

Aging-dependent Functional Alterations of Mitochondrial DNA (mtDNA) from Human Fibroblasts Transferred into mtDNA-less Cells*

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To investigate the role that aging-dependent accumulation of mitochondrial DNA (mtDNA) mutations plays in the senescence processes, mitochondria from fibroblasts of 21 normal human individuals between 20 weeks (fetal) and 103 years of age were introduced into human mtDNA-less (ρ^0) 206 cells by cytoplasm \times ρ^0 cell fusion, and 7–31 transformant clones were isolated from each fusion. A slight cell donor age-dependent decrease in growth rate was detected in the transformants. Using an O_2 consumption rate of 1 fmol/min/cell, which was not observed in any transformant among 158 derived from individuals 20 weeks (fetal) to 37 years of age, as a cut-off to identify respiratory-deficient clones, 11 such clones were found among 198 transformants derived from individuals 39–103 years of age. Furthermore, conventional and nonparametric analysis of the respiratory rates of 356 clones revealed a very significant decrease with donor age. In other analyses, a very significant age-dependent decline in the mtDNA content of the clones was observed, without, however, any significant correlation with the decrease in O_2 consumption rate in the defective transformants. These observations clearly indicate the occurrence in the fibroblast-derived transformants of two independent, age-related functional alterations of mtDNA, presumably resulting from structural damage to this genome.

aging and degenerative diseases, due to the high mutation rate of mtDNA, which is 10 times higher than that of single-copy nuclear genes (9), to the size and compactness of the mitochondrial genome, to the lack of histones, to the lack or inefficiency of repair mechanisms, and to the somatic segregation of mitochondrial genomes during cell division (6). Substantial support for this suggestion has come from biochemical, histochemical, and immunohistochemical evidence of a progressive deterioration with aging of the respiratory capacity of different tissues, which exhibits a characteristic intercellular mosaicism of mitochondrial dysfunction (10–16), as well as from the demonstration of aging-related mtDNA damage in the form of large deletions (17–23), small deletions and insertions (24), and oxidative adducts of DNA (25–28). However, it has been impossible thus far to directly relate the aging-related respiratory decline to mtDNA damage.

A major difficulty in these studies, which has also been encountered in the identification of the genetic origin of the mitochondrial dysfunctions of the oxidative phosphorylation apparatus which cause disease in man (29), is the large contribution of nuclear genes to mitochondrial biogenesis and function (30). Another difficulty as to the direct correlation of mitochondrial lesions with the disease or aging phenotype is the heterogeneity of the mtDNA population. Pathogenetic mtDNA mutations are often heteroplasmic. Similarly, each one of the very large multiplicity of aging-related structural alterations of mtDNA that have been identified by different investigators occurs in a small fraction of the mtDNA population, and it has therefore been impossible to obtain a picture of the overall mtDNA damage. Furthermore, in experiments in which mitochondria from patient's cells carrying a pathogenetic mtDNA mutation had been transferred into human mtDNA-less cells (31), it has been found that the presence of as little as 6% of wild-type mtDNA is sufficient to protect transformed cells from the phenotypic effects of the mutation (32). The degree of heteroplasmy of the mtDNA population and the extent of complementation between genes carrying aging-related lesions and wild-type genes are also expected to play a major role in the manifestation of the aging phenotype.

Recent advances in mammalian mitochondrial genetics have resulted in the development of a novel technique, which has proven to be extremely valuable in the analysis of the pathogenetic role of disease-associated mtDNA mutations (32–35). This technique also has the potential to allow a correlation of mtDNA lesions with aging-dependent mitochondrial dysfunctions. In this approach, human cell lines devoid of mtDNA (ρ^0), isolated by long term exposure to low concentrations of ethidium bromide, are used for mitochondria-mediated transformation (31). Due to the lack of a functional respiratory chain, these cell lines have become auxotrophic for pyrimidines

Since the free radical theory of aging was first proposed (1), a voluminous amount of data has accumulated indicating that free radicals contribute to the degeneration of biological systems (2–4). Over 90% of the oxygen consumed by mammalian cells is utilized in mitochondria, and up to 4% of this oxygen is transformed into excited oxygen species (5). It is assumed that oxygen-free radicals are produced in mitochondria at a rate proportional to cellular metabolism, and that reaction with these free radicals may cause progressive damage to mitochondrial macromolecules during the life of the organism, contributing to the phenotypic effects of aging (3, 6–8). In particular, it has been hypothesized by Linnane and collaborators (6) that accumulation of mtDNA¹ mutations is a major contributor to

