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Efficient Selection and Characterization of Mutants of a Human Cell Line Which Are Defective in Mitochondrial DNA-Encoded Subunits of Respiratory NADH Dehydrogenase

GÖTZ HOFHAUS AND GIUSEPPE ATTARDI*

Division of Biology, California Institute of Technology, Pasadena, California 91125

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The mitochondrial NADH dehydrogenase (complex I) in mammalian cells is a multimeric enzyme consisting of ~40 subunits, 7 of which are encoded in mitochondrial DNA (mtDNA). Very little is known about the function of these mtDNA-encoded subunits. In this paper, we describe the efficient isolation from a human cell line of mutants affected in any of these subunits. In the course of analysis of eight mutants of the human cell line VA_2B selected for their resistance to high concentrations of the complex I inhibitor rotenone, seven were found to be respiration deficient, and among these, six exhibited a specific defect of complex I. Transfer of mitochondria from these six mutants into human mtDNA-less cells revealed, surprisingly, in all cases a cotransfer of the complex I defect but not of the rotenone resistance. This result indicated that the rotenone resistance resulted from a nuclear mutation, while the respiration defect was produced by an mtDNA mutation. A detailed molecular analysis of the six complex I-deficient mutants revealed that two of them exhibited a frameshift mutation in the ND4 gene, in homoplasmic or in heteroplasmic form, resulting in the complete or partial loss, respectively, of the ND4 subunit; two other mutants exhibited a frameshift mutation in the ND5 gene, in near-homoplasmic or heteroplasmic form, resulting in the ND5 subunit being undetectable or strongly decreased, respectively. It was previously reported (G. Hofhaus and G. Attardi, EMBO J. 12:3043–3048, 1993) that the mutant completely lacking the ND4 subunit exhibited a total loss of NADH:Q_1 oxidoreductase activity and a lack of assembly of the mtDNA-encoded subunits of complex I. By contrast, in the mutant characterized in this study in which the ND5 subunit was not detectable and which was nearly totally deficient in complex I activity, the capacity to assemble the mtDNA-encoded subunits of the enzyme was preserved, although with a decreased efficiency or a reduced stability of the assembled complex. The two remaining complex I-deficient mutants exhibited a normal rate of synthesis and assembly of the mtDNA-encoded subunits of the enzyme, and the mtDNA mutation(s) responsible for their NADH dehydrogenase defect remains to be identified. The selection scheme used in this work has proven to be very valuable for the isolation of mutants from the VA_2B cell line which are affected in different mtDNA-encoded subunits of complex I and may be applicable to other cell lines.

NADH:ubiquinone oxidoreductase (complex I) is the largest and most complicated of the enzymes of the mitochondrial respiratory chain (15, 31, 35). With a molecular mass of ~900 kDa and the presence of about 40 different subunits, the complexity of mammalian complex I approaches that of the ribosome. The reason for this level of complexity is unknown. Complex I from mammalian cells and *Neurospora crassa* contains seven subunits that are encoded in mitochondrial DNA (mtDNA) (2, 3, 35). Despite the fact that the primary structures of the mammalian mtDNA-encoded subunits were determined 13 years ago (1), still little is known about their function. The subunit encoded in the mitochondrial ND1 gene binds rotenone, a specific inhibitor of complex I (6), and interacts with ubiquinone, the natural electron acceptor (10). In addition, the ND1 gene product may be involved in proton translocation, because it binds *N*,*N*′-dicyclohexylcarbodiimide (37). An insight into the function of the mtDNA-encoded subunits of complex I has come from their localization within complex I. By electron microscopy, the enzyme from *N. crassa* has been shown to have an overall L-shaped structure (17), and it is likely that this model applies also to the mammalian enzyme. One arm of the L is buried in the inner mitochondrial membrane, while the other protrudes into the matrix. The membrane arm has been isolated and shown to contain all the mtDNA-encoded subunits, while the matrix arm contains most of the nucleus-encoded subunits and most of the prosthetic groups that are involved in the redox reactions between NADH and ubiquinone (35). Complex I uses the energy generated by these reactions to pump protons out of the mitochondrial matrix, thereby generating an electrochemical gradient across the inner mitochondrial membrane. The above observations, therefore, support involvement of the membrane arm of the enzyme and of the mtDNA-encoded subunits in proton translocation. The importance of the mtDNA-encoded subunits is further emphasized by the fact that the much simpler bacterial counterpart of complex I contains subunits homologous to all seven mtDNA-encoded polypeptides of the mammalian enzyme (34, 38).

In the past few years, the mtDNA-encoded subunits of complex I have attracted increasing attention as a result of the discovery that mutations in the genes for these subunits can cause a disease in humans. In particular, it has been shown that mutations in the ND4 gene (33), the ND1 gene (18–20), and the ND6 gene (21) can be the primary cause of Leber's hereditary optical neuropathy, with mutations in other ND genes contributing to the severity of the syndrome (32). Furthermore, for some of these mutations, a reduction in the activity of the enzyme has been reported (24, 25, 28). It is also conceivable that some of these mutations affect proton transloca-

* Corresponding author.

tion. A deeper understanding of the functions of the mtDNA-encoded subunits of complex I will be necessary to explain at the molecular level the pathogenetic mechanism(s) of these mutations and other possible disease-causing mutations affecting these subunits. Progress in this understanding has been hampered by the complexity of the enzyme and the scarcity of mutants available.

Recently, in the course of an analysis of rotenone-resistant variants of the human cell line VA_2B, a complex I-deficient mutant lacking the mitochondrial ND4 gene product because of a frameshift mutation in the gene has been identified and characterized (16). In the present study, an extension of the analysis to seven other rotenone-resistant mutants has, surprisingly, revealed that five of these mutants also exhibited a defect in any of the mtDNA-encoded subunits of respiratory chain NADH dehydrogenase. Furthermore, among these defective mutants, one carried, in heteroplasmic form, the same ND4 frameshift mutation previously identified, while two others contained a frameshift mutation in the ND5 gene either in near-homoplasmic or in heteroplasmic form. An analysis of the mutant almost completely lacking the ND5 subunit showed that this subunit is essential for complex I activity, but, in contrast to the ND4 subunit, the absence of the ND5 subunit does not prevent the assembly of the membrane arm of the complex. The mutant selection scheme applied in this study to VA_2B cells has thus proved to be a valuable means for the isolation from this cell line of mutants affected in different mtDNA-encoded subunits of the enzyme.

MATERIALS AND METHODS

Cell lines and media. The human cell line VA_2B (27) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 3 μg of 8-azaguanine per ml. The 143B.TK$^-$ cell line was grown in DMEM supplemented with 5% fetal bovine serum and 100 μg of bromodeoxyuridine (BrdU) per ml. The $\rho^0$206 cell line (23), a derivative of 143B.TK$^-$ cells, was grown in DMEM supplemented with 5% fetal bovine serum, 50 μg of uridine per ml, and 100 μg of BrdU per ml.

Isolation of rotenone-resistant VA_2B cell mutants. Ten plates of VA_2B cells ($\sim 4 \times 10^5$ cells per plate) in DMEM with 10% calf serum and 3 μg of 8-azaguanine per ml were exposed to 0.4 μM rotenone and maintained continuously in the presence of this concentration of the drug, with frequent medium changes (every 3 or 4 days). After 3 weeks, ~1,400 clones were obtained from the 10 plates. Ten independent rotenone-resistant clones (picked one from each plate) were subjected to increasing concentrations of the drug, and eight clones were adapted to grow in medium containing 1 μM drug (after 1 to 3 months from their isolation, with the exception of C7, which was maintained for 7 months in 0.4 μM rotenone and thereafter proved to be resistant to 1 μM rotenone). They were then maintained for 8 to 17 months (4 weeks for C7) at this concentration of the drug. To test the VA_2B cells and the VA_2B-derived clones for their rotenone resistance, cell cultures (3×10^5 cells per 10-cm-diameter dish) were exposed for 5 days to the indicated concentrations of the drug, and then cells were counted.

Transfer of mitochondria from rotenone-resistant mutants into $\rho^0$206 cells. ρ^0 cell transformation by cytoplast fusion was carried out as previously described (23), using clones that had been maintained in 1 μM rotenone for 8 to 17 months (4 weeks for C7). Transformants were isolated in DMEM supplemented with 5% dialyzed fetal bovine serum and 100 μg of BrdU per ml. Four to six weeks after their isolation, the BrdU was omitted from the medium. Rotenone resistance of the 143B.TK$^-$ cells and of the ρ^0 transformants was tested as described in the previous paragraph, except that the cells were plated at 2×10^5 per dish.

