Nuclear background determines biochemical phenotype in the deafness-associated mitochondrial 12S rRNA mutation

Min-Xin Guan¹,²+, Nathan Fischel-Ghodsian³ and Giuseppe Attardi¹

¹Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA, ²Division of Human Genetics, Children’s Hospital Medical Center and Department of Pediatrics, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229, USA and ³Ahmanson Department of Pediatrics, Steven Spielberg Pediatric Research Center, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

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The pathogenetic mechanism of the human mitochondrial 12S rRNA gene mutation at position 1555, associated with non-syndromic deafness and aminoglycoside-induced deafness, has been investigated in 33 transformants obtained by transferring mitochondria from lymphoblastoid cell lines into human mitochondrial DNA (mtDNA)-less (p⁺) cells. In this nearly constant nuclear background, 15 transformants derived from five symptomatic individuals from a large Arab-Israeli family, carrying this mutation in homoplasmic form, exhibited significant decreases compared with nine control transformants in the rate of growth in a medium containing galactose instead of glucose, as well as in the rates of mitochondrial protein synthesis and of substrate-dependent respiration. Most significantly, these decreases were very similar to those observed in nine transformants derived from three asymptomatic members of the family. This result in transmitochondrial cybrids is in contrast to the differences in the same parameters previously demonstrated between the original lymphoblastoid cell lines derived from the symptomatic and asymptomatic members of the Arab-Israeli family. In addition, the intragroup variability in biochemical dysfunction among the lymphoblastoid cell lines from different symptomatic or asymptomatic or control individuals was significantly reduced in the derived mitochondrial transformants carrying the same nuclear background. These observations provide strong genetic and biochemical evidence in support of the idea that the nuclear background plays a determinant role in the phenotypic manifestation of the non-syndromic deafness associated with the A1555G mutation.

INTRODUCTION

Hearing loss is the most frequent sensory disorder. One in 1000 children is born deaf, an equal number lose their hearing by adulthood and half the population experience significant hearing impairment by the age of 65 years (1,2). These hearing impairments are either associated with other dysfunctions or syndromes (syndromic) or isolated (non-syndromic). Deafness can be due to genetic or environmental causes or a combination of both. About 50% of the deafness cases have a genetic etiology or predisposition, with autosomal dominant, autosomal recessive, X-linked or mitochondrial patterns of inheritance, which are presently being rapidly defined at the molecular level (3).

Over the last several years, some forms of sensorineural hearing loss, both syndromic and non-syndromic, have been found to be associated with mutations in mitochondrial DNA (mtDNA) (4). In particular, several non-syndromic deafness-causing mtDNA mutations have been identified. These include a T→C transition at position 7445 in the precursor of the tRNAser(UCA) gene (5,6), a C nucleotide insertion at position 7472 in the tRNAser(UCA) gene (7), a T→C transition at position 7511 in same tRNA gene (8) and a homoplasmic A→G transition at position 1555 in the 12S rRNA gene (9–14). The A1555G mutation is one of most common causes of aminoglycoside-induced deafness and non-syndromic deafness (4,14). The A1555G mutation is located in a region of the small rRNA highly conserved from bacteria to mammals (15), which is an essential part of the decoding site of the small ribosomal subunit (16). The same region is important for the action of aminoglycosides (17,18). In fact, the new G–C pair in 12S rRNA created by the A1555G transition facilitates the binding of aminoglycosides (11,19), accounting for the fact that exposure to aminoglycosides causes hearing loss in individuals carrying this mutation (4,14). In the absence of aminoglycosides, the A1555G mutation produces a clinical phenotype that ranges from severe congenital deafness, through moderate progressive hearing loss of later onset (14), to completely normal hearing (9,14). No abnormalities in any

*To whom correspondence should be addressed at: Division of Human Genetics, Children’s Hospital Medical Center, 3333 Burnet Avenue, TCHRF 1042, Cincinnati, OH 45229-3039, USA; Tel: +1 513 636 3337; Fax: +1 513 636 2261; Email: guar6n@chmcc.org
other organ, including the vestibular system, have been observed (20).