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¹ The abbreviations used are: mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BrdUrd, bromodeoxyuridine.

and pyruvate, and the lack of either requirement can be used as a selectable marker for repopulation of the ρ^0 cells with exogenous mitochondria (31). In the present work, this approach has been applied to investigate the role of mtDNA alterations in the aging processes using as mitochondria donors fibroblasts from individuals 20 weeks (fetal) to 103 years of age. Among 356 transformants analyzed, 11 of 198 clones derived from individuals 39–103 years of age exhibited a clearly respiratory-deficient phenotype, as contrasted to their absence among 158 transformants derived from the younger age group. Furthermore, both conventional and nonparametric statistical analysis showed a highly significant decrease with cell donor age in respiration rate, as well as a significant age-dependent decrease in mtDNA content of the clones, without, however, a significant correlation between the two parameters.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—The 143B. ρ^0 206 cell line was derived from 143B.TK⁻ cells by long term exposure to a low concentration of ethidium bromide (31), and a fast-growing clone (ρ^0 206.C3) was isolated, greatly expanded (36) and used in the experiments described below. Fibroblasts from individuals of varying age, to be used as a source of transforming mitochondria, were either obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) or derived from biopsies of old individuals at the Clinical Neurology Institute of the University of Milan (Milan, Italy). The latter set of individuals, all from retirement homes, were free of any neurological or muscular pathology. ρ^0 206.C3 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 50 μ g/ml uridine and 100 μ g/ml 5-bromodeoxyuridine (BrdUrd), and supplemented with 10% fetal calf serum (FBS). Fibroblasts were cultured in DMEM supplemented with 10% FBS.

Fibroblast Mitochondria-mediated ρ^0 Cell Transformation—The enucleated cell \times ρ^0 cell fusion technique was utilized to construct cybrids derived from skin fibroblasts of differently aged individuals, using the 143B. ρ^0 206.C3 cell line as a recipient. Transmitochondrial cell lines were derived from 21 individuals ranging in age from 20 weeks (fetal) to 103 years.

Enucleation of the fibroblast cultures by centrifugation in the presence of cytochalasin B and fusion of the cytoplasts with ρ^0 206.C3 cells in the presence of polyethylene glycol were carried out using standard techniques (37, 38). In each fusion experiment, cytoplasts from 3,000–4,000 cells, from individuals below 48 years of age, to 30,000–60,000 cells from individuals above 48 years of age, and 5×10^5 ρ^0 206.C3 cells were used. The fusion products were resuspended in 20 ml of DMEM, containing 5 μ g/ml uridine and 100 μ g/ml 5-bromodeoxyuridine (BrdUrd), and supplemented with 5% dialyzed FBS. Ten ml of this suspension were further diluted 1:2 with the same medium. The concentrated and diluted cell suspensions were then plated on four 96-well microtiter plates. Three days later, the medium was changed to selective medium, *i.e.* the same medium lacking uridine. Transformants were selected by taking advantage of the pyrimidine auxotrophy of the ρ^0 cells (31); the transformants containing exogenous functional mitochondria are in fact able to grow in the absence of added pyrimidines. The cell line ρ^0 206.C3, like its parent 143B.TK⁻, because of the lack of a functional thymidine kinase, is capable of growing in the presence of BrdUrd. The inclusion of BrdUrd in the selective medium prevented the growth of donor cells that had not been enucleated. Two to three weeks after plating, microtiter wells containing single transformant colonies were identified, and the transformants isolated and expanded for analysis. To obtain a representative sampling, 7–31 of the cybrid transformants were isolated and analyzed from each cytoplast \times ρ^0 cell fusion.

Analysis of Transmitochondrial Cell Lines—Total oxygen (O_2) consumption rate determinations were performed as described previously (31). Each value is the average of two separate determinations. 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 previously detailed (39). The mtDNA content of the transformants was quantified, at the time of the O_2 consumption measurements, by DNA transfer hybridization of total cell DNA samples carried out with a slot blot apparatus, using a mtDNA specific probe (mtDNA clone pTZ18/K4, containing a mtDNA fragment from positions 41 to 2578) and, for normalization purposes, a 28 S nuclear rRNA probe (40), as detailed earlier (41). Each value is the average of three determinations. Population doubling times were de-