O_2 consumption measurements. VA_2B-derived mutant cell lines were grown in rotenone-free medium for 24 h prior to the measurements. O_2 consumption determinations for intact cells were carried out as previously described (23). For measurements of O_2 consumption by digitonin-permeabilized cells (13), about 10^7 cells were harvested by trypsinization and resuspended in 1 ml of buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.1], 10 mM $MgCl_2$, 250 mM sucrose), and then 100 to 150 μg of digitonin (1 to 1.5 μl of a 10% dimethyl sulfoxide solution) was added while the suspension was being mixed; after addition of 9 ml of buffer, the cells were pelleted and resuspended in ~100 μl of buffer. After introduction of the cells into the chamber of a Gilson 5/6 Oxygraph containing 1.7 ml of respiration medium (20 mM HEPES [pH 7.1], 250 mM sucrose, 2 mM KP_i, 10 mM $MgCl_2$, and 1.0 mM ADP), two small aliquots were removed to determine the exact number of cells in the chamber. Substrates and inhibitors from 100× stock solutions (neutralized with NaOH when necessary) were added with Hamilton syringes. The final concentrations were as follows: malic acid, 5 mM; glutamic acid, 5 mM; succinic acid, 5 mM; Na_2 glycerol-3-phosphate, 5 mM; ascorbic acid, 11 mM; *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), 0.2 mM; rotenone, 100 nM; antimycin, 4 nM; and KCN, 1 mM.

NADH:Q_1 and NADH:$K_3Fe(CN)_6$ oxidoreductase activity measurements. The mitochondrial fraction was isolated from ~0.5 ml of packed cells as described elsewhere (30), resuspended in 8 ml of 10 mM Tris (pH 7.5) at 4°C, and sonicated for 40 s (four 10-s pulses separated by 15-s intervals) in a polycarbonate tube immersed in ice water. Mitochondrial membranes were pelleted by centrifugation at 39,000 rpm in a Beckman Ty 65 fixed-angle rotor for 60 min and resuspended in the buffer described above at a protein concentration (determined by the Bio-Rad assay) of about 5 mg/ml. The oxidoreductase activities were measured, at protein concentrations of 85 to 120 μg/ml for Q_1 reduction and 20 to 35 μg/ml for $K_3Fe(CN)_6$ reduction, in a mixture containing 20 mM Tris (pH 7.5), 1 mM KCN, 100 μM NADH, and 50 μM Q_1 (Eisai Co., Japan) or 1 mM $Fe(CN)_6$. The reaction was monitored at 275 nm for the reduction of Q_1 ($\varepsilon = 12{,}250\ M^{-1}\ cm^{-1}$) and at 410 nm for the reduction of $K_3Fe(CN)_6$ ($\varepsilon = 1000\ M^{-1}\ cm^{-1}$). The NADH:$Q_1$ oxidoreductase activity of the VA_2B control was >95% sensitive to 100 nM rotenone.

Mitochondrial protein synthesis analysis. Labeling was performed as described by Chomyn et al. (4). Samples of 10^6 VA_2B or ρ^0 transformant cells were plated on 5-cm-diameter dishes and grown overnight. After the cells were washed with methionine-free DMEM, 5 ml of the same medium containing 100 μg of emetine per ml was added to each plate. After 10 min of incubation, [^{35}S]methionine (0.1 mCi) was added and the plates were incubated for 2 h at 37°C. The cells were then trypsinized, washed, and lysed in 1% sodium dodecyl sulfate (SDS). Samples containing 26 to 150 μg of protein were electrophoresed through an SDS–15 to 20% exponential gradient polyacrylamide gel.

Immunoprecipitation experiments. As described by Chomyn et al. (3), samples of 2×10^6 cells were plated on 10-cm-diameter dishes, grown for 22 h in the presence of 40 μg of chloramphenicol per ml to accumulate nucleus-encoded subunits of complex I, washed with methionine-free DMEM to remove chloramphenicol and methionine, incubated for 10 min in the same medium with 100 μg of cycloheximide per ml, and then exposed for 2 h to 1 mCi of [^{35}S]methionine. Thereafter, the cells were washed and subjected to a 15-h chase in complete unlabeled medium to allow the incorporation of the labeled mtDNA-encoded subunits into complex I. The cells were then trypsinized, washed, and mixed with 0.1 ml of packed HeLa cells, and the mitochondrial fraction was isolated by homogenization and differential centrifugation and lysed in 0.5% Triton X-100. Samples containing 120 μg of protein were incubated at 4°C with 72 μg of gamma globulins from an antiserum against the 49-kDa subunit of bovine complex I (2), from an antiserum against the C-terminal undecapeptide of subunit II of human cytochrome *c* oxidase (COII) (26), or from normal rabbit serum. The mitochondrial proteins of transformant C6T exhibited, after pulse-chasing, about one-third the specific activity observed in VA_2B cells and in the other transformants (see below). Therefore, an approximately threefold-larger amount of protein (380 μg) was used in the immunoprecipitation experiments. Immunocomplexes were bound to formaldehyde-fixed *Staphylococcus aureus* (Zysorbin; Zymed) (2), spun down, and washed several times. The final pellets were dissolved in 1% SDS and separated on an SDS–15 to 20% exponential gradient polyacrylamide gel.

DNA analysis. Total DNA was isolated from cells by direct lysis in PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl, 1 mM $MgCl_2$) containing 100 μg of proteinase K per ml and 0.5% Tween 20 (22). Quantification of mtDNA in total cell DNA samples was carried out by slot blot hybridization, by using the clone pTZ18-K4 (containing the *Eco*RI-*Kpn*I fragment of human mtDNA between positions 41 and 2578), labeled by random priming, as a probe. To correct for possible quantitative variations among different DNA samples, the same membrane was probed with a nuclear 28S rRNA gene fragment, constructed by PCR amplification using oligonucleotides corresponding to positions 1503 to 1522 and 1981 to 2000 in the 28S rRNA gene (12), purified on an agarose gel, and ^{32}P labeled by random priming. For DNA sequencing, the segments of the VA_2B, C4T, and C5T mtDNAs between positions 10420 and 12200, which contain the ND4L and the ND4 genes, were amplified by PCR with appropriate oligonucleotides in two overlapping portions 989 and 896 bp in length. Similarly, the segments of VA_2B, C8T, and C9T mtDNAs between positions 12282 and 14177, which contain the ND5 gene, and the segments of VA_2B and C6T mtDNAs between positions 3274 and 4329, which contain the ND1 gene, were amplified (the ND5-containing segment in two overlapping portions of 978 and 1111 bp). The PCR products were electrophoresed on an agarose gel in Tris-acetate-EDTA, eluted from the gel, and ethanol precipitated, and the purified double-stranded fragments were directly sequenced by the chain termination method (29) using CircumVent DNA polymerase (New England Biolabs) and ^{35}S-dATP. The products of the sequencing reactions were separated on a 0.4-mm-thick 6% polyacrylamide–7 M urea gel.

