at the >99% confidence level. The cell lines from the asymptomatic individuals showed also a decrease in their average growth rate in galactose medium relative to that of the control cell lines, which was significant at the 95% confidence level. However, among the asymptomatic individual-derived cell lines, F6C, which had a high mtDNA content, and F12H exhibited DT values in galactose medium comparable with the mean control value. There was a clear difference in average growth rate between the cell lines from

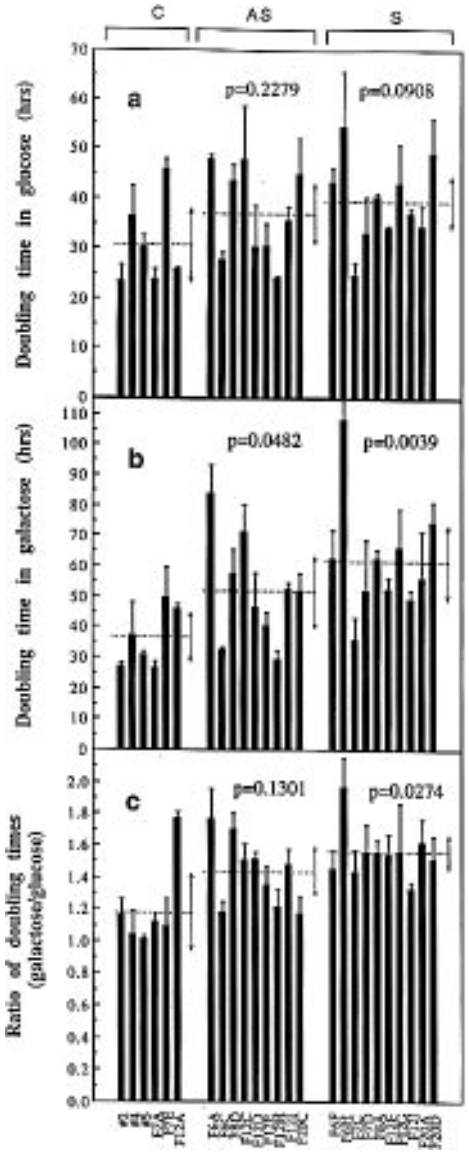

Figure 2. Growth properties of lymphoblastoid cell lines. The population doubling time (DT) during 72 h of growth was determined in special DMEM-glucose (**a**), or in special DMEM-galactose (**b**). (**c**) shows the ratios of DTs (in galactose- versus glucose-containing medium). The average of two to five determinations for each cell line is shown, with error bars representing two standard errors of the mean (SE). C: controls; AS: asymptomatic individuals; S: symptomatic individuals. The horizontal dashed lines represent the average value for each group, and the vertical arrows, two SE; p indicates the significance, according to the *t*-test, of the differences between AS mean and C mean, and between S mean and C mean.

symptomatic and asymptomatic individuals, although this difference did not reach a statistically significant level.

The variability in growth rates among the different lymphoblastoid cell lines within each group (control, asymptomatic and symptomatic), which was remarkably well reproduced in the cultures grown in glucose and in those grown in galactose (Fig. 2a and b), probably reflected mostly differences in nuclear background among the different cell lines, with a possible contribution of differences in mtDNA haplotype in the control group. In order to reduce this background variability, the ratios of DTs in galactose medium to those in glucose medium were calculated. As shown in Figure 2c, an analysis of these ratios revealed that the variations among cell lines within each group were decreased, whereas the differences between the groups persisted, although somewhat reduced in their statistical significance, as expected (see Discussion). Specifically, the cell lines from symptomatic individuals exhibited DT ratios from 1.1 to 1.6 times higher than the average value found for the control cell lines, while the cell lines from asymptomatic individuals exhibited DT ratios varying between the average control value and a 1.5 times higher value. To be noticed is the unusually high DT ratio of F12A among the control cell lines.

Mitochondrial protein synthesis

It was expected that the 1555 mutation would affect mitochondrial protein synthesis. Figure 3 shows typical electrophoretic patterns of the organelle-specific translation products of the mutant and control lymphoblastoid cell lines after labeling the cells for 30 min with [^{35}S]methionine. The patterns of the mitochondrial translation products from the mutation-carrying probands appeared to be qualitatively identical, in terms of both electrophoretic mobility and relative rate of labeling of the various polypeptides, to those of the six control lymphoblastoid cell lines and 143B.TK$^-$ cells. However, all cell lines carrying the mutation, derived from symptomatic (Fig. 3a and b) or asymptomatic individuals (Fig. 3a, b and d), showed a clear tendency to a decrease in the total rate of labeling of the mitochondrial translation products relative to the control cell lines (Fig. 3a and c). Among the latter, F12A, which was mentioned above for its unusually high galactose/glucose DT ratio, exhibited also an abnormally low protein labeling rate (Fig. 4). The decrease in rate of labeling of the mitochondrially synthesized polypeptides, relative to the mean value measured in the control cell lines, in the mutant cell lines from the symptomatic individuals ranged between 27 and 64%, with an average of 48%, statistically significant at the 97% confidence level (Fig. 4). Among the cell lines from the asymptomatic individuals, all, except lines F6C and F12H (which had also normal DT values in galactose medium), exhibited likewise an appreciable reduction in the rate of protein labeling, relative to the mean control value, ranging between 21 and 61%; the average decrease in rate of labeling of the mitochondrial translation products in this group of cell lines was 28%, but reached only the 85% confidence level (Fig. 4). It is particularly noteworthy that the difference in average protein labeling rate between the cell lines from the symptomatic and asymptomatic individuals reached the 94% confidence level.