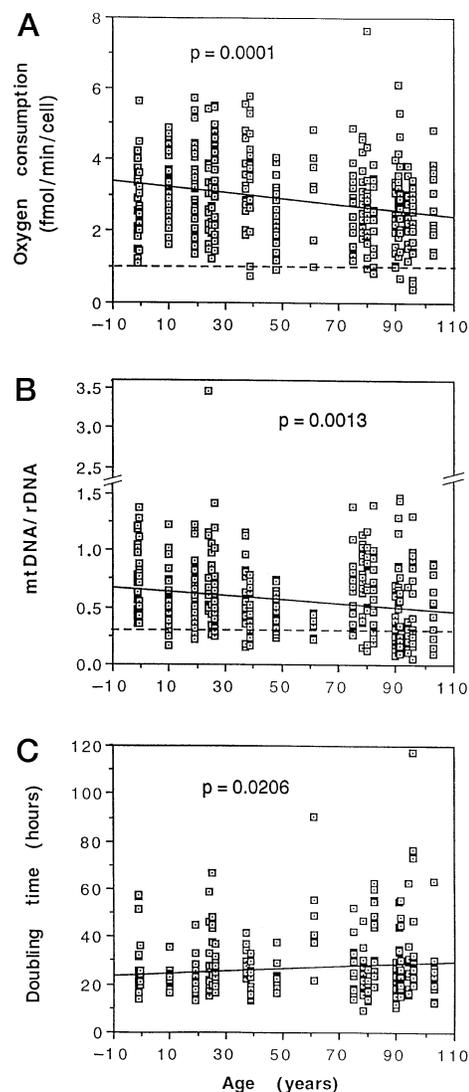


FIG. 1. Relationship between mitochondria donor age and cellular characteristics of mitochondria-mediated transformants.

The plots represent the rate of O_2 consumption (A), the normalized mtDNA content (B), and the doubling time (C) of 356 fusion clones derived from fibroblasts obtained from individuals of varying age. In panels A, B, and C, the solid lines represent standard regression analysis of the data from the transformant populations derived from the various individuals ($p = 0.0001$, $p = 0.0013$, and $p = 0.0206$, respectively). In A and B, the dashed lines represent the levels below which the values are considered to be outside the range of normal variation. See text for details.

termined as described previously (41). Statistical analysis was carried out using StatView SE+ version 1.03 software (Abacus Concepts, Berkeley, CA).

RESULTS

Respiratory Capacity of Transformant Clones—To screen the clones for possible mtDNA functional alterations, the total cell O_2 consumption rate in each isolated transformant was determined 8–18 weeks after fusion. As shown in Fig. 1A, a considerable variation in O_2 consumption was observed among the clones derived from the same individual. Furthermore, a cell donor age-dependent trend to a shift toward lower values in the range and average of the cellular respiration rates was found in the clones derived from different individuals. Since, among the transformants derived from individuals 20 weeks (fetal) to 37 years of age, no clone exhibited a rate of O_2 consumption lower than 1 fmol/min/cell, this level was chosen as a cut-off to identify clearly respiratory-deficient transformants. As shown in

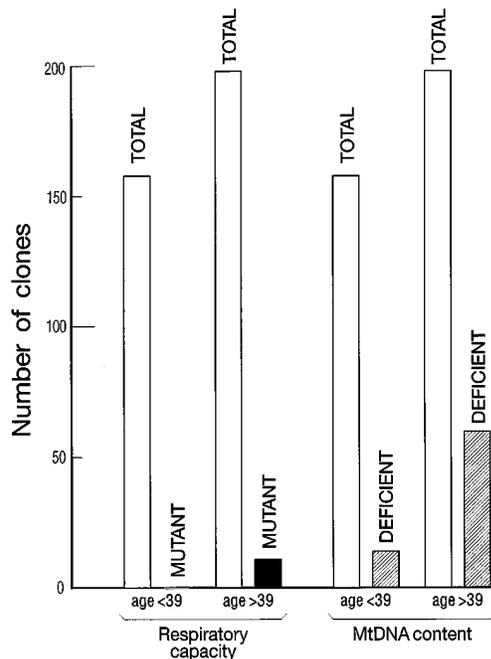


FIG. 2. Summary of age-dependent changes in respiratory capacity and mtDNA content of transformant clones.

Fig. 1A, a significant number of clones derived from individuals 39–103 years of age exhibited respiration rates below the chosen cut-off level. In particular, 11 of 198 transformants appeared to be respiration deficient, as contrasted to their absence among 158 transformants derived from the younger age group (Fig. 2). Conventional regression analysis showed that the observed cell donor age-dependent decrease in cellular respiration was statistically very significant ($p = 0.0001$). A non-parametric analysis according to Kruskal-Wallis (42) of the overall distribution of the O_2 consumption values of the transformant populations confirmed the high significance of the age-dependent variation among the clone populations derived from the various individuals ($p = 0.0001$). Pairwise comparison of the populations derived from different individuals using the Mann-Whitney U test (42) showed that the difference between the populations representing the extremes of the age range was greater than the differences between the clone populations derived from individuals of equal or similar ages. This analysis confirmed the significance of the Kruskal-Wallis test as indicating a donor age-dependent trend in the decline in the respiratory capacity of the clones.