Quantification of the mtDNA frameshift mutations was carried out by allele-specific termination of primer extension, which is illustrated schematically in Fig. 1a and b for the ND4 and ND5 gene mutations, respectively. A 346-bp segment between positions 10762 and 11108, containing the ND4 mutation (a C insertion in a row of six C residues at positions 10947 to 10952), and a 359-bp segment of mtDNA between positions 12282 and 12641, containing the ND5 mutation (an A insertion in a row of eight A residues at positions 12417 to 12424), were amplified

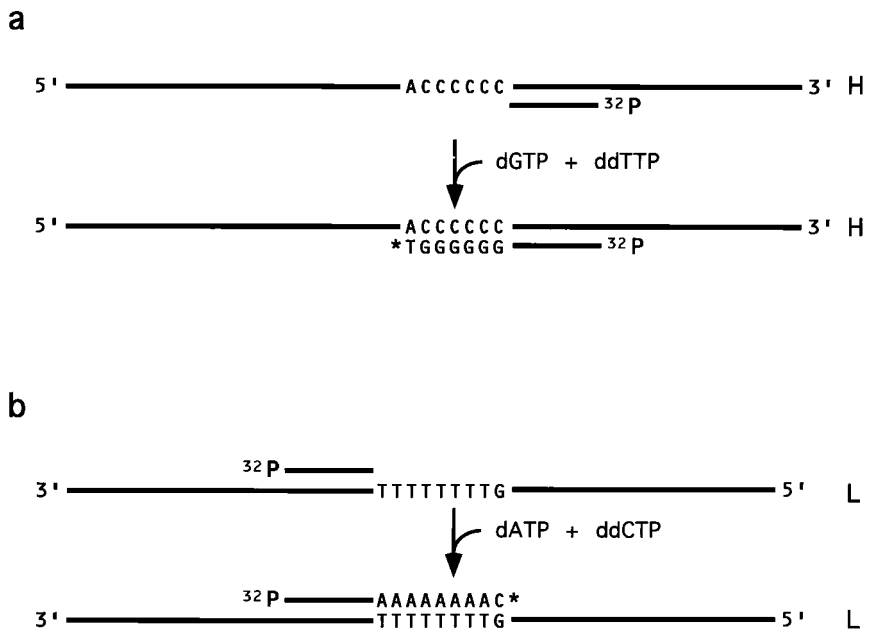

FIG. 1. Illustration of the approach used for the quantification of the insertion of a C residue in the ND4 gene (a) and of an A residue in the ND5 gene (b). A dideoxynucleoside triphosphate (asterisk) is indicated. H, heavy strand; L, light strand.

with *Taq* polymerase, using 10^6 to 10^7 template molecules. The two amplified fragments were separated from free nucleotides on a 1% Tris-acetate-EDTA–agarose gel, eluted from the gel overnight, and ethanol precipitated. Solutions in Sequenase buffer that contained the amplified DNA fragment and a corresponding 5′-^{32}P-labeled primer (of 24 bp [positions 10976 to 10953) for the ND4 gene mutation and 20 bp [positions 12398 to 12427] for the ND5 gene mutation) in a 1:1 molar ratio were prepared. Nucleotide concentrations were 60 μM dGTP and 500 μM ddTTP for the quantification of the C insertion in the ND4 gene and 60 μM dATP and 250 μM ddCTP for the quantification of the A insertion in the ND5 gene. The mixtures were heated to 95°C for 3 min and chilled on ice. After addition of 1 μl of diluted Sequenase, they were incubated for 5 min at 45°C. The products (31 and 32 bp for the ND4 mutation and 29 and 30 bp for the ND5 mutation) were denatured and separated on a 50-cm-long 20% polyacrylamide–6 M urea gel. The correct sizes of the products were checked separately by electrophoresis in a sequencing gel. Quantification of the intensity of the bands was done by using a phosphorimager and the IMAGE-QUANT program.

Quantification of a G→A transition in the ND1 gene at position 3496, which causes the disappearance of an *Aci*I site, was carried out by amplifying a 354-bp segment of mtDNA between positions 3274 and 3628, which encompasses the mutation, digesting it with *Aci*I, and analyzing it by electrophoresis through an 8% polyacrylamide gel and scanning the ethidium-bromide stained gel with an LKB laser densitometer. The amplified fragment of wild-type mtDNA contains two *Aci*I cutting sites separated from each other by 30 nucleotides. Cutting at the 3′-end-proximal site cleaves off a 104-bp fragment, which serves as an internal control for completion of digestion. The remaining 251-bp fragment has an additional *Aci*I site that disappears as a result of the G→A transition. In wild-type mtDNA, this fragment is shortened by 30 nucleotides because of cleavage at the second *Aci*I site. The ratio between the 221- and the 251-bp fragments was used for the quantification of the mutation.

RESULTS

Six of eight rotenone-resistant mutants show a decreased complex I activity due to an mtDNA mutation. The original goal of these experiments was to isolate mutants of the human cell line VA_2B (27) which were resistant to rotenone, a specific inhibitor of complex I. Since the ND1 gene encodes the subunit which binds rotenone (6), it was hoped that, at least in some of the isolated mutants, the resistance may have been due to a mutation in this mtDNA gene. Preliminary experiments showed that both the growth capacity in mass culture and the plating efficiency of VA_2B cells were significantly affected by rotenone concentrations as low as 0.2 μM, with complete inhibition by 1 μM drug (Fig. 2a). An intriguing observation was that the growth of mtDNA-less $\rho^0$206 cells was also severely inhibited by 0.25 μM rotenone and totally inhibited by 0.5 to 1.0 μM concentrations of the drug (Fig. 2b).

After ~4×10^6 VA_2B cells were exposed to 0.4 μM rotenone for 3 weeks, ~1,400 clones were obtained. Ten randomly chosen independent clones were subjected to stepwise-increased concentrations of rotenone, starting from 0.4 μM, and eight clones (designated C2 and C4 to C10) were adapted to grow in the presence of a 1 μM concentration of the drug, with doubling times of between 26 and 171 h (Fig. 2a). Interestingly, the growth rates of several transformants tested (C4, C5, and C6) were found to be increased substantially (by a factor of about 2) by addition of 50 μg of uridine per ml to the medium. This observation pointed to the occurrence in these transformants of a respiration defect, induced by a mutation or by rotenone action, which affected the pyrimidine synthesis capacity of the cell; in fact, it is known that the activity of dihydroorotate dehydrogenase, a key enzyme in this biosynthetic pathway, depends on a functional respiratory chain (14).

Support for the suggestion of the presence of a respiratory defect in at least some of the original VA_2B mutants was provided by a direct investigation of their respiratory capacity. In fact, a preliminary analysis of some of these mutants (C4, C6, C8, and C10) revealed decreased O_2 consumption (0.5 to 1.2 fmol/min per cell, compared with values of 3.0 to 3.5 fmol/min per cell obtained for VA_2B cells). The residual respiratory capacity of the mutants appeared to be sensitive to 0.1 μM rotenone, as was the respiratory capacity of VA_2B cells (data not shown). By using digitonin-permeabilized cells (13), it was possible to measure the activity of the individual components of the mitochondrial respiratory chain in the rotenone-resistant mutants. Of the eight mutants, six (C2, C4, C5, C6, C8, and C9) showed, surprisingly, a specific reduction of complex I activity, with complexes III and IV being apparently functionally normal (Fig. 3a). Clone C10 showed a decreased activity of all three enzyme complexes of the respiratory chain and was

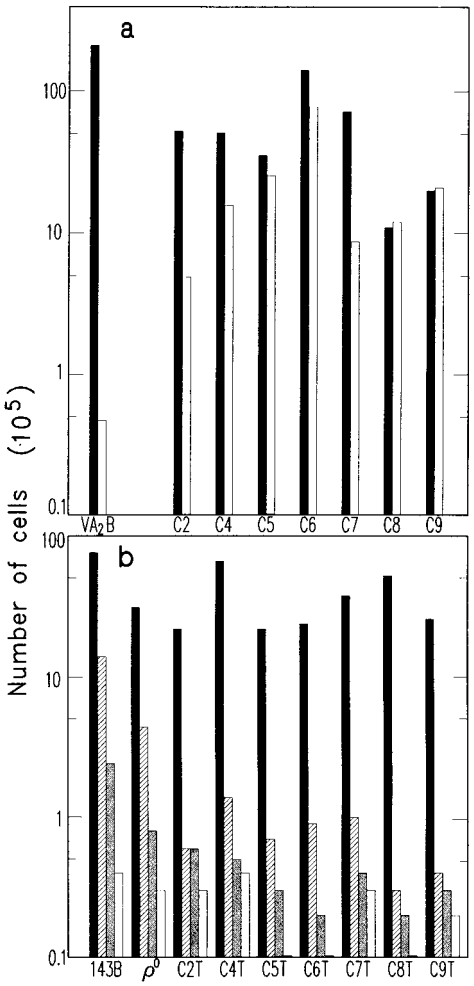

FIG. 2. Growth of VA_2B cells and the indicated rotenone-resistant mutants (a) and of ρ^0 and 143B.TK$^-$ cells and the indicated transformants (b) in the presence of different concentrations of rotenone. The total numbers of cells in plates seeded with 3×10^5 (a) or 2×10^5 (b) cells after 5 days of growth in media containing 0 (■), 0.25 (▨), 0.50 (▩), or 1.0 (□) μM rotenone are shown.