In previous studies, the phenotypic effects of the mtDNA A1555G mutation associated with non-syndromic deafness and aminoglycoside-induced deafness were analyzed in 19 lymphoblastoid cell lines derived from members of a large Arab-Israeli family carrying this mutation in homoplasmic form and from six control individuals (21). This investigation revealed a variable decrease in the rates of mitochondrial protein synthesis and respiration and in the rate of growth in medium containing galactose instead of glucose in the cell lines carrying the A1555G mutation, compared with control cell lines, strongly suggesting that this mutation is responsible for the biochemical defects associated with the deafness phenotype. However, the severity of mitochondrial dysfunction in the mutant cell lines was found to be correlated with the presence or absence of hearing loss in the donor individuals, pointing to some other factor(s) playing an important role in the phenotypic manifestation of the mutation (21). The latter conclusion was in agreement with the evidence indicating that the maternally inherited deafness associated with the A1555G mutation requires additional environmentally or genetically induced changes for phenotypic expression (22,23). The observation that the greater severity of biochemical defects in the lymphoblastoid cell lines derived from symptomatic individuals, compared with those from asymptomatic individuals, occurred in a cell type totally unrelated to the auditory function, pointed specifically to some differences in either nuclear gene content or activity as underlying the diversity in biochemical phenotype between symptomatic and asymptomatic individual-derived cell lines.

If the additional factors required for the manifestation of the biochemical phenotype of the A1555G mutation involved the nuclear background, transfer of mitochondria carrying the A1555G mutation into the constant nuclear background of a mtDNA-less cell line would be expected to eliminate the biochemical differences observed between the lymphoblastoid cell lines derived from the symptomatic individuals and those derived from the asymptomatic individuals. It also seemed important to verify this prediction in view of its significant implications in relation to the development of genetic approaches for identifying the putative nuclear factor(s) and to point to some other factor(s) playing an important role in the phenotypic manifestation of the mutation (21). The latter conclusion was in agreement with the evidence indicating that the maternally inherited deafness associated with the A1555G mutation requires additional environmentally or genetically induced changes for phenotypic expression (22,23). The observation that the greater severity of biochemical defects in the lymphoblastoid cell lines derived from symptomatic individuals, compared with those from asymptomatic individuals, occurred in a cell type totally unrelated to the auditory function, pointed specifically to some differences in either nuclear gene content or activity as underlying the diversity in biochemical phenotype between symptomatic and asymptomatic individual-derived cell lines.

If the additional factors required for the manifestation of the biochemical phenotype of the A1555G mutation involved the nuclear background, transfer of mitochondria carrying the A1555G mutation into the constant nuclear background of a mtDNA-less cell line would be expected to eliminate the biochemical differences observed between the lymphoblastoid cell lines derived from the symptomatic individuals and those derived from the asymptomatic individuals. It also seemed important to verify this prediction in view of its significant implications in relation to the development of genetic approaches for identifying the putative nuclear factor(s) and to possible therapeutic interventions. Therefore, in the present work, transmitochondrial cell lines were constructed by enucleating lymphoblastoid cell lines derived from symptomatic and asymptomatic members of the Arab-Israeli family and from control individuals and by fusing the cytoplasts thus obtained with cells of the human mtDNA-less cell line ρ°206 (24).

RESULTS

Construction of transmitochondrial cybrid cell lines

The lymphoblastoid cells derived from three control individuals (#4, F7A and F7E), three asymptomatic members (F6C, F12D and F12H) and five symptomatic members (F6H, F7C, F7D, F12G and F12J) of the Arab-Israeli family (21,25) were enucleated and subsequently fused to a large excess of mtDNA-less human ρ°206 cells, derived from the bromodeoxyuridine (BrdU)-resistant 143B.TK− cell line (24). The transmitochondrial cybrid clones were isolated by growing the fusion mixtures in selective Dulbecco’s modified Eagle’s medium (DMEM) containing BrdU and lacking uridine (24). Between 25 and 45 days after fusion, 10–15 presumptive mitochondrial transformants derived from each of the different donor cell lines were isolated and subsequently analyzed for the presence and level of the A1555G mutation. The results confirmed the absence of the mutation in the controls and its presence in homoplasmic form in all transformants derived from the mutant cell lines. Three transformants derived from each donor cell line were used for the biochemical characterization described below.