Oxygen consumption rate measurements

The respiration capacity of the various cell lines was measured by determining the oxygen consumption rate in intact cells. As can be seen in Figure 5, the rate of oxygen consumption of the 10 mutant cell lines from the symptomatic individuals revealed a variable decrease relative to the mean value measured in wild-type cell lines, ranging from 10 to 47%, with an average reduction of 30%, statistically significant at the 95% confidence level. Again to be noticed, among the control cell lines, F12A, which exhibited an abnormally low oxygen consumption rate. Among the nine cell lines from the asymptomatic individuals, all, except F6C (already mentioned for its high mtDNA content, normal DT value in galactose medium and normal protein

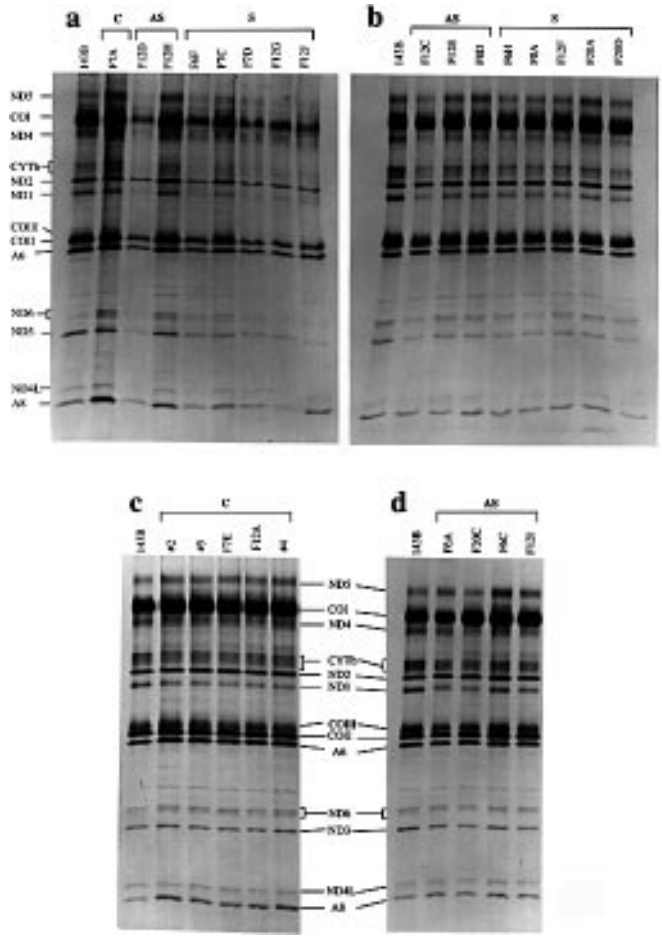

Figure 3. Electrophoretic patterns of the mitochondrial translation products of the lymphoblastoid cell lines and of 143B.TK$^-$ cells labeled for 30 min with [^{35}S]methionine in the presence of 100 μg of emetine per ml. Samples containing equal amounts of protein (30 μg), except the 143B.TK$^-$ sample, which contained only 15 μg protein, after correction for differences in radiation decay after labeling, were run in SDS/polyacrylamide gradient gels. The four panels represent electrophoretic patterns obtained in separate gel runs, each one including the 143B.TK$^-$ control for normalization purposes. 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. Quantification of the intensities of the bands was done by densitometric analysis of appropriate exposures of the fluorograms.