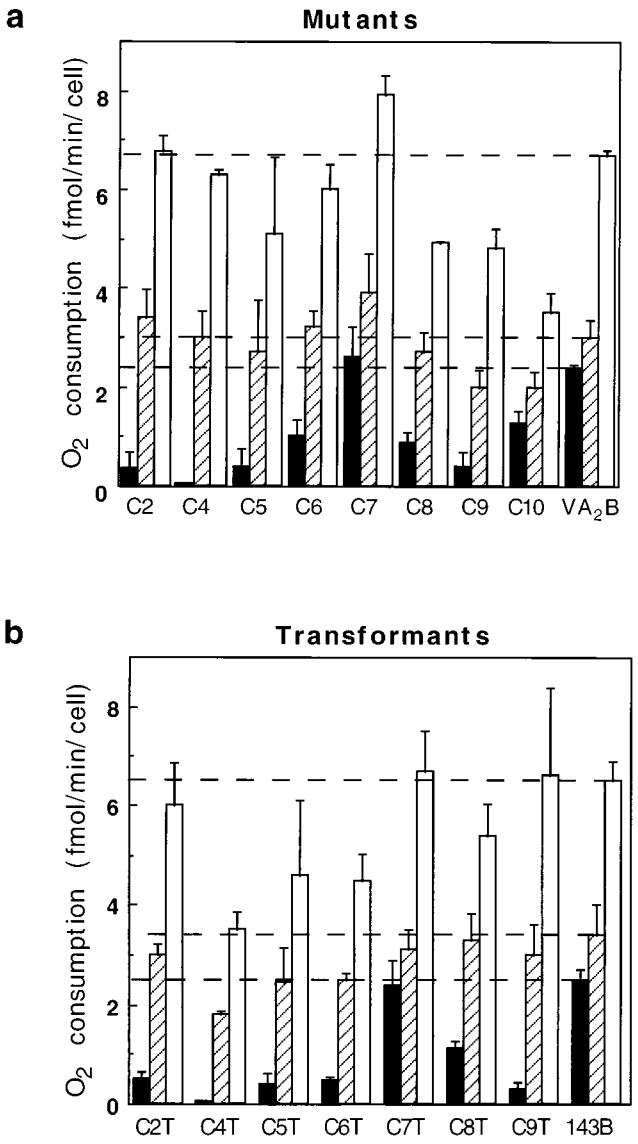

FIG. 3. Activities of the enzymes of the mitochondrial respiratory chain in the mutant clones (a) and the corresponding ρ^0 cell transformants (b). By using $\sim 5 \times 10^6$ digitonin-permeabilized cells, the activities of the various components of the respiratory chain were determined as respiration dependent on malate-glutamate (■), glycero-3-phosphate–succinate (▨), and ascorbate-TMPD (□). Error bars represent 2 standard errors.

excluded from further analysis; clone C7 showed a normal activity of the three enzymes and was utilized as a control.

In order to investigate the genetic origins of the rotenone resistance and of the respiratory defect of the isolated VA_2B variants and the relationship between the two phenomena, advantage was taken of the possibility of repopulating mtDNA-less (ρ^0) 143B.206 cells with exogenous mitochondria (23). This approach, in fact, is a very convenient means of testing whether the rotenone resistance and the respiratory defect of the isolated variants were due to a mutation(s) in a nuclear or a mitochondrial gene(s). By using enucleated cells (cytoplasts), the mitochondria from seven of the mutants (C2 and C4 to C9) were transferred into ρ^0 cells, thus producing transmitochondrial cell lines containing the mtDNA of the original isolates in the constant nuclear background of the ρ^0 cells (23). Surprisingly, it was found that all transformants showed an even greater sensitivity to rotenone in their growth capacity than 143B.TK$^-$ and ρ^0 cells (Fig. 2b). Thus, it was concluded that the drug resistance of the original mutants was due to a nuclear mutation(s).

In contrast to the rotenone resistance, both the decrease in overall respiratory capacity and the specific complex I defect of the six respiration-deficient mutants (C2, C4, C5, C6, C8, and C9) were transferred into ρ^0 cells together with their mtDNA (Fig. 3b). These results were consistent with the idea that a mitochondrial mutation was the cause of the reduction in respiration and complex I activity. This putative mutation did not produce rotenone resistance of complex I, since the residual respiration and complex I activity of the transformants were found to be rotenone sensitive, like those of the original mutants tested. An analysis of the mtDNA contents of the transformants showed that they did not differ significantly (between −12 and +28%) from the 143B.TK$^-$ value, except for the content of C4T, which was ~50% of the 143B.TK$^-$ value (data not shown). It seemed unlikely that a defect in the transport of the substrates or in the activity of the corresponding dehydrogenases was responsible for the low complex I activity, since

TABLE 1. Measurement of NADH:Q_1 and NADH:$Fe(CN)_6$ oxidoreductase activities in mitochondrial membranes isolated from various transformants and VA_2B cells

Cell line	Avg nmol/min/mg (2 SE)[a]		NADH:Q_1/NADH:$Fe(CN)_6$ (10^2)
	NADH:Q_1 oxidoreductase	NADH:$Fe(CN)_6$ oxidoreductase	
VA_2B	7.6 (0)	210 (15)	3.6 (0.3)
C2T	2.1 (0.3)	263 (13)	0.80 (0.12)
C4T	0	281 (35)	0
C5T	0.2 (0)	343 (61)	0.06 (0.01)
C6T	3.9 (0.1)	485 (21)	0.80 (0.04)
C7T	4.9 (0.3)	253 (58)	1.9 (0.5)
C8T	1.5 (0.3)	240 (10)	0.63 (0.13)
C9T	0.3 (0.1)	264 (5)	0.11 (0.04)

[a] Determinations were made in duplicate.

the enzymes involved in substrate transport and the dehydrogenases are nucleus encoded. To verify the above conclusion, the activity of complex I in partially purified mitochondrial membranes was measured, using NADH and a water-soluble ubiquinone analog (Q_1) to circumvent any transport problem (Table 1). The NADH:$K_3Fe(CN)_6$ oxidoreductase activity of the membranes was also measured. The latter reaction is catalyzed by the nucleus-encoded flavoprotein fragment of the enzyme (11). If in mammalian cells the matrix arm of complex I, which includes the flavoprotein fragment, is assembled independently from the membrane arm, as has been shown to be the case for *N. crassa* (35), it seemed likely that the NADH:$K_3Fe(CN)_6$ oxidoreductase activity would not be affected by an mtDNA mutation. This lack of effect of the mutation on the NADH:$K_3Fe(CN)_6$ oxidoreductase activity had indeed been shown previously for the C4T mutation (16) and appeared to be true also for the mtDNA mutations of the other rotenone-resistant clones (Table 1). Thus, this activity could be used to correct for differences in mitochondrial content among the crude mitochondrial membrane preparations isolated from different clones. The corrected activities are shown in Table 1. The results of the spectrophotometric analysis confirm the decreases in complex I activity detected by the polarographic measurements for the transformants.

Protein synthesis defects in complex I-deficient transformants. In order to obtain some indications as to the possible sites of the mutations responsible for the complex I defect in the six mutant cell lines and in the derived transformant cell lines described above, the mitochondrial translation products of the transformants were labeled for 2 h with [^{35}S]methionine in the presence of emetine (to block cytoplasmic protein synthesis). As can be seen in Fig. 4, there was no significant difference in the overall labeling of the mitochondrial translation products between any of the transformants and VA_2B cells. However, several transformants exhibited some differences in the relative labeling of the various mtDNA-encoded polypeptides in comparison with the pattern of the VA_2B cell line. One striking difference was the previously reported complete disappearance of the ND4 gene product in the pattern from the C4T transformant (16), while the labeling of the other mtDNA-encoded complex I subunits of this transformant appeared to be normal. Similarly, there was a specific decrease in the labeling of the ND4 subunit in the C5T transformant. Figure 4 also shows that the translation product corresponding to the ND5 subunit was not detectable in the C9T transformant and was strongly decreased in the C8T transformant. By contrast, transformants C2T and C6T showed no difference from VA_2B cells in the relative labeling or electrophoretic