Mitochondrial protein synthesis defect in the transformant cell lines

To examine whether a defect in mitochondrial protein synthesis occurred in the cell lines carrying the A1555G mutation, cells from each transmitochondrial line were labeled for 30 min with [35S]methionine in the presence of 100 µg/ml emetine. Samples containing equal amounts of protein (20 µg) were run in SDS–polyacrylamide gradient gels (A and B). 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, respectively; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, subunits 1, 2, 3, 4, 4L, 5 and 6 of the respiratory-chain NADH dehydrogenase, respectively; A6 and A8, subunits 6 and 8 of the H+-ATPase, respectively; CYTb, apocytochrome b.

Figure 1. Electrophoretic patterns of the mitochondrial translation products of the transformant cell lines and of 143B.TK− cells labeled for 30 min with [35S]methionine in the presence of 100 µg/ml emetine. Samples containing equal amounts of protein (20 µg) were run in SDS–polyacrylamide gradient gels. (A and B) 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, respectively; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, subunits 1, 2, 3, 4, 4L, 5 and 6 of the respiratory-chain NADH dehydrogenase, respectively; A6 and A8, subunits 6 and 8 of the H+-ATPase, respectively; CYTb, apocytochrome b.
electrophoretic mobility and relative rate of labeling of the various polypeptides, to those of the three control transformant cell lines and of 143B.TK– cells (Fig. 1). However, the transformant cell lines carrying the mutation showed a clear tendency to a decrease in the total rate of labeling of the mitochondrial translation products relative to the control cell lines. Figure 2 illustrates a quantification of the results of a large number of labeling experiments and electrophoretic runs, which was carried out by densitometric analysis of appropriate exposures of the fluorograms and normalization to the data obtained for the 143B.TK– sample included in each gel. It appears that the overall rate of labeling of the mitochondrial translation products in the mutant cell lines from symptomatic individuals decreased relative to the mean value measured in the control cell lines by 29–46%, with an average of 37% ($P = 0.0005$). Similarly, the reduction in the rate of labeling of the same products in the mutant cell lines from asymptomatic individuals ranged from 31–42%, with an average of 35% ($P = 0.0038$).

**Respiration defects in the transformant cell lines**

The endogenous respiration rates of three transmitochondrial cell lines derived from each of three control individuals and each of three asymptomatic and five symptomatic members of the Arab-Israeli family were measured by determining the $O_2$ consumption per cell. As can be seen in Figure 3A, the rate of total $O_2$ consumption in the transmitochondrial cell lines derived from the five symptomatic individuals exhibited a variable decrease, ranging between $\sim 34$ and 42%, relative to the mean value measured in the control cell lines, with an average reduction of $\sim 38$ ($P = 0.0004$). The transmitochondrial cell lines from the three asymptomatic individuals also exhibited a variable decrease in $O_2$ consumption rate, ranging between 31 and 38%, when compared with the mean control value, with an average reduction of $\sim 33$ ($P = 0.0097$). The variations in overall respiration rate among the individual mutant cell lines derived from symptomatic members of the Arab-Israeli family...
Table 1. Mitochondrial protein labeling rate, respiration rates and doubling times of lymphoblastoid and transmitochondrial cell lines

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<th>Average percent related to control</th>
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<td>Lymphoblastoid cell lines&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Mitochondrial protein labeling rate</td>
<td>Asymptomatic individuals</td>
<td>72 ± 17</td>
<td>52 ± 7</td>
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<td>Symptomatic individuals</td>
<td>75 ± 9</td>
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<td>Endogenous O&lt;sub&gt;2&lt;/sub&gt; consumption rate</td>
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<td>50 ± 25</td>
<td>43 ± 9</td>
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<td>57 ± 17</td>
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<td>Mal/Glu-dependent respiration rate</td>
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<td>72 ± 15</td>
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<td>Succ/G3P-dependent respiration rate</td>
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<td>87 ± 17</td>
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<td>COX-dependent respiration rate</td>
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<td>143 ± 32</td>
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<td>DT in glucose medium</td>
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<td>60 ± 9</td>
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<td>DT in galactose/glucose</td>
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<td>90 ± 1</td>
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<td></td>
<td>Symptomatic individuals</td>
<td>119 ± 3</td>
<td>112 ± 1</td>
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<sup>a</sup>From Guan et al. (21).

and, similarly, among the mutant cell lines derived from asymptomatic members, compared with the individual control cell lines, showed a very significant correlation with the corresponding variations in rate of mitochondrial protein synthesis ($r = 0.98$, $P < 0.001$; $r = 0.99$, $P < 0.001$, respectively).