To determine whether the age of the mitochondrial donor had an effect on the degree of variation observed among the clones derived from a single individual, the variance (σ^2) of the O_2 consumption values obtained for the clones derived from fibroblasts of each individual was determined. The variance was found to be substantially constant as a function of the mitochondrial donor age (Fig. 3).

mtDNA Content and Growth Rate of Transformant Clones—It has been shown that the repopulation of ρ^0 cells with exogenous mtDNA by mitochondria-mediated transformation can be slow and proceed at a variable rate, depending upon the donor cell type (43), the recipient cells, and other factors. It was, therefore, conceivable that a lower than normal mtDNA content of the transformants could have affected their respiration capacity, being responsible for the interclonal variability within the clone distribution from the same individual. To investigate this possibility, the mtDNA content of the transformants was quantified at the time of the O_2 consumption measurements. DNA transfer hybridization analysis of total DNA

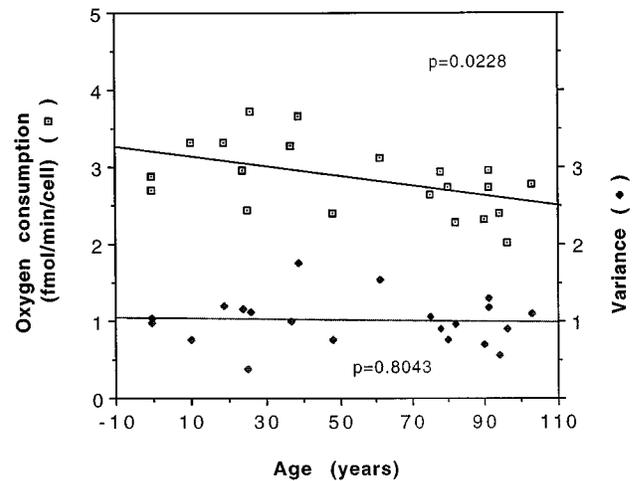


FIG. 3. Averages and variances (σ^2) of the rates of O_2 consumption in transformant populations derived from individuals of varying age. The upper solid line represents standard regression analysis of the average O_2 consumption rates in various transformant populations (open squares) ($p = 0.0001$). The lower solid line represents standard regression analysis of the corresponding variance values (filled diamonds) ($p = 0.8043$).

samples revealed a considerable variability in mtDNA levels among the clones derived from the same individual, as shown in Fig. 1B, where the mtDNA contents are plotted relative to the value for the ρ^0 cell parent 143B.TK⁻ (equivalent to 9100 molecules/cell; Ref. 31) taken as 1. It should be noted that the mtDNA content of one transformant, exhibiting an O_2 consumption rate of 3.04 fmol/min/cell, exceeded the 143B.TK⁻ level by a factor of approximately 3.5, a phenomenon that possibly reflected a compensatory mechanism, as previously observed (41). There was a clear tendency of the mtDNA content of the clones to decrease with the age of the cell donor, as concerns the ranges and averages of the individual distributions ($p = 0.0013$) (Fig. 1B). A mtDNA content of 30% of the level in 143B.TK⁻ cells, which does not occur in any of 30 clones derived from fetal individuals, was taken as the minimum compatible with a respiration rate within the normal range of variation under the present experimental conditions, in agreement with previous observations (36). Using this cut-off level, 60 of 198 transformants derived from individuals 39–103 years of age appeared to be mtDNA-deficient, as contrasted to 15 of 158 transformants derived from the younger age group (Fig. 2). A non-parametric analysis according to Kruskal-Wallis confirmed the high significance of the age-dependent decline of the mtDNA content of the clones ($p = 0.0001$).