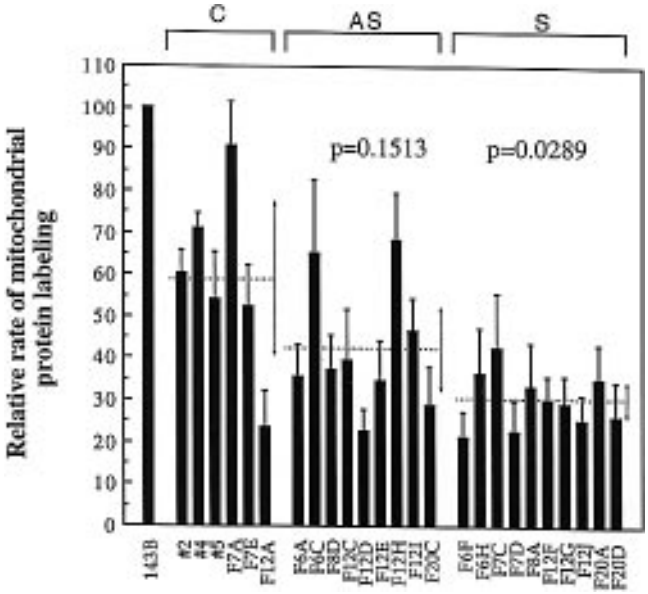

Figure 4. Quantification of the rates of labeling of the mitochondrial translation products, after a 30 min [^{35}S]methionine pulse, in different lymphoblastoid cell lines. The rates of mitochondrial protein labeling, determined as detailed in Materials and Methods, are expressed as percentages of the value for 143B.TK$^-$ in each gel, with error bars representing two SE. Two to four independent labeling experiments and two to four electrophoretic analyses for each labeling were carried out for each cell line. Graph details and symbols are explained in the legend to Figure 2.

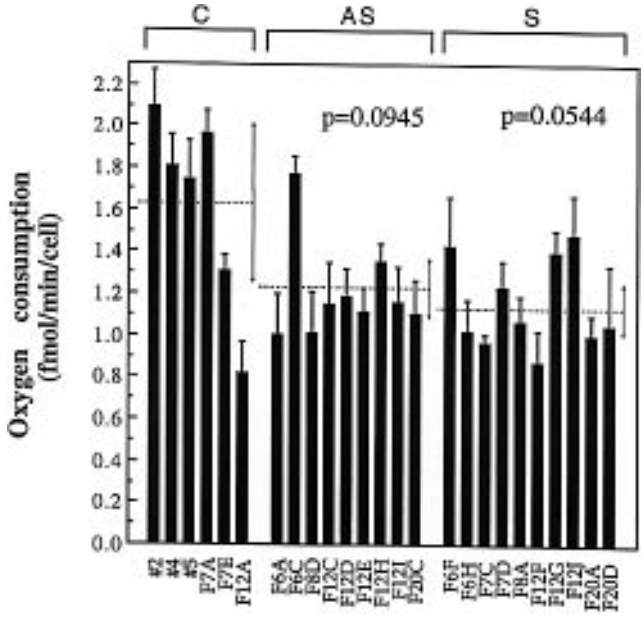

Figure 5. The average rates of oxygen consumption per cell measured in different lymphoblastoid cell lines are shown, with error bars representing two SE. Four to six determinations were made for each cell line. Graph details and symbols are explained in the legend to Figure 2.

labeling rate), exhibited also a variable decrease in oxygen consumption rate, ranging between 17 and 38%, when compared with the mean control value. The average reduction in respiratory capacity in this group of cell lines was ~25%, which approached, but did not reach the 95% confidence level. Similarly, there was an evident difference in the mean respiration rate between the cell lines from symptomatic and asymptomatic individuals, but this was not statistically significant.

In order to investigate which of the enzyme complexes of the respiratory chain was affected in the cell lines carrying the 12S rRNA 1555 mutation, oxygen consumption measurements were carried out in digitonin-permeabilized cells from three control cell lines, five cell lines from symptomatic individuals and five cell lines from asymptomatic individuals, using different substrates and inhibitors. As illustrated in Figure 6, in the cell lines derived from symptomatic individuals, the rate of malate/glutamate-driven respiration, normally reflecting the activity of NADH:ubiquinone oxidoreductase (complex I), was decreased, relative to the control cell