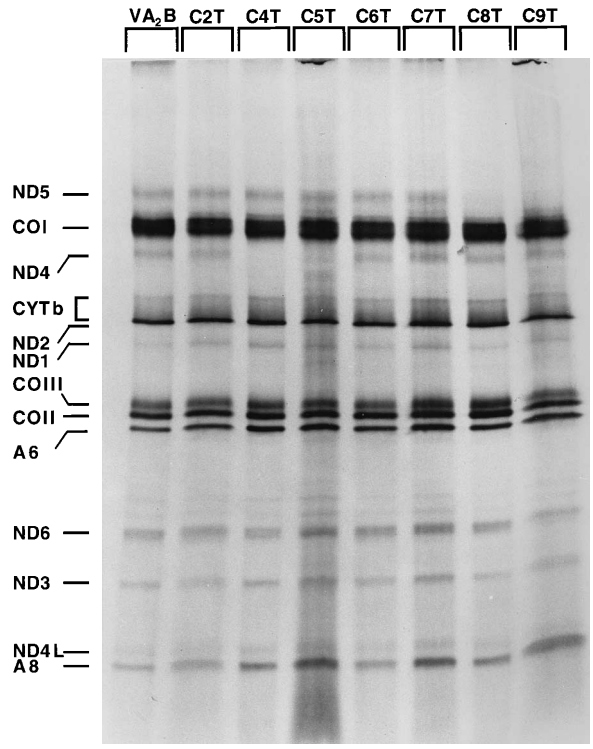

FIG. 4. Electrophoretic patterns of newly synthesized mitochondrial translation products in VA_2B cells and different transformants. Shown are the fluorograms, after polyacrylamide gradient gel electrophoresis, of samples of total cell lysates from VA_2B (48 μg of protein), C2T (94 μg), C4T (26 μg), C5T (100 μg), C6T (81 μg), C7T (78 μg), C8T (44 μg), and C9T (150 μg) cells exposed to [^{35}S]methionine for 2 h in the presence of 100 μg of emetine per ml. The individual mitochondrial translation products were identified according to reference 4. 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 respiratory chain NADH dehydrogenase, respectively; A6 and A8, subunits 6 and 8 of the H^+-ATPase, respectively; CYTb, apocytochrome *b*.

migration of their mitochondrial translation products. Experiments in which the transformants were subjected to short (20- or 40-min) [^{35}S]methionine pulses showed that the rate of labeling of their mitochondrial translation products did not differ significantly from that observed in VA_2B cells, except for a 50 to 60% decrease in the case of the C2T and C9T transformants.

Four complex I-deficient mutants exhibit frameshift mutations in their mtDNA. In previous work (16), by PCR amplification and subsequent sequencing of the mtDNA segment encompassing the ND4 gene, the mutation in the C4T transformant had been identified as an insertion of a cytidine residue in a stretch of six C's at positions 10947 to 10952 (Fig. 5a). As described in the previous paper (16), the insertion causes a frameshift and the creation of a stop codon ~150 bp downstream; this results in the formation of a premature termination product of ~13 kDa, which appears to be rapidly degraded. In the present work, PCR amplification and DNA sequencing analysis showed that the C5T transformant carried the same mutation present in C4T but in a heteroplasmic form (Fig. 5a). This heteroplasmicity produced the appearance of double bands in the region of the sequencing gel downstream of the insertion point (Fig. 5a).

In order to quantify the mutation in the C4T and C5T cell lines, an assay based on allele-specific termination of primer extension was developed (Fig. 1a). A 24-bp-long primer ending

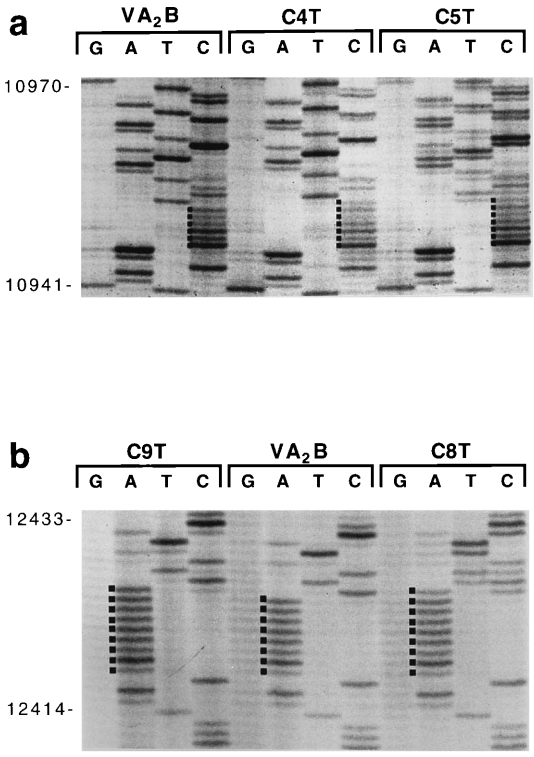

FIG. 5. (a) Portion of the sequence of the ND4 gene from VA_2B, C4T, and C5T cells (between positions 10941 and 10970), showing the insertion of a C residue in the C4T and C5T sequences. The single-stranded PCR product corresponding to the coding (light) strand was used as a template for sequencing by the chain termination method, and the sequence obtained was converted to the light-strand sequence for convenience. (b) Portion of the sequence of the ND5 gene from C9T, VA_2B, and C8T cells (between positions 12411 and 12434), showing the insertion of an A residue in the C9T and C8T sequences.

just at the nucleotide preceding the six C's was synthesized and ^{32}P labeled at the 5′ end. A portion of the ND4 gene (350 bp) encompassing the mutation was amplified by PCR of total DNA samples from VA_2B and various transformants and purified over a 1% agarose gel. The primer was annealed to the PCR product, and the mixture was then incubated with Sequenase. Only dGTP and ddTTP were present in the mixture, so that the enzyme could attach six or seven G residues to the primer and then incorporate ddTTP, which terminated the elongation. This resulted in products that were 31 or 32 bp long, depending on the number of C residues in the template, and that could be separated on a 20% polyacrylamide–6 M urea sequencing gel (Fig. 6a). A control assay using a cloned fragment of mtDNA containing the C stretch showed that the PCR amplification step did not produce detectable amounts of the C insertion. Besides a few artifacts of unknown origin, the control assay with the cloned fragment yielded a clear band corresponding to six C's (Fig. 6a). The correct size of the product was checked by electrophoresis on a sequencing gel (data not shown). VA_2B cells showed no detectable mutant mtDNA, whereas the C4T transformant exhibited no trace of wild-type DNA; by contrast, transformant C5T contained about 34% wild-type DNA. Transformant C6T showed a weak signal corresponding to the wild-type 6-C band, but even after longer exposures of the autoradiogram, no signal corresponding to the 7-C band could be detected.

PCR amplification and subsequent sequencing of an mtDNA segment encompassing the ND5 gene led to the discovery that the mtDNA of C9T carries an insertion in the 5′-end-proximal portion of the ND5 gene. A stretch of eight A's, starting at position 12417 of the mtDNA, was found to be elongated by an additional A in the transformant (Fig. 5b). Again, this insertion causes a frameshift and leads to the formation of a premature termination product of ~6.9 kDa. No band corresponding to such a product was visible in the protein labeling pattern of the C9T transformant (Fig. 4), probably because of its rapid degradation, as observed in a previous study (16) for the premature termination product of transformant C4T. A primer extension assay similar to that described above was used to quantify the mutation, this time using dATP and ddCTP (Fig. 1b). The experiment (Fig. 6b) again showed no detectable mutant mtDNA in VA_2B cells. By contrast, C9T exhibited a very small amount of wild-type mtDNA (~4%); this amount of wild-type mtDNA could account for the residual complex I activity of the transformant, which was estimated to be ~12% of the wild-type activity by polarography and ~3% by enzymatic assays. However, the expression of the very small amount of wild-type mtDNA did not lead to a detectable level of ND5 labeling in the 2-h [^{35}S]methionine pulse-labeling experiment (Fig. 4). The same A insertion in the ND5 gene present in C9T was found in the C8T transformant but in heteroplasmic form (Fig. 5b). This heteroplasmicity was responsible for the appearance of double bands in the region of the sequencing gel downstream of the insertion point. By the primer extension assay, it was determined that C8T contains about 28% wild-type mtDNA (Fig. 6b).