The observation that the ranges and averages of both O_2 consumption rate and mtDNA content of the clone distributions obtained from differently aged individuals tended to shift to lower values with the age of the cell donor raised the possibility that the defect of the respiratory-deficient transformants was due to their abnormally low mtDNA content. Indeed, when the pooled data of O_2 consumption rates for the various clone sets derived from different individuals were compared with the corresponding mtDNA content values, a positive correlation ($p = 0.0001$) was found (Fig. 4A). However, when the analysis was limited to clones exhibiting mtDNA content values lower than 30% of the 143B.TK⁻ level, a decrease in the significance of the correlation with the corresponding O_2 consumption rates was observed ($p = 0.0226$). Furthermore, an analysis comparing the O_2 consumption rates with the corresponding mtDNA content values in the clearly respiratory-deficient transformants (with an O_2 consumption rate < 1 fmol/min/cell) failed to reveal a significant correlation ($p = 0.3742$) (Fig. 4B). These

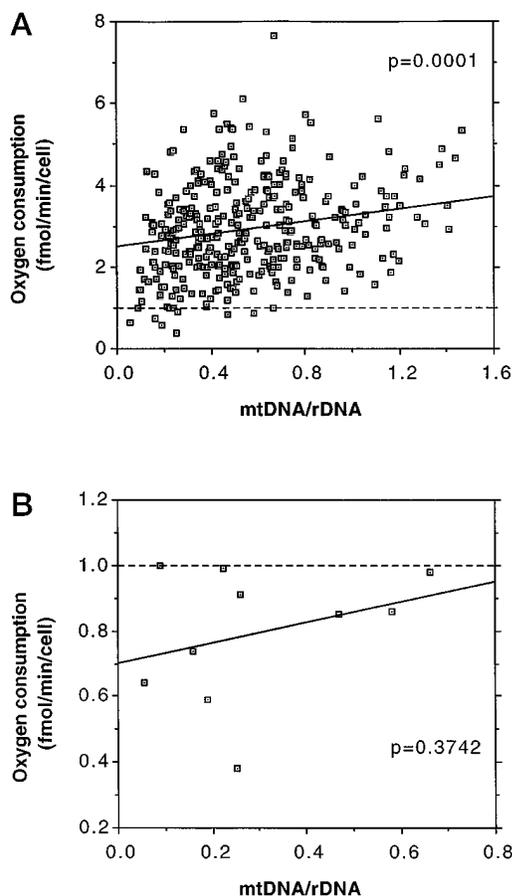


FIG. 4. Relationship between O_2 consumption and mtDNA content of individual clones. *A*, pooled data pertaining to the clones from differently aged individuals. One point, corresponding to O_2 consumption of 3.04 fmol/min/cell and mtDNA/rDNA of 3.47, was not included in the plot, but taken into consideration in the statistical analysis. *B*, data pertaining to the respiratory-deficient transformants. In *A* and *B*, the *solid lines* represent standard regression analysis of the transformant populations derived from the various individuals ($p = 0.0001$ and $p = 0.3742$, respectively). The *dashed lines* represent the levels below which the O_2 consumption rates are considered to be outside the range of normal variation.

findings strongly suggested that the low O_2 consumption rate did not necessarily result from a low mtDNA content.

A slight, but significant, age-dependent decrease in growth rate of the ρ^0 cell transformants was observed, clones with abnormally high doubling time being found only in the advanced age range of the cell donors (Fig. 1C). However, the observed age-dependent increase in doubling time appeared to be dependent upon the contribution of a small number of extreme values obtained from older individuals, suggesting that it might not reflect the behavior of the majority of the observations. To address this concern, the statistical analysis was repeated excluding from the analysis all transformants with doubling times exceeding 60 h ($n = 9$). As suspected, the correlation between donor age and doubling time lost its statistical significance ($p = 0.3713$). By contrast, even after excluding the values for O_2 consumption and mtDNA content from these outliers from the data pools, the negative correlation between donor age and O_2 consumption rate or mtDNA content remained significant ($p = 0.0001$ and $p = 0.0026$, respectively). Similarly, when excluding from the analysis the O_2 consumption rates below 1 fmol/min/cell ($n = 11$) and the values of mtDNA content below 30% of the 143B.TK⁻ level ($n = 75$), the negative correlation between O_2 consumption rate and age remained statistically significant ($p = 0.0001$ and $p = 0.0017$,