In order to investigate which of the enzyme complexes of the respiratory chain was affected in the mutant cell lines, O<sub>2</sub> consumption measurements were carried out on digitonin-permeabilized cells, using different substrates and inhibitors (26). As illustrated in Figure 3B, in the transmitochondrial cell lines derived from the five symptomatic individuals, the rate of malate/glutamate-driven respiration, which depends on the activities of NADH:ubiquinone oxidoreductase (Complex I), ubiquinol-cytochrome c reductase (Complex III) and cytochrome c oxidase (Complex IV), but usually reflects the rate-limiting activity of Complex I, was very significantly decreased, relative to the average rate in the control cell lines, by 35–60% (∼31% on average; $P = 0.0003$). Similarly, the rate of succinate/glycerol-3-phosphate (G3P)-driven respiration, which depends on the activities of Complex III and Complex IV, but usually reflects the rate-limiting activity of Complex III, was significantly affected, relative to the average rate in the control cell lines, by 25–45% (∼34% on average; $P = 0.0003$); furthermore, the rate of N,N,N′,N′-tetramethyl-p-phenylene diamine (TMPD)/ascorbate-driven respiration, which reflects the activity of Complex IV, exhibited a 25–40% reduction in Complex IV activity (∼32% on average; $P = 0.0003$). The corresponding rates in the transmitochondrial cell lines from asymptomatic individuals were also reduced relative to those from the control cell lines. These decreases were only slightly lower than those observed in the cell lines from symptomatic individuals, i.e. 32–51%, with ∼41% on average ($P = 0.0064$), for malate/glutamate-promoted respiration; 31–36%, with ∼32% on average ($P = 0.0004$), for succinate/G3P-promoted respiration; and 28–35%, with ∼31% on the average ($P = 0.0012$), for TMPD/ascorbate-promoted respiration. The variations in the rates of malate/glutamate-driven, succinate/G3P-driven and TMPD/ascorbate-driven respiration among the individual mutant cell lines derived from symptomatic subjects, compared with the individual control cell lines, showed a significant correlation with the corresponding variations in rate of mitochondrial protein synthesis ($r = 0.93$, $P < 0.001$; $r = 0.95$, $P < 0.001$; and $r = 0.91$, $P < 0.001$, respectively). Likewise, the variations in the rates of malate/glutamate-driven, succinate/G3P-driven and TMPD/ascorbate-driven respiration among the individual mutant cell lines derived from asymptomatic subjects, compared with the individual control cell lines, showed a significant correlation with the corresponding variations in the rate of mitochondrial protein synthesis ($r = 0.98$, $P < 0.001$; $r = 0.96$, $P < 0.001$; and $r = 0.93$, $P < 0.001$, respectively).

Growth defects in the transformant cell lines

Figure 4 shows the analysis of the growth properties of the different cell lines in glucose- or galactose-containing medium. The average doubling times (DTs) in glucose-containing medium of the transformant cell lines carrying the A1555G mutation were increased relative to those of the control cell lines (Fig. 4A). In particular, the DTs of the cell lines derived from the five symptomatic individuals ranged from 24.3 to 36.8 h, with an average of 30.2 h, the latter thus being significantly higher ($P = 0.0256$) than the mean value (DT = 21.2 h) measured in the control cell lines. The DTs of the cell lines from the asymptomatic individuals exhibited similar increases, ranging from 25.8 to 30.9 h, with an average of 28.2 h ($P = 0.0185$).

It has been shown that cell lines deficient in oxidative metabolism have an impaired growth capacity in media containing galactose instead of glucose (21,27–30). In fact, galactose is not utilized efficiently by mammalian cells as a glycolytic substrate and, therefore, in such media, the cells are forced to rely almost exclusively on oxidative phosphorylation for ATP production. Figure 4B shows the DTs of the transmitochondrial cell lines investigated here in a medium containing galactose and an increased concentration of pyruvate relative to the regular DMEM. In this medium, the DTs of the cell lines derived from the symptomatic subjects ranged from 86.9 to 125 h, with an average of 101.9 h, being therefore very significantly higher ($P = 0.0024$) than the DTs of the control cell lines, which ranged from 33.3 to 55.4 h, with an average of 44 h.
The variations among the individual transmitochondrial cell lines derived from asymptomatic subjects, compared with the individual control cell lines, also exhibited a significant negative correlation with the corresponding variations in their DTs in galactose-containing medium ($r = 0.88$, $P < 0.05$ and $r = 0.94$, $P < 0.001$, respectively).