The mtDNA mutations associated with the two remaining complex I-deficient mutants (C2 and C6) have not yet been identified. Sequencing of the ND1 gene of the C6T transformant revealed the presence of three differences from the Cambridge sequence. Two of these differences were synonymous, apparently homoplasmic base substitutions present also in VA_2B and presumably represented mtDNA polymorphisms. The third difference, a G-to-A transition at position 3496 which produced the change of a fairly conserved Ala to an Asn in the protein, was present in heteroplasmic form in the parental mtDNA. The changed amino acid is at the beginning of the second conserved predicted membrane-spanning helix (8), and the additional charge may in principle hinder the appropriate folding of the ND1 subunit. As shown in Fig. 7, this mutation, which causes the disappearance of an *Aci*I site and can therefore be easily detected, was found to occur in heteroplasmic form with predominance of the wild-type gene in VA_2B and C8T and with predominance of the mutant gene in C9T and in homoplasmic form in C2T, C4T, C5T, C6T, and C7T. The mutation does not appear to affect complex I activity, as shown most clearly by its presence in homoplasmic form in C7T, which has a normal complex I activity (Fig. 3b).

Analysis of the assembly of complex I in the transformants. In order to address the question of whether the assembly of complex I is affected in the various transformants analyzed in this study, antibodies against the nucleus-encoded 49-kDa iron-sulfur subunit of the bovine enzyme, which is part of the matrix arm, were used to immunoprecipitate whole complex I (2). In these experiments, only the mtDNA-encoded subunits that were assembled into complex I were expected to be coprecipitated. The mtDNA translation products of several transformants, pregrown for 22 h in the presence of chloramphenicol (to accumulate nucleus-encoded subunits of complex I), were labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide (to inhibit cytosolic protein synthesis) and then chased for 15 h in the absence of inhibitors. The mitochondrial fraction was then isolated from each transformant. One portion of it was utilized to make an SDS lysate for analysis of the

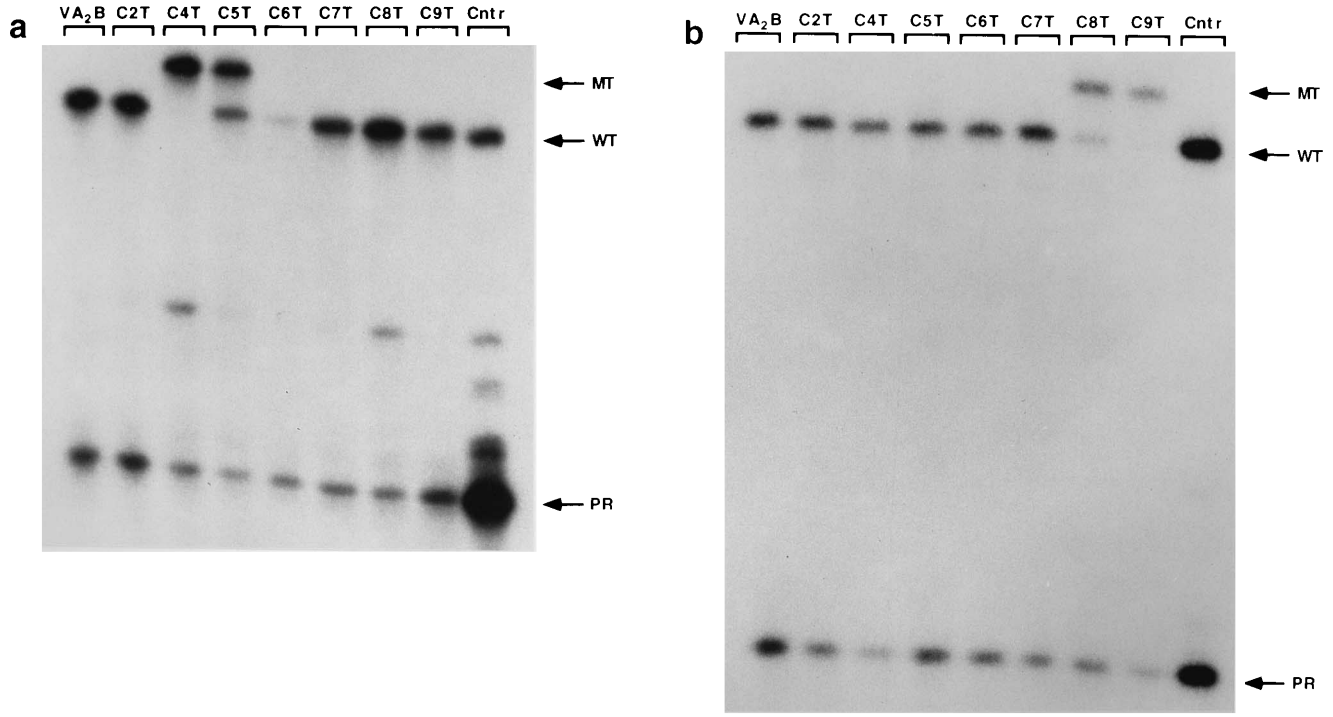

FIG. 6. Results of the quantification of the insertions in the ND4 (a) and ND5 (b) genes in VA_2B cells, in various transformants, and in cloned template DNA (lanes Cntr), carried out by using allele-specific termination of primer extension. The products of analysis were separated on a 20% polyacrylamide–7 M urea sequencing gel. MT, mutant product; WT, wild-type product; PR, unextended primer.

mitochondrial translation products. Another portion was lysed with Triton X-100, and equal samples of it were incubated with gamma globulins from an antiserum against the purified bovine 49-kDa subunit to precipitate the assembled complex I, from an antiserum against human COII to precipitate the assembled cytochrome *c* oxidase complex (26), or from normal rabbit serum. Proteins of the SDS mitochondrial lysate and of the Zysorbin-adsorbed and washed immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and the gels were subjected to fluorography.

As shown in Fig. 8a, the electrophoretic pattern of the SDS mitochondrial lysate from pulse-chased VA_2B cells revealed all the mitochondrial translation products, with a few extra bands in the high-molecular-weight region, representing cytoplasmic proteins labeled during the chase (2). The presence of closely migrating contaminating bands made the recognition of the ND5 subunit in the VA_2B pattern, as well as in the patterns for the transformants, difficult. In the pattern for C4T cells, as previously reported (16), the labeled mtDNA-encoded subunits of complex III, complex IV, and H^+-ATPase were present in the same amount as in the VA_2B pattern; by contrast, several of the mtDNA-encoded subunits of NADH dehydrogenase were apparently absent (ND4) or strongly reduced (ND2, ND6, and ND3), a result which pointed to their instability, presumably resulting from defective assembly. Only the ND1 and ND4L subunits were present in C4T in normal amounts (Fig. 8a). In the pattern from C9T, the ND5 subunit was apparently absent, as expected, while the ND4, ND2, ND1, ND3, and ND4L subunits were present in comparable or only slightly decreased amounts relative to those in the VA_2B pattern. Only the ND6 subunit appeared to be significantly decreased (Fig. 8a). In the C6T pattern, there was a general decrease in the labeling of the mitochondrial translation products to about one-third of the levels observed in VA_2B cells and the other transformants, but the relative labeling of the various mtDNA-encoded subunits of NADH dehydrogenase seemed to be comparable to that observed in the VA_2B pattern (Fig. 8a). A repeat of the pulse-chase experiment confirmed the reduction in the labeling of the mitochondrially synthesized polypeptides in this transformant. Since, as mentioned above, the labeling of the mitochondrial translation products in C6T

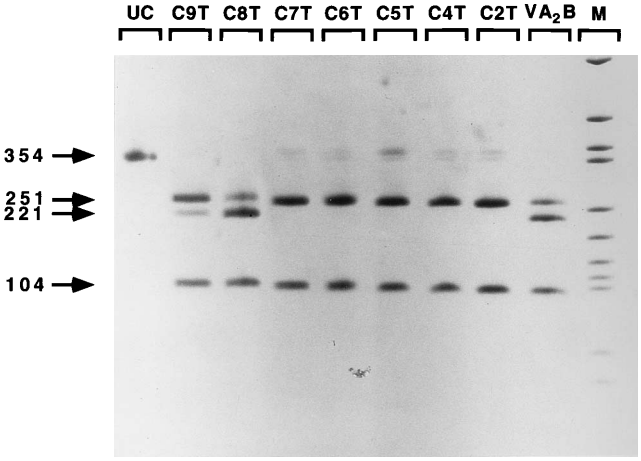

FIG. 7. Detection of ND1 gene mutation at position 3496 in total DNA samples from VA_2B cells and various transformants. The sizes of the uncut (UC) PCR product (354 bp) and of the three fragments produced by *Aci*I digestion of the VA_2B PCR product (251, 221, and 104 bp) are indicated. M, molecular weight marker (*Msp*I-digested pBluescript KS+ DNA; fragment sizes: 710, 489, 404, 367, 242, 190, 147, 118, 110, 67, and 57 bp).