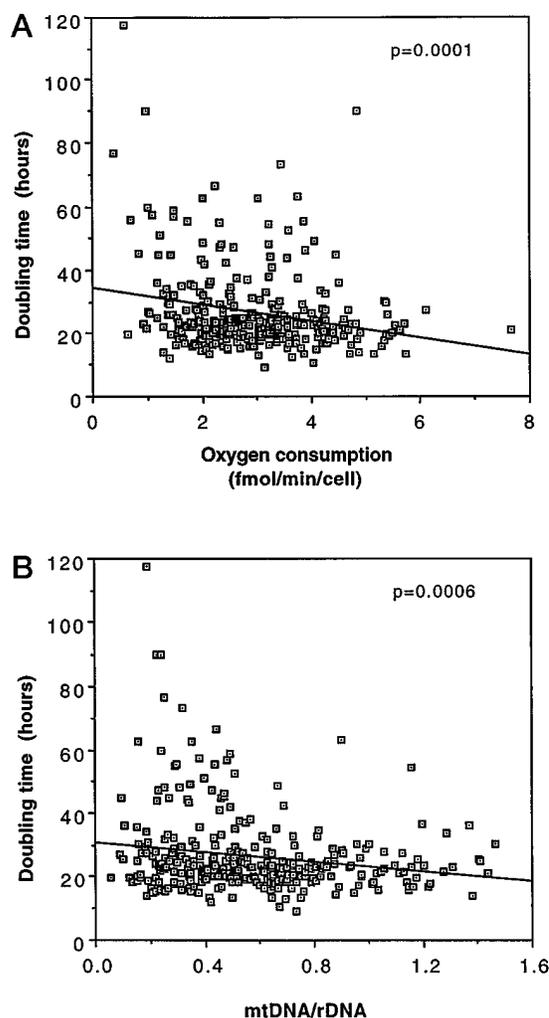


FIG. 5. Relationship between doubling time and other cellular characteristics of individual transformants. *A*, doubling time plotted against O_2 consumption rate. *B*, doubling time plotted against mtDNA content. In *A* and *B*, the *solid lines* represent standard regression analysis of the data from the transformant populations ($p = 0.0001$ and $p = 0.0006$, respectively). In *B*, one point, corresponding to mtDNA/rDNA of 3.47, was not included in the plot, but taken into consideration in the statistical analysis.

respectively). A similar analysis of the negative correlation between mtDNA content and age in the transformants, after the exclusion of the lower O_2 consumption rate subset or of the lower mtDNA content subset, yielded values of $p = 0.0035$ and $p = 0.6356$, respectively. The loss of statistical significance of the negative correlation between mtDNA content and age, after exclusion of the lower mtDNA content subset, was not unexpected, since the values excluded from consideration represented over 20% of the data collected.

To determine whether there was a correlation between respiratory capacity and growth rate, the values obtained from doubling time measurements were plotted against the O_2 consumption rate. Both with and without the inclusion of the data from the transformants representing the upper extreme values in doubling time, as defined above, a statistically significant negative correlation was observed ($p = 0.0001$) (shown for all the transformants in Fig. 5A). When a comparison was made between doubling time and mtDNA content, a significant negative correlation was observed both for all the transformants ($p = 0.0006$, Fig. 5B) and for the lower doubling time subset ($p = 0.0132$; data not shown).

An analysis of the average cell size of the transformants,

performed on trypsinized cells by microscopy using a micrometer eyepiece, failed to reveal any age-related variation (data not shown).

Long Term Behavior of Transformant Clones—Following the initial determination of O₂ consumption, a number of transformants which had been found to be respiration-deficient, then grown further for several weeks and subsequently frozen, were thawed and maintained in culture for an additional 30 days, and the determination was repeated at different times after thawing. The majority of the clones tested showed a significant increase in O₂ consumption, while the others maintained a low respiratory capacity (Fig. 6A), without, however, any consistent correlation with changes in mtDNA content. When the clones that initially exhibited a low rate of O₂ consumption were analyzed by polarography, to test the activity of the respiratory complexes, the samples that maintained their respiratory-deficient phenotype at the time of analysis revealed a general decrease in the activity of all the complexes (Fig. 6B).

DISCUSSION

The main result of the present study is the demonstration of aging-dependent functional alterations of the mtDNA of a differentiated cell type, as tested in a common nuclear background. By the use of an approach based on mitochondria-mediated transformation of mtDNA-less cells, this work has for the first time linked mtDNA to an aging-related respiratory decline. A considerable variation in O₂ consumption rate and mtDNA content was observed among transformants derived from the same individual, from 20 weeks (fetal) to 100 years of age. This variation did not represent experimental noise, since the differences between duplicate or triplicate measurements were very small. In fact, the mean standard deviation of replicate measurements of O₂ consumption rate in the whole population of transformants was 0.29 fmol/min/cell, and that of replicate measurements of mtDNA content in a representative subset of 83 clones was 6.2% of the 143B.TK⁻ level. On the other hand, the constancy with the donor age in the extent of variation observed in O₂ consumption rate within the population of transformants derived from each individual suggested that age alone was not a major factor in the observed variability. In view of the observed highly significant positive correlation between O₂ consumption rate and mtDNA content in the total transformant population, it is very likely that the heterogeneity in respiration capacity detected among the ρ^0 cell transformants derived from the same individual reflected in part the very slow and variable process of repopulation of ρ^0 cells with fibroblast-derived mtDNA, which has been described recently (43). However, it is also reasonable to assume that possible differences in nuclear gene content and/or activity among the recipient ρ^0 cells (36) played a significant role in the observed variability in respiratory capacity among individual donor-derived transformants.