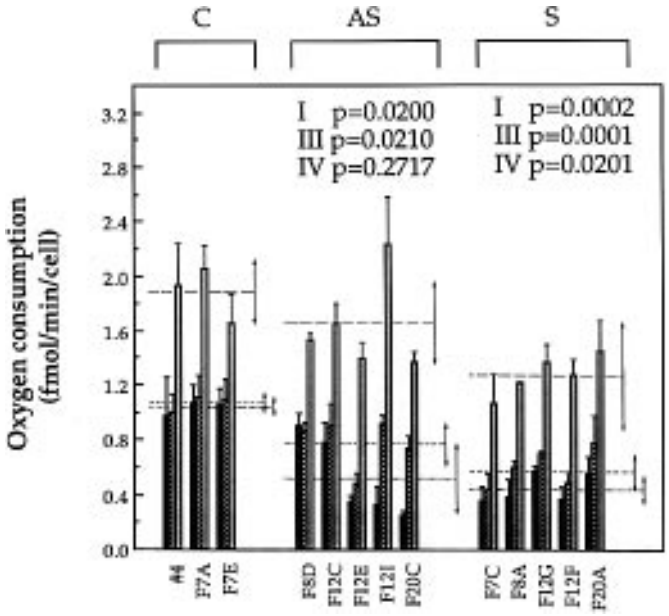

Figure 6. Activity of the enzymes of the mitochondrial respiratory chain in lymphoblastoid cell lines from symptomatic (S) and asymptomatic (AS) members of the Arab-Israeli family carrying the 12S rRNA 1555 mutation and from control individuals (C). By using ~10^7 digitonin-permeabilized cells, the activities of the various components of the respiratory chain were determined as respiration dependent on malate-glutamate (filled bars; group averages: dotted lines), G-3-P-succinate (hatched bars; group averages: short-dash lines) and ascorbate-TMPD (shaded bars; group averages: long-dash lines). Three to six determinations were carried out for each cell line. Graph details and symbols are explained in the legend to Figure 2. I: complex I; II: complex II; III: complex III.

lines, by an average of 56% ($P = 0.00016$), the rate of succinate/glycerol-3-phosphate (G-3-P)-driven respiration, normally reflecting the activity of ubiquinol:cytochrome *c* reductase (complex III), by an average of 44% ($P = 0.00014$), and the rate of N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate-driven respiration, reflecting the activity of cytochrome *c*: oxygen oxidoreductase (complex IV), by an average of 32% ($P = 0.020$). The corresponding rates for the cell lines from asymptomatic individuals were also reduced relative to those from the control cell lines, but these decreases were lower than those observed for the cell lines from symptomatic individuals (50%, $P = 0.020$ for complex I, 27%, $P = 0.021$ for complex III, and 13%, $P = 0.2717$ for complex IV).

Sensitivity of cell lines to neomycin and paromomycin

An analysis of the growth characteristics in glucose-containing medium of different lymphoblastoid cell lines in the presence of 100 μg of neomycin or paromomycin per ml [which are the concentration (paromomycin) or twice the concentration (neomycin) recommended for animal cell culture to eliminate contaminating microorganisms (17)] revealed no obvious effects of these aminoglycosides on the growth rate of any of the cell lines derived from the control, symptomatic or asymptomatic individuals (data not shown). On the contrary, with 10 times the recommended concentrations of antibiotics, all mutant cell lines exhibited, when compared with the wild-type cell lines, sensitivity to the drugs. These results were consistent with the possibility that the mitochondrial inner membrane is not very permeable to these antibiotics, and that a certain minimum concentration of aminoglycosides in the medium is needed to reach an effective accumulation inside the organelles.

As shown in Figure 7, in the presence of 0.5 mg neomycin or 1 mg paromomycin per ml, the growth rates of the lymphoblastoid cell lines tested derived from control individuals, as well as that of the 143B.TK$^-$ cell line, were not affected, as shown by the ratio of ~1.0 of the DTs in the presence or absence of the antibiotic. By contrast, under the same conditions, the growth rates of the cell lines tested, derived from both symptomatic and asymptomatic individuals, showed a very significant average decrease relative to the growth rates of the control lymphoblastoid cell lines and of 143B.TK$^-$ cells. In particular, the DT ratios in the presence or absence of neomycin were increased on average by ~16% in both groups of mutant cell lines ($P \approx 0.01$), while the DT ratios in the presence or absence of paromomycin were increased by 30% ($P \approx 0.001$) and 26% ($P < 0.001$) in the symptomatic-derived and asymptomatic-derived cell lines, respectively. It is clear from these data that the sensitivities to the aminoglycosides tested of the growth rate of the cell lines derived from symptomatic and asymptomatic individuals were substantially identical. It should also be noted that the DT ratios for neomycin and paromomycin in ρ°206 cells were similar to those found in 143B.TK$^-$ cells and other control cell lines, supporting the hypothesis that the main target of the antibiotics is the mitochondrial 12S rRNA.

DISCUSSION

The 1555 mutation is responsible for the biochemical defects of the lymphoblastoid cell lines

The 1555 mutation occurs in a region of the small rRNA molecule region which is highly conserved in different organisms, from bacteria to mammals (18). The equivalent region in the bacterial 16S rRNA forms an essential part of the decoding site in the small ribosomal subunit (10). In bacteria, base changes at a position of 16S rRNA equivalent to the 1555 position of the mitochondrial 12S rRNA have been shown to have severe effects on ribosome function, resulting in reduced growth rate or even lethality (7). In view of the molecular genetic evidence pointing to a ribosomal dysfunction caused by the 1555 mutation as being responsible for the non-syndromic deafness in the Arab-Israeli family, in the present work, we have examined the effects of this mutation on mitochondrial protein synthesis in lymphoblastoid cells derived from members of that family and from control individuals. The relative labeling and the electrophoretic mobility of the mitochondrial translation products in the mutant cell lines did not differ from those observed in control cell lines, suggesting that this mutation did not cause misincorporation of amino acids affecting the stability or electrophoretic mobility of the mitochondrially synthesized polypeptides. There was, however, strong evidence of an overall decrease in the rate of mitochondrial protein labeling in the mutant cell lines after a short [^{35}S]methionine pulse, with an average reduction of 48 or 28% in the cell lines derived from symptomatic or asymptomatic individuals, respectively, as compared with the mean for the control cell lines. Furthermore, pulse–chase experiments failed to show evidence of differences in protein stability among the cell lines (data not shown). In view of the labeling conditions used, which involved methionine starvation prior to the pulse, and of