The variability in growth rates among the different transmitochondrial cell lines within each group (control, asymptomatic and symptomatic), which was in general well reproduced in the cultures grown in glucose and in those grown in galactose (Fig. 4A and B), may have reflected nuclear heterogeneity among the recipient $p^{"206}$ cells (31) or differences in mtDNA copy number among the transformants (32). 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 4C, an analysis of these ratios revealed that the variations among cell lines within each group, except the control group, were decreased, whereas the differences between the groups persisted. Specifically, the cell lines from symptomatic individuals exhibited DT ratios from 1.39 to 1.72 times higher [on average 1.58 times higher ($p = 0.0053$)] than the mean value found for the control cell lines, whereas the cell lines from asymptomatic individuals exhibited DT ratios varying between 1.46 and 1.61 times higher [on average 1.53 times higher ($p = 0.0283$)] than the mean control value.

**DISCUSSION**

The control of phenotypic expression of the homoplasmic mitochondrial 12S rRNA A$\rightarrow$G transition at position 1555 has been the object of intensive investigations in recent years. In previous work, to account for the variable severity of hearing impairment in the members of the Arab-Israeli family who carried the A1555G mutation, a model involving the simultaneous inheritance of the homoplasmic mtDNA mutation and an autosomal recessive mutation had been proposed (33). The subsequent observation that the manifestation or lack of manifestation of the clinical phenotype correlated with the severity of biochemical defects in lymphoblastoid cell lines, i.e. a cell type totally unrelated to the auditory function, from symptomatic or asymptomatic individuals, was fully consistent with the model of a two locus disease mentioned above. However, a genome-wide screen by parametric analysis of the Arab-Israeli family failed to identify a single major nuclear modifier gene. It was therefore concluded that the penetrance of the mtDNA mutation depends on additive effects of several nuclear genes (34). In contrast to this conclusion, very recently, a genome-wide screen by non-parametric analysis of the Arab-Israeli family, as well as of 19 Spanish families and two Italian families with matrilineal hearing loss, has led to the identification of a candidate locus for a nuclear modifier gene. It was therefore concluded that the penetrance of the mtDNA mutation depends on additive effects of several nuclear genes (34). In contrast to this conclusion, very recently, a genome-wide screen by non-parametric analysis of the Arab-Israeli family, as well as of 19 Spanish families and two Italian families with matrilineal hearing loss, has led to the identification of a candidate locus for a nuclear modifier gene. It was therefore concluded that the penetrance of the mtDNA mutation depends on additive effects of several nuclear genes (34). 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transformant cell lines lacking the mutation, has unambiguously shown that the A1555G mutation is the primary factor responsible for the protein synthesis defect observed in the original lymphoblastoid cell lines and the resulting substantial reduction in rate of assembly of functional respiratory complexes. However, the central observation in the present work has been that the transmitochondrial cell lines derived from symptomatic and asymptomatic mitochondria-donors from the Arab-Israeli family exhibited nearly identical average degrees of mitochondrial dysfunction. This is in striking contrast to the observations made in previous work on lymphoblastoid cell lines derived from the two groups of individuals (21).

Table 1 shows the quantification of the mitochondrial defects detected in the previous and the present work. It appears that, in the earlier studies, the A1555G mutation caused an overall decrease in the rate of mtDNA-encoded protein labeling in the mutant lymphoblastoid cell lines from symptomatic individuals, which was significantly higher than the decrease observed in the cell lines derived from asymptomatic individuals (21). In contrast, in the present work, in the nearly constant nuclear background of mtDNA-less p<sup>206</sup> cells (24), almost identical average reductions in the rate of mitochondrial protein labeling have been found in the cell lines derived from five symptomatic and three asymptomatic individuals of the Arab-Israeli family. A comparison of the behavior of the other parameters tested in the lymphoblastoid and transmitochondrial cell lines revealed the same trend. In fact, the defects tended to be generally more pronounced in the lymphoblastoid cell lines from symptomatic individuals than in the cell lines from asymptomatic individuals, the differences of some critical parameters [rates of succinate/G3P- or COX-dependent respiration, DT ratio (galactose/glucose)] approaching statistical significance. In contrast, the defects observed in the cybrids from the two groups of individuals were identical or closely similar.