In the present work, the lack of correlation between the cell donor age-related decrease in O₂ consumption rate and mtDNA content of the highly defective ρ^0 cell transformants suggests the occurrence of two independent functional alterations of mtDNA. No information has been obtained in the present work as concerns the molecular basis of these functional alterations. However, the most plausible interpretation is that they reflect the accumulation of age-dependent mtDNA mutations resulting from free radical damage. In particular, a broad range of mtDNA mutations may underlie the age-related respiratory deficiency of the transformants, including structural changes in genes encoding subunits of the oxidative phosphorylation apparatus, or in genes encoding components of the mitochondrial translational machinery, or in critical mtDNA sequences controlling the transcription process. The age-related decrease

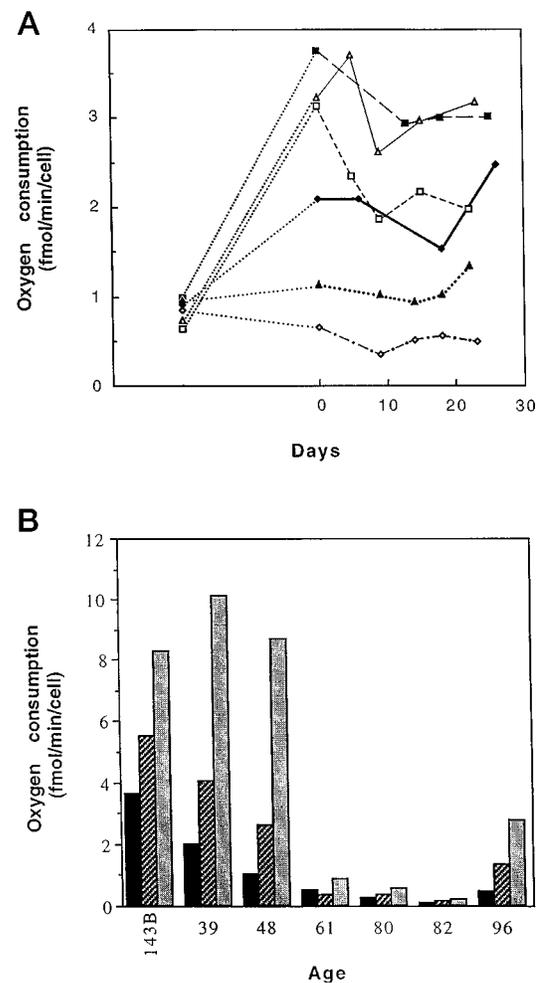


FIG. 6. Characterization of some representative respiratory-deficient transformant clones. A, O₂ consumption rate of respiratory-deficient clones after extended culture time. The initial value represents the first O₂ consumption determination, after which the cells were maintained in culture an additional 1–11 weeks, then frozen. Clones were thawed and expanded for additional analysis, the first measurement (time 0 on the *abscissae* axis) being made 7–9 days after thawing, except for the 39-year old individual-derived transformant (81 days after thawing). Cell donor age was as follows: *open triangle*, 39 years; *open square*, 96 years; *filled diamond*, 48 years; *filled square*, 61 years; *filled triangle*, 80 years; *open diamond*, 82 years. B, activities of the enzymes of the mitochondrial respiratory chain in the clones represented in Fig. 4A and in 143B.TK⁻ cells. By using $\sim 5 \times 10^6$ digitonin-permeabilized cells, the activities of the various components of the respiratory chain were determined as respiration dependent on malate/glutamate (*filled bars*), glycero-3-phosphate/succinate (*hatched bars*), and ascorbate/*N,N,N,N'*-tetramethyl-*p*-phenylenediamine (*shaded bars*). The values for the 39-, 48-, and 96-year-old individual-derived transformants were obtained after partial or total recovery of the total O₂ consumption rate as compared to that of the parental line (~ 4 fmol/min/cell). The total O₂ consumption rates of these clones at the time of the polarographic measurements corresponded approximately to the rates of the malate/glutamate-dependent respiration. The values for the 61-, 80-, and 82-year-old individual-derived transformants were obtained when the total O₂ consumption rate was at the initial levels.