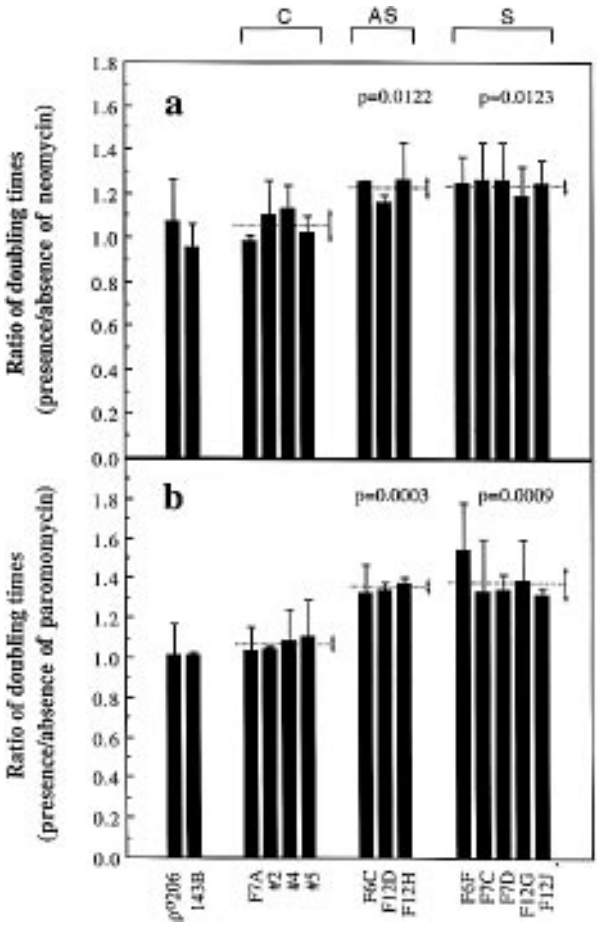

Figure 7. Analysis of growth capacity of different lymphoblastoid cell lines in the presence of neomycin (**a**) or paromomycin (**b**) in special DMEM-glucose. The ratios of DTs in the presence of 10-fold the concentration of neomycin (a) or 10-fold the concentration of paromomycin (b) recommended for animal cell culture (16), and in the absence of antibiotics are shown, with error bars representing two SE. Graph details and symbols are explained in the legend to Figure 2.

the lack of evidence of differential mitochondrial protein turnover which could have affected the intramitochondrial methionine pool, the most plausible interpretation of the results is that the labeling of the mitochondrial translation products after a short pulse reflected the rate of synthesis of these products.

A reasonable interpretation of the respiratory deficiency observed in the mutant cell lines is that it was due to the substantial reduction in the rate of assembly of functional respiratory complexes as a result of the protein synthesis defect. Indeed, the degree of respiratory deficiency in the cell lines derived from the symptomatic and asymptomatic members of the pedigree correlated well with the reduction in their rate of mitochondrial protein synthesis. The decreases in activity of the respiratory complex I, complex III and complex IV in the mutant cell lines are consistent with the observation of a general defect of the translation apparatus affecting the synthesis of all mtDNA-encoded subunits of these complexes.

The moderate decrease in growth rate in glucose-containing medium, relative to the controls, observed in the cell lines derived from the symptomatic and asymptomatic individuals, presumably reflected the limited extent to which ATP production was affected by the oxidative phosphorylation defect in this medium, which supported an adequate level of glycolysis. By contrast, it is reasonable to assume that the marked decrease in growth rate in galactose medium observed, relative to the control cell lines, in the symptomatic individual-derived and, to a lower degree, in the asymptomatic individual-derived cell lines was due to the severe consequences of the oxidative phosphorylation defect under conditions where glycolysis was seriously compromised by the slow conversion of galactose to glucose. A plot of the ratios of growth rates in glucose and galactose medium showed that the differences between groups, which presumably reflected the differences, among the three sets of cell lines, in their dependence on oxidative phosphorylation for ATP production, were, as expected, somewhat reduced.