As shown in from Table 1, an exception to the general trend of more marked defects in the lymphoblastoid cell lines from symptomatic individuals, compared with those from asymptomatic individuals, was the small difference in endogenous respiration rate between the two groups of cell lines. This result may reflect a different degree of coupling of mitochondria in the two types of cells. Another exception, i.e. the relatively small difference in malate/glutamate respiration rate, could conceivably be due to some compensatory changes in the lymphoblastoid cell lines from symptomatic individuals or, alternatively, to a decrease in the rate of transport of the chosen substrates and/or in the activity of the corresponding dehydrogenases in the lymphoblastoid cell lines from asymptomatic individuals. It should be mentioned that the endogenous respiration rate and the malate/glutamate-dependent respiration rate are two parameters involving a multiplicity of nuclear-controlled steps and, therefore, more susceptible to compensatory phenomena. Quite different results were obtained in the analysis of other types of substrate-dependent respiration in the same cell lines. In particular, the most significant observations were the striking differences in succinate-dependent or TMPD/ascorbate-dependent respiration rate between the two groups of lymphoblastoid cell lines, which were observed at identical concentrations of exogenously provided substrates and in uncoupled cells (35). In agreement with the finding that transfer of mitochondria from mutant cell lines into the constant nuclear background of a mtDNA-less cell line all but eliminated the differences previously observed between lymphoblastoid cell lines from symptomatic and asymptomatic individuals in degree of mitochondrial dysfunction (21), was the observation that the intragroup variability in growth or biochemical properties among the original lymphoblastoid cell lines derived from different symptomatic or asymptomatic or control individuals was in general significantly reduced in the derived mitochondrial transformants carrying the same nuclear background.

On the basis of the evidence obtained in this work, it is reasonable to conclude that the pathogenetic mechanism of the A1555G mutation of the mitochondrial 12S rRNA involves a respiratory deficiency caused by the mitochondrial translation defect and resulting in a decline in ATP production in the cochlear cells (hair cells and/or stria vascularis), which are essential for hearing function (9). However, although the A1555G mutation is clearly the primary factor underlying the development of deafness, it is not sufficient to produce the clinical phenotype. Some difference(s) in either nuclear gene content or activity appears to contribute significantly to the biochemical defect responsible for the penetrance of non-syndromic deafness associated with this mutation. 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 the mutation, so as to produce the clinical phenotype, or suppress it so as to maintain a normal hearing phenotype (21). To further elucidate the mechanism leading to the non-syndromic deafness associated with the A1555G mutation, it will obviously be essential to identify and characterize the nuclear modifier gene(s) involved in the development of the deafness phenotype.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

A total of 11 human immortalized lymphoblastoid cell lines derived from members of the Arab-Israeli family [five symptomatic individuals (F6H, F7C, F7D, F12J and F12G), three asymptomatic members (F6C, F12D and F12H) and three genetically unrelated control individuals (#, F7A and F7E) (21,25)] were grown either in a specially made DMEM containing 1 mg/ml glucose, 0.11 mg/ml pyruvate and 0.18 mM CaCl<sub>2</sub>, supplemented with 10% fetal bovine serum (FBS), or in the same medium lacking glucose, but containing 0.9 mg/ml galactose and 0.5 mg/ml pyruvate (21) (hereafter referred to as special DMEM-galactose), supplemented with 10% dialyzed FBS. The BrdU-resistant 143B.TK – was grown in regular DMEM containing 4.5 mg/ml glucose and 0.11 mg/ml pyruvate, supplemented with 100 µg/ml BrdU and 5% FBS. The mtDNA-less p<sup>206</sup> cell line, derived from 143B.TK – (24), was grown under the same conditions as the parental line, except for the addition of 50 µg/ml uridine. All transmitochondrial cell lines constructed with enucleated lymphoblastoid cell lines were maintained in the same medium as the 143.TK – cell line.
Mitochondria-mediated ρ°206 cell transformation