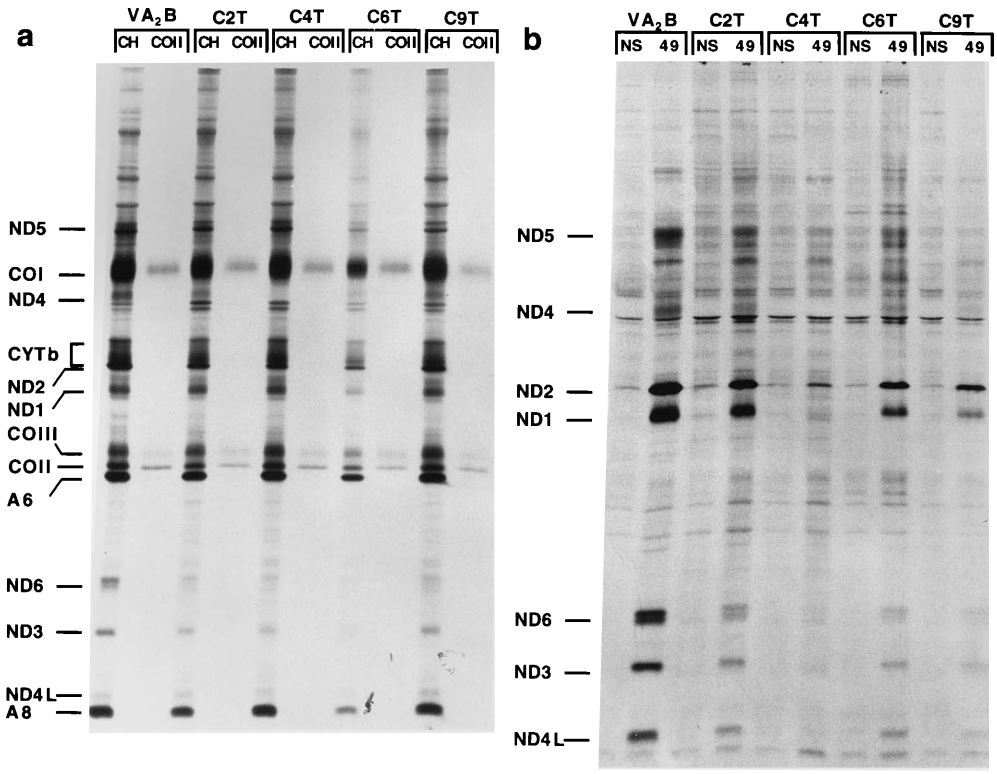

FIG. 8. (a) Electrophoretic patterns of samples of SDS mitochondrial lysates of [^{35}S]methionine-pulse-chased VA_2B, C2T, C4T, C6T, and C9T cells (lanes CH) and of immunoprecipitates obtained by incubating samples of 0.5% Triton X-100 mitochondrial lysates of the pulse-chased cells with gamma globulins from an antiserum against the human COII subunit (lanes COII). (b) Electrophoretic patterns of immunoprecipitates obtained by incubating samples of 0.5% Triton X-100 mitochondrial lysates of [^{35}S]methionine-pulse-chased VA_2B, C2T, C4T, C6T, and C9T cells with gamma globulins from an antiserum against the bovine 49-kDa subunit (lanes 49) (2) or from normal rabbit serum (lanes NS). See Materials and Methods for details.

after a short pulse or a 2-h [^{35}S]methionine pulse was not significantly different from that observed in VA_2B cells, it seems likely that the decreased protein labeling in the pulse-chased cells reflected a lesser stability of the mitochondrial translation products, possibly due to the particular nuclear background of this transformant. To correct for this difference in labeling, an approximately threefold-greater amount of the C6T lysate, compared with the other lysates, was used in the immunoprecipitation experiments.

A control experiment in which the whole cytochrome *c* oxidase was precipitated from Triton X-100 mitochondrial lysates with antibodies against the C-terminal undecapeptide of subunit II of the human enzyme (26) showed no significant difference in extent of enzyme complex immunoprecipitation between the lysate from VA_2B cells and the lysates from the transformants, as judged from the intensities of the COI, COII, and COIII bands (Fig. 8a). The anti-49-kDa-subunit antibodies coprecipitated from the VA_2B Triton X-100 mitochondrial lysate all mtDNA-encoded subunits of complex I (Fig. 8b); the electrophoretic pattern of the immunoprecipitate exhibited a small amount of spurious bands, most of which were, however, present also in the precipitate obtained with the normal-serum-treated lysate. The immunoprecipitates from the C2T and C6T transformants exhibited all the mtDNA-encoded subunits of complex I, although in reduced amounts compared with those of the VA_2B pattern (Fig. 8b). In contrast, as reported earlier (16), the immunoprecipitate obtained with the C4T Triton X-100 mitochondrial lysate completely lacked the ND4 subunit and contained only trace amounts of the other mtDNA-encoded subunits (Fig. 8b).

The immunoprecipitate from the C9T transformant exhibited a different pattern, characterized by the apparent absence of the ND5 subunit above the background level present in the normal-serum control (Fig. 8b). The remaining mtDNA-encoded complex I subunits were present in the immunoprecipitate (the ND1 and ND2 subunits being relatively abundant), but they appeared to be significantly reduced in amount in comparison with those in the VA_2B pattern. The above observations strongly suggest that the lack of the ND5 subunit does not prevent the assembly of the membrane arm of complex I but reduces the efficiency of this process or affects the stability of the complex in Triton X-100.

DISCUSSION

The main conclusion of this study is that selection for resistance to high concentrations of rotenone, a specific inhibitor of respiratory NADH dehydrogenase, can be an efficient means for isolating from the human cell line VA_2B mutants affected in any of the mtDNA-encoded subunits of the first respiratory enzyme. Originally devised as an approach for the isolation of mutants affected in the ND1 gene, which encodes the complex I subunit that binds rotenone (6), the selection scheme used here yielded, surprisingly, a variety of mutants altered in different subunits of the enzyme. Considering the fact that six of eight mutants showed an mtDNA-encoded complex I defect, it is clear that this system will prove invaluable for the genetic dissection of complex I and for understanding the still-elusive functional role of the mtDNA-encoded subunits of this important enzyme. This is particularly significant in view of the in-

creasingly clear involvement of complex I in human diseases (32). Furthermore, the present technology for transferring mtDNA mutations into ρ^0 cells and the availability of null mutants lacking one of the mtDNA-encoded subunits of complex I open exciting possibilities for investigation.

Possible mechanism of selection. The lack of transfer into ρ^0 cells of the rotenone resistance together with the mitochondria from the drug-resistant mutants implied that a nuclear mutation was responsible for the cell resistance to high concentrations of rotenone. The VA_2B cells used for selection had not been mutagenized, and one must therefore assume that the initial nuclear mutation producing resistance to 0.4 μM rotenone preexisted in the VA_2B cell population. The observation that even ρ^0 cells, which lack a respiratory chain, were sensitive to the high concentrations (0.4 to 1 μM) of rotenone used for the isolation of drug-resistant mutants indicated that the drug affected another vital cell activity besides respiratory function. As to the nature of the nuclear mutation that conferred resistance to high concentrations of rotenone upon the original VA_2B mutants investigated in this study, considering the chemical structure of this drug, it seems possible that it involved an overexpression and/or amplification of the genes encoding the P-glycoproteins, the cell membrane-associated, energy-dependent drug efflux pumps which are responsible for the multi-drug resistance phenotype (7).