On the basis of the evidence obtained in this work, it is reasonable to assume that the pathogenetic mechanism of the 12S rRNA 1555 mutation involves a respiratory deficiency caused by the primary mitochondrial translation defect and resulting in a decline in ATP production in the cochlear cells (hair cells and/or stria vascularis), with dramatic effects on the ion pumps and, therefore, the ion balance, which is essential for the hearing function (3). It is interesting that the decrease in rate of mitochondrial protein synthesis which was associated with a significant reduction in oxygen consumption and growth rate in galactose medium was about 50%. This threshold level in the mitochondrial protein synthetic capacity capable of supporting a normal respiratory phenotype is consistent with the evidence indicating that a reduction of more than 50–60% in the copy number of the tRNA genes comprised within the 5 kbp mtDNA deletion associated with chronic external ophthalmoplegia (15), or with the Pearson syndrome (19), or a 50–60% decrease in aminoacylation capacity of the mitochondrial $tRNA^{Lys}$ associated with the 8344 mutation causing the MERRF syndrome (20) produces a dramatic decrease in mitochondrial translation rate and respiratory capacity. The above observations indicate that, in mammalian cells, there is not a large excess of mitochondrial protein synthesis capacity over the minimum required for cell viability or normal cell function. Therefore, in cell types with high oxidative phosphorylation demands, like cochlear cells, or under situations of energetic stress, a 50% decrease in mitochondrial protein synthesis rate, as observed in the present work in the symptomatic individual-derived cell lines, can have disastrous consequences for the cell.

As concerns the variability in biochemical properties and doubling times which was observed among the cell lines derived from different subjects within the same group, i.e. control, asymptomatic and symptomatic, it can be excluded that it was due to experimental noise. In fact, the differences observed among the cell lines in each group were in general consistent for the different parameters measured, as illustrated by the striking reproducibility in growth rate differences among the cell lines of each group in glucose medium and in galactose medium. The most plausible interpretation of the variation in biochemical properties and growth rates within the same group is that it reflects mainly nuclear differences among the individual donors. That nuclear variability plays a major part in the mtDNA-linked phenotype is not surprising, considering that >90% of the mitochondrial proteins, including all proteins involved in mtDNA replication and transcription and in mitochondrial translation and the majority of the subunits of the OXPHOS apparatus, are encoded

in nuclear genes (21). Such an important role of the nucleus in the mtDNA-linked functions has been amply documented in yeast. Even among mtDNA-less (ρ°) cell transformants carrying the same mtDNA in a relatively constant nuclear background, a considerable variability in respiratory capacity has been recently shown, by sequential mitochondrial transfer into different ρ° cells (22), to reflect variability in nuclear gene content or activity among the original ρ° cell recipients.

Apart from differences in nuclear background, in the case of the control cell lines, which did not share the same mtDNA as the cell lines from asymptomatic and symptomatic individuals, one cannot exclude a role of the mtDNA haplotype in producing phenotypic variability. This possibility applies particularly to the cell line F12A, which exhibited an unusually high galactose/glucose DT ratio and abnormally low mitochondrial protein synthesis and oxygen consumption rates, as compared with the other control cell lines. In the case of the cell line F6C, the significantly higher than average rates of protein synthesis and respiration, and significantly lower than average doubling time may be related to its unusually high mtDNA content described above, which possibly reflects a compensatory phenomenon, as previously observed (23).

The nuclear and mtDNA background-related variability among cell lines derived from different individuals which was discussed above illustrates the need for investigating a relatively large number of subjects, when analyzing the biochemical defects, frequently moderate (20), caused by pathogenetic mtDNA mutations. In the present work, the availability of a large family carrying the A1555G mutation with or without clinical phenotype has permitted the analysis of much larger groups of individuals from the same family than previously possible in other mtDNA-linked diseases. It is indeed remarkable that, in spite of the occurrence of occasional outliers, a clear trend has emerged from the present work, which has permitted significant conclusions to be drawn.

Nuclear gene involvement in the non-syndromic deafness of the Arab-Israeli family

A formal segregation analysis of the Arab-Israeli family has predicted that the disease phenotype of the non-syndromic deafness of this family is caused by the simultaneous inheritance of a homoplasmic mtDNA mutation and an autosomal recessive mutation (24). Indeed, the variable severity of the deafness phenotype in different members of the family strongly suggested that, besides the 1555 mtDNA mutation, other factors, i.e. nuclear background or environmental factors, played an important part in the development of deafness. Thus, it is conceivable that, by interacting with the mutated 12S rRNA or a ribosomal protein binding to the mutation site, the product(s) of a putative nuclear gene(s) could enhance the effect of mutation so as to produce the clinical phenotype, or suppress it so as to make the hearing normal.