Transformation by cytoplasts of mtDNA-less ρ°206 cells was carried out as described by King and Attardi (24,36). Briefly, 5 × 10⁷ lymphoblastoid cells from the mutant or control individuals were harvested by centrifugation and resuspended in 5 ml of 12.5% Ficoll in regular DMEM with 5% FBS containing 10 µg/ml cytochalasin B (Sigma) (from a 2 mg/ml stock solution in dimethyl sulfoxide). The Ficoll step gradients were prepared as described by King and Attardi (36) in sterile cellulose nitrate centrifuge tubes fitting the SW41 rotor for the Beckman ultracentrifuge, by carefully filling them with the following layers of Ficoll solutions: 2 ml of 25%, 2 ml of 17%, 0.5 ml of 16%, 0.5 ml of 15% and 2 ml of 12.5%, all in DMEM containing 10 µg/ml cytochalasin B. Then, 4 ml of the freshly prepared cell suspension was applied on the top of each step gradient. The gradients were centrifuged in the SW41 rotor in an L 3–50 ultracentrifuge for 60 min at 25 000 r.p.m. at 37°C. The centrifuge and rotor were prewarmed to 37°C. After centrifugation, three bands of cellular material appeared in the centrifuge tube. Two bands of cytoplasts tended to be located between the 15 and 16% Ficoll layers, and the karyoplasts, between the 17 and 25% layers. Cytoplast bands were collected by removing each layer from the top, diluted 10-fold and centrifuged at 1000 r.p.m. for 5 min to remove the Ficoll and cytochalasin B. Then the pellet of cytoplasts was suspended in 5 ml of cytoplast medium (DMEM with 20% FBS and 50 µg/ml uridine), centrifuged as described above and resuspended in 2 ml of the same medium. The suspension was incubated at 37°C for 30 min to allow the cytoplasts to recover and resume spherical morphology.

Cell fusion was carried out by mixing the cytoplasts with 1 × 10⁶ ρ°206 cells in regular DMEM, centrifuging them and resuspending the pellet in 1 ml 40% 1500 polyethylene glycol (PEG, BDH) solution. After 1 min incubation at room temperature, the fusion mixture was appropriately diluted with regular DMEM containing 50 µg/ml uridine and supplemented with 10% FBS, plated into 96-well microplates at 44 cells/well and incubated at 37°C. After changing the medium with regular DMEM supplemented with 5% dialyzed FBS and 100 µg/ml BrdU, and further incubation, the transfomers were isolated, 25–45 days after fusion, from wells containing single clones.

Growth measurements

These were made by plating samples of 1 × 10⁵ cells on 10 cm Petri dishes in 10 ml of regular DMEM or special DMEM-galactose, supplemented with 5% dialyzed FBS, incubating them at 37°C for 3 days and performing cell counts at daily intervals. The population DTs of the cell lines were determined from the growth curves or by using the formula: DT = (t – t₀)log2/(log N – log N₀) where, t and t₀ are the times at which the cells were counted and N and N₀ are the cell numbers at times t and t₀, respectively (37).

Mitochondrial DNA analysis

The analysis of the presence and level of the A1555G mutation in the mitochondrial 12S rRNA gene was carried out as previously described (21).

Analysis of mitochondrial protein synthesis

Pulse-labeling of the cell lines for 30 min with [³⁵S]methionine–[³⁵S]cysteine in methionine-free DMEM in the presence of emetine, electrophoretic analysis of the translation products, and quantification of radioactivity in the whole electrophoretic patterns or in individual well-resolved bands was carried out as detailed by Chomyn (38).

O₂ consumption measurements

Rates of O₂ consumption in intact cells were determined with a YSI 5300 oxygraph (Yellow Springs Instruments) on samples of 5 × 10⁶ cells in 1.5 ml of special DMEM-glucose lacking glucose, supplemented with 10% dialyzed FBS (24). 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 by Hofhaus et al. (26).

Computer analysis

Variance analysis was performed by the single factor analysis of variance (ANOVA) test contained in the Microsoft Excel program for Macintosh (version 5), entering individual replicate values. Correlation analysis was performed using the curve fitting in CA-Cricket Graph III™ program for Macintosh (version 1.5.2).

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