In light of the observations discussed above, the respiration defects transmissible with mitochondria into ρ^0 cells which were detected in nearly all the rotenone-resistant mutants investigated in this study must have resulted from a selection of mtDNA mutations conferring respiration deficiency during the prolonged exposure of the cells to high concentrations of rotenone. It seems reasonable to assume that, in the presence of the increasingly high rotenone concentrations used for selection, relatively small amounts of the drug may have accumulated in the cells, approaching or surpassing the 0.1 or 0.2 μM level, which was shown to be sufficient to inhibit complex I activity and partially affect cell growth. Under these conditions, any mtDNA mutation which had made a cell respiration deficient, with an accompanying adaptation to exclusive or predominant glycolytic ATP production (5), would have also made that cell relatively respiration independent and provided it with a growth advantage, even if slight. The single clone, among the eight isolated, that exhibited normal respiration and normal complex I activity may have represented a case in which a nuclear mutation producing a higher level of resistance to rotenone prevented the accumulation in the cell of damaging concentrations of the drug. A possible mechanism for the establishment of an mtDNA mutation in the rotenone-resistant clones might have involved a replicative advantage of the mutated mtDNA molecules, such as that recently described for mtDNA carrying the mutation associated with MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode syndrome) (39). In the absence of rotenone selection, such hyperreplication of the mutated mtDNA would not be favored, because intercellular selection would tend to eliminate the cells more severely compromised in their respiratory activity by the mutation. By contrast, in the presence of rotenone, which strongly reduces the respiratory activity in all cells, the cells with amplified mutant molecules would not be selected against but would, on the contrary, be selected for because of the progressive adaptation to glycolytic metabolism accompanying the mutation amplification.

In regard to the origin of the mtDNA mutations, it is likely that, like the mutation conferring rotenone resistance, they existed in the VA_2B cell population prior to the rotenone selection. The evidence concerning the occurrence of two distinct mtDNA frameshift mutations, each found in two independent clones, supports this conclusion, and this probably also applies to the mtDNA mutations of C2T and C6T which have not yet been identified. The $ND1_{3496}$ mutation detected in the present work provides a clear example of a mutation preexisting in heteroplasmic form in VA_2B cells which subsequently shifted towards homoplasmy to a variable extent in different rotenone-resistant clones. However, in this case, the shift may have been caused by random segregation, since there was no evidence of a deleterious effect of this mutation on the cell respiration capacity. It should be mentioned that in a recently completed study, in which single mitochondria from HeLa cells were microinjected into $\rho^0$206 cells, about 30% of the transformants were shown to be respiration deficient because of mtDNA mutations in complex I subunits (1a). This work strongly suggests that untreated HeLa cells carry mtDNA mutations in genes encoding subunits of complex I. It is a reasonable hypothesis that mutations of mtDNA arise continuously during the growth of established cell lines and that the mutations may be either diluted out or selected for if appropriate growth conditions are applied. Furthermore, it can be expected that treatment of the cells with mutagens prior to rotenone treatment would increase the frequency of isolation of desired mtDNA mutations affecting subunits of complex I. Obviously, more work is needed to understand the selection mechanism at work in the VA_2B system studied here, as well as to determine whether the same mechanism operates in other established cell lines.

Six of eight rotenone-resistant mutants exhibit a specific mtDNA-linked complex I defect. Polarographic analysis of the seven rotenone-resistant, respiration-deficient mutants isolated here revealed, surprisingly, that six of them exhibited a specific complex I defect. Furthermore, the transfer of this defect with mitochondria into ρ^0 cells clearly indicated that an mtDNA mutation underlay the enzyme deficiency. According to the selection mechanism proposed above, any mtDNA mutation affecting a subunit of complex III or IV so as to compromise respiration should have also been selected for. A possible explanation for the preferential targeting by mutations of the genes for complex I subunits in the mutants analyzed here is that deleterious mutations in any subunit of complex III or IV or in any mtDNA-encoded component of the mitochondrial translation apparatus would have compromised the cell capacity for pyrimidine biosynthesis because of the dependence of dihydroorotate dehydrogenase activity on a functional respiratory chain downstream of NADH dehydrogenase (14). It thus appears that, under the conditions used in the present work, complex I activity is not indispensable for the growth of the cells.

The measurements of NADH:Q_1 oxidoreductase activity in the partially purified mitochondrial membranes from the ρ^0 cell transformants derived from the rotenone-resistant mutants investigated in this study gave results which were reasonably consistent with the polarographic data. As previously observed for the transformant derived from the C4 mutant, lacking the ND4 subunit (16), the NADH:$K_3Fe(CN)_6$ oxidoreductase activity was unaffected in the transformants derived from the five other complex I-deficient mutants, pointing to the normal assembly and association with the membrane of the nucleus-encoded flavoprotein fragment (11). The mitochondrial membranes from transformant C6T exhibited an unusually high NADH:$K_3Fe(CN)_6$ oxidoreductase activity and a higher NADH:Q_1 oxidoreductase activity than expected from the polarography data; it is possible that this result reflects a higher mitochondrial content in the C6T membrane preparation.

Frameshifts in mtDNA of complex I-deficient mutants. A remarkable observation made in the present work is that four of the six complex I-deficient mtDNA mutants investigated exhibited frameshifts in the DNA that created stop codons downstream and caused the formation of unstable, prematurely terminated translation products. As discussed above, there is reason to believe that the frameshift mutations preexisted in the VA_2B cell population, although in very small amounts not detectable in our assays. In human mtDNA, there are 35 homopolymeric sequences of six or more identical residues (1). These homopolymeric stretches could be hot spots for frameshifts because of stuttering by the DNA polymerase. It seems very likely that, during the long rotenone selection, there was a shift towards homoplasmy of the ND4 and ND5 frameshift mutations detected in the present work, either because of a growth advantage of cells with higher levels of frameshifts or because of a replicative advantage of frameshifted molecules, as discussed above. This process resulted in a homoplasmic or near-homoplasmic genotype in C4T and C9T and a heteroplasmic genotype in C5T and C8T.

Frameshift mutations in a run of five T residues leading to premature termination of translation have been previously described for the *Saccharomyces cerevisiae* mitochondrial gene encoding subunit COII of cytochrome *c* oxidase (9). By contrast, it is intriguing that among the mtDNA mutations in protein-coding genes associated with diseases in humans, no frameshift has been described so far (32).

Functional effects of the ND4 and ND5 frameshift mutations. There is considerable evidence to support the conclusion that the ND4 and ND5 gene frameshifts were responsible for the complex I defect detected in the C4T and C9T transformants, respectively. Although one cannot absolutely exclude the presence of deleterious mutations in mtDNA genes encoding other subunits of NADH dehydrogenase in these transformants, the presence of these subunits in normal relative amounts and with the expected electrophoretic mobilities in the protein labeling patterns, after short and long pulses, of C4T and C9T makes the lack of the ND4 and ND5 gene products, respectively, the most plausible cause of the complex I defect. Also, the occurrence of the ND4 and ND5 gene frameshifts in heteroplasmic form in C5T and C8T, respectively, which exhibited residual complex I activities of 25 and 46% and a reasonably proportional wild-type gene content (34 and 28%, respectively), is consistent with the exclusive pathogenetic role of these frameshifts. It should be noted that neither frameshift mutation was detectable in VA_2B cells or in the five other transformant clones analyzed. These observations, as well as the apparent absence so far of deleterious mutations affecting complexes III and IV, indicate that the occurrence of mtDNA mutations causing a defective phenotype was relatively rare in the transformants analyzed here. This is consistent with the low overall frequency of mtDNA mutations producing respiration defects which were detected in the present work, as well as in a previous screening of VA_2B cells for the respiration-deficient phenotype (36). Therefore, it seems justified to conclude that the probability that any one of the mutants analyzed here, in particular, any one of the frameshift mutants, carries a second deleterious mutation is very low.

The total lack of complex I activity and the failure of the mtDNA-encoded subunits of this enzyme to assemble in the C4T transformant, which completely lacks the ND4 subunit, had been previously described (16). A new, significant result reported here is the substantial lack of complex I activity in the transformant C9T, which exhibited a near-complete absence of the ND5 subunit. This is the first evidence reported that indicates that the ND5 subunit is essential for complex I activity in mammalian cells. In contrast to the situation observed with the C4T transformant, antibodies against the 49-kDa subunit precipitated from a C9T mitochondrial lysate all mtDNA-encoded subunits, except ND5, although in reduced amounts, compared with those from VA_2B, C2T, and C6T. Thus, the absence of the ND5 subunit did not prevent the assembly of the remaining mtDNA-encoded subunits. The reduced level of immunoprecipitation of these subunits from the C9T lysate may indicate a lower efficiency of assembly or an instability of the membrane arm of the enzyme in the absence of the ND5 subunit in the C9T transformant.

The immunoprecipitate obtained from the C2T and C6T mitochondrial lysates exhibited all the mtDNA-encoded subunits, including ND4 and ND5, indicating that the mutation causing the complex I defect did not affect the assembly of the membrane arm of the enzyme in these transformants.

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