In the present investigation, the central observation has been that the lymphoblastoid cell lines derived from asymptomatic individuals exhibited a lower degree of mitochondrial dysfunction than those derived from symptomatic individuals, although all cell lines carried the same mtDNA with the 12S rRNA 1555 mutation. The difference in rate of protein synthesis between the two groups of cell lines was statistically significant at the 94% confidence level. The differences between the two sets of cell lines in rate of oxygen consumption and rate of growth in galactose medium, although showed clearly the same trend, did not reach statistical significance, presumably due to experimental noise. It is interesting, however, that when the rates of mitochondrial protein synthesis, oxygen consumption and growth in galactose medium of the cell lines from either the symptomatic or the asymptomatic group were expressed as percentages relative to the average control value for the same parameter and pooled together, the overall difference between the two groups became significant at the 99% confidence level. Consistent with these results was the observation that the activity of the three respiratory enzyme complexes which are under the control of mtDNA was more significantly decreased in the cell lines derived from symptomatic individuals, relative to the control cell lines, than in the cell lines derived from asymptomatic individuals. These results are particularly remarkable when contrasted with the lack of difference in sensitivity to aminoglycosides of the cell lines derived from the asymptomatic and symptomatic individuals, and strongly suggest that the factors underlying the manifestation or lack of manifestation of the clinical phenotype in the individuals carrying the 1555 mutation have also an effect in determining the severity of the biochemical defects in the lymphoblastoid cell lines derived from those individuals, i.e. in a cell type totally unrelated to the auditory function. These observations are not easily reconcilable with a possible role of environmental factors, and rather point to some difference(s) in either nuclear gene content or activity, which occurs in the cochlear cells as well as in other cell types, as being responsible for the observed variability in the mtDNA mutation-associated clinical and biochemical phenotype. These results are, therefore, consistent with the model of two-locus disease proposed for the non-syndromic deafness (24).

The conclusion reached in this paper that some difference(s) in either nuclear gene content or activity underlies the diversity in biochemical phenotype between symptomatic and asymptomatic individual-derived lymphoblastoid cell lines leads to the prediction that transfer of mitochondria from these cell lines into the constant nuclear background of a mtDNA-less cell line (14) would eliminate the observed differences between the two groups of cell lines. Experiments to test this prediction are in progress. In a general context, the approach utilized in the present work should be valuable for the analysis of the nuclear involvement in other mtDNA-linked diseases, like the Leber's hereditary optical neuropathy (12). Furthermore, this approach provides a paradigm for testing the role of the nuclear background in the putative pathogenetic effects of mtDNA mutations associated with Alzheimer's and Parkinson's diseases (25).

Aminoglycoside sensitivity of the lymphoblastoid cell lines from the Arab-Israeli family

Aminoglycoside antibiotics are known to exert their antibacterial effects at the level of the ribosome, inducing in general codon mis-reading and, therefore, misincorporation of amino acids. A considerable amount of evidence indicates that sensitivity to aminoglycosides in bacteria involves their binding to a base pair at the base of the penultimate helix (4–7). In particular, in wild-type *E.coli* sensitive to several aminoglycosides, the nucleotide at position 1491 (G) in 16S rRNA is base-paired with a C at position 1409; mutation or methylation of the 1491 nucleotide disrupts the G–C base pairing, resulting in resistance to aminoglycosides (5,7). The nucleotide at position 1555 in the human 12S rRNA (equivalent to position 1491 in the *E.coli* 16S rRNA) in wild-type cells is A, which, when mutated to a G, as in the Arab-Israeli family, would be expected to pair with the C at position 1494, thus facilitating aminoglycoside binding and sensitivity (3). Therefore, it was

anticipated that the mutant lymphoblastoid cell lines from this family would be sensitive to aminoglycosides. Indeed, the evidence obtained has indicated that all mutant cell lines, when compared with the wild-type cell lines, exhibited a fairly uniform, highly significant reduction in growth rate in glucose medium in the presence of high concentrations of paromomycin or neomycin. There was no significant difference in sensitivity to aminoglucosides between the cell lines derived from asymptomatic individuals and those derived from symptomatic individuals. These results provide the first direct evidence that aminoglycosides have a toxic effect on mammalian cells in culture, which appears to be dependent on the presence of the 1555 12S rRNA mutation. In view of the fact that five genetically independent cell lines were used as controls, it seems very unlikely that the sensitivity to aminoglycosides of the mutant cell lines is due to the particular mtDNA haplotype of the maternally inherited Arab-Israeli family mtDNA. However, more extensive comparisons with control cell lines will have to be carried out to exclude completely this possibility. Similarly, further work is needed to determine the biochemical defect underlying the decrease in growth rate of the mutant cell lines, and in particular to test whether a protein synthesis defect is involved.