in mtDNA content of the transformants may, in turn, reflect the occurrence of mutations that affect the interactions between mtDNA and the nuclear-encoded gene products involved in mtDNA replication. A previous analysis of human fibroblasts in culture had shown that there was a significant increase in mtDNA copy number per cell with the age of the donor (44). It is, therefore, unlikely that the observed age-dependent decrease in mtDNA content is a result of a decrease in the number of mtDNA molecules transferred, during the fusion process, from fibroblasts of older individuals. The recovery of

respiratory capacity in some of the transformants upon prolonged culturing, similar to that observed previously in fibroblast cultures harboring deletions resulting in respiratory deficiency (45), may have resulted from mitotic segregation and selection against cells carrying mutant mtDNA or from nuclear suppression phenomena. The clones that maintained the mutant phenotype presumably represented stable heteroplasmic transformants, as described previously (41).

The observed strong correlation between doubling time and O_2 consumption rate suggests that the respiratory capacity of the transformants may determine or greatly influence the growth rate of the transformants. The link between growth rate and respiratory capacity may in turn account for the observed relationship between doubling time and mtDNA content, in view of the positive correlation described above between O_2 consumption rate and mtDNA content. Through the comparison of O_2 consumption rate, mtDNA content, and growth rate, it seemed plausible to expect that a subset of transformants might be identified which would exhibit the highest values of all three parameters. Although a certain degree of overlap was observed, the occurrence of an aging-dependent deficiency in one of the parameters examined did not strictly correlate with deficiencies in the other parameters in the same transformants. This observation is consistent with the presence in the transformants of variable mtDNA structural alterations and of a variable degree of heteroplasmy; this variation was expected to occur in the donor fibroblasts and to be amplified as a result of the random transfer of mtDNA during the enucleation and fusion processes.

As to the possibility of analyzing the aging-related structural alterations of mtDNA underlying the functional defects reported in the present work, the use of sensitive assays for the detection of the major deletions found in tissues of older individuals (19, 20) and the application of rapid screening methods for point mutations, like the psoralen-clamp modification (46) of denaturing gradient gel electrophoresis (47) and the single-strand conformation polymorphism assay (48), may allow the identification of the prevalent mtDNA mutations occurring in the respiration-deficient mutants. Experiments in this direction are in progress.

Recently, a report has appeared which claimed a nuclear, but not mitochondrial genome involvement in aging-related mitochondrial dysfunction of human fibroblasts (49). However, the evidence presented failed to discriminate between *in vitro* senescence of fibroblasts from the older individuals analyzed and *in vivo* aging-related phenomena. In addition, in the experiments showing a restoration of cytochrome *c* oxidase activity by transfer of the HeLa cell nucleus to fibroblasts from aged donors, the possibility of a selection of respiratory-competent fibroblasts in the cell fusion step was not excluded. Additionally, in the reported mtDNA transfer experiments, the very small number of cybrids analyzed would have prevented the identification of mutants occurring with the frequency found in the present work. Therefore, none of the data presented argue convincingly against the occurrence of aging-related mtDNA damage.

The aging-dependent functional alterations of mtDNA found in the ρ^0 cell transformants analyzed in the present work appear to be more significant if one considers the type of cells utilized as mtDNA donors. In fact, due to the probable rapid renewal rate of fibroblasts, one would expect that selection would occur *in vivo* against the cells most damaged by aging-dependent mutations. Moreover, further loss of the cells most deficient in respiratory capacity would occur during the fusion process and during the selection and expansion of the transformants. A decrease in the efficiency of mitochondria-mediated

transformation as a function of the mitochondria donor age has been observed.² Furthermore, the evidence presented above on the tendency of most of the respiratory-deficient clones to re-acquire, upon prolonged culturing, their respiratory capacity clearly points to the facility whereby the mtDNA-linked defective phenotype can be lost in cultured cell systems by mtDNA segregation and selection or by nuclear suppression. On the basis of these considerations, it seems plausible to assume that the defective transformants observed here represent only a small fraction of the mutant fibroblasts occurring *in vivo*. The development of early screening methods for respiratory-deficient mutants among the ρ^0 cell transformants, which is presently being pursued in our laboratory, should significantly facilitate the detection of aging-related mtDNA damage. Furthermore, one would expect that post-mitotic cells, like nerve cells, muscle fibers, and heart cells, would accumulate aging-dependent mutations at a much higher rate than fibroblasts. In another context, the findings reported in this paper are particularly significant in view of the emerging evidence pointing to the contributing role of aging-related mtDNA damage to the incidence of neurodegenerative diseases and other degenerative disorders (28, 50).

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