MATERIALS AND METHODS

Cell cultures

A total of 25 human immortalized lymphoblastoid cell lines derived from 22 members of the Arab-Israeli family [nine individuals carrying the 1555 mutation in the 12S rRNA gene, but lacking a clinical phenotype, 10 individuals exhibiting both the mutation and hearing loss, and three individuals lacking the mutation ('married-in')] and from three other, genetically unrelated, control individuals were grown in either a specially made Dulbecco's modified Eagle medium (DMEM) containing 1 mg of glucose per ml, 0.11 mg pyruvate per ml and 0.18 mM $CaCl_2$ (hereafter referred to as special DMEM-glucose) supplemented with 10% fetal bovine serum (FBS), or the same medium lacking glucose, but containing 0.9 mg galactose per ml and 0.5 mg pyruvate per ml (hereafter referred to as special DMEM-galactose), supplemented with 10% dialyzed FBS. The bromodeoxyuridine (BrdU)-resistant 143B.TK$^-$ cell line was grown in regular DMEM (containing 4.5 mg of glucose and 0.11 mg pyruvate per ml) supplemented with 100 µg of BrdU per ml and 5% FBS. The mtDNA-less ρ°206 cell line, derived from 143B.TK$^-$, was grown under the same conditions as the parental line, except for the addition of 50 µg of uridine/ml. To test the various cell lines for sensitivity to aminoglycosides, cells were grown for 4 days in special DMEM-glucose, supplemented with 10% FBS, in the presence of 0.5 mg of neomycin per ml or of 1 mg of paromomycin per ml.

The population doubling time (DT) of the cell lines in special DMEM-glucose or special DMEM-galactose, both supplemented with 10% dialyzed FBS, was determined from the growth curves or by using the formula (26):

$$DT = (t\text{-}t_0)\log 2/(\log N - \log N_0)$$

where DT is the doubling time, t and t_0 are the times at which the cells were counted, and N and N_0 are the cell numbers at time t and t_0, respectively.

Mitochondrial DNA analysis

For the detection of the A to G transition in the mitochondrial 12S rRNA gene at position 1555 in the Cambridge human mtDNA sequence (1), an mtDNA fragment containing the mutation was amplified by PCR using oligodeoxynucleotides corresponding to positions 1326–1355 and 1684–1704. The amplified segments were digested with *Bsm*AI in the presence of the 2.96 kb *Hin*dIII fragment of pBluescript KS$^+$ DNA as an internal marker for completion of digestion (26). Equal amounts of the various digested samples were then analyzed by electrophoresis through a 1% agarose gel. The proportions of digested and undigested PCR product were determined by laser densitometry after ethidium bromide staining.

Total DNA samples were isolated from the cultured lymphoblastoid cell lines and from 143B.TK$^-$ with an Applied Biosystems 340A DNA extractor. The quantification of DNA was performed by slot blot hybridization, using the clone pTZ18-K4 (containing the *Eco*RI-*Sac*I fragment of human mtDNA between positions 41 and 2578), ^{32}P-labeled by random priming (27), as a probe. To correct for the possible quantitative variations among different samples, the same membrane was probed with a nuclear 28S rRNA gene fragment, which was constructed by PCR amplification using oligodeoxynucleotides corresponding to positions 1503–1522 and 1981–2000 in the 28S rRNA gene (28), purified on agarose gel, and ^{32}P-labeled by random priming. Quantification of the hybridization was carried out by scanning the autoradiograms with an LKB laser densitometer or by analyzing the slot blot in a Phosphorimager (Molecular Dynamics). For comparison of the data from different blots, the values obtained for the lymphoblastoid cell lines in each blot were normalized to the values obtained for the 143B.TK$^-$ sample in the same blot.

Analysis of mitochondrial protein synthesis

Samples of cultures of the mutant or control lymphoblastoid cell lines (~5×10^6 cells) and of 143B.TK$^-$ (6–8×10^5 cells) were labeled with [^{35}S]methionine-[^{35}S]cysteine (1175 Ci/mmol methionine; in total, 50 µCi/ml of medium) for 30 min in the presence of 100 µg of emetine per ml in methionine-free special DMEM-glucose supplemented with 10% dialyzed FBS. After labeling, the cultures were centrifuged down to remove the medium, the cells were then washed twice with buffered saline lacking Mg^{2+} and Ca^{2+} (TD), finally resuspended in TD and frozen in aliquots. After protein determination, made by the Bradford method (29), samples containing equal amounts of protein (30 µg) from different lymphoblastoid cell lines or 15 µg protein from 143B.TK$^-$ cells were run on SDS-exponential polyacrylamide gradient gels (26). The gels were treated with DMSO/PPO, dried and exposed for fluorography. Quantification of radioactivity was made by scanning five well-resolved peaks (ND1, ND2, ND3, COII or COII + COIII, A6) in appropriate exposures of the autoradiograms with an LKB laser densitometer. For comparison of the data from different gels, the densitometric data obtained for the samples of lymphoblastoid cell lines in each gel were normalized to the data obtained for the 143B.TK$^-$ sample in the same gel.

Oxygen consumption measurements

Rates of oxygen consumption were measured with a Gilson 5/6 oxygraph on samples of 1×10^7 cells in 1.85 ml of special DMEM-glucose lacking glucose, supplemented with 10% dialyzed FBS (30). Polarographic analysis of digitonin-permeabilized cells, using different respiratory substrates and inhibitors, to test the activity of the various respiratory complexes, was carried out as detailed elsewhere (31).

Computer analysis

Statistical analysis was carried out using the unpaired, two-tailed Student's *t*-test contained in the Microsoft-Excel program for Macintosh (version 4).

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