*Infect. Immun.* **33**, 820 (1981).
19. A. Bartoloni *et al.*, *Biotechnology* **6**, 709 (1988).
20. M. T. De Magistris, M. Romano, A. Bartoloni, R. Rappuoli, A. Tagliabue, *J. Exp. Med.* **169**, 1519 (1989).
21. M. Mariani *et al.*, *J. Immunol. Methods* **92**, 189 (1986); M. Mariani *et al.*, *ibid.* **71**, 43 (1984); G. Antoni and M. Mariani, *ibid.* **61**, 61 (1985).

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# Human Cells Lacking mtDNA: Repopulation with Exogenous Mitochondria by Complementation

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**Two human cell lines (termed $\rho^0$), which had been completely depleted of mitochondrial DNA (mtDNA) by long-term exposure to ethidium bromide, were found to be dependent on uridine and pyruvate for growth because of the absence of a functional respiratory chain. Loss of either of these two metabolic requirements was used as a selectable marker for the repopulation of $\rho^0$ cells with exogenous mitochondria by complementation. Transformants obtained with various mitochondrial donors exhibited a respiratory phenotype that was in most cases distinct from that of the $\rho^0$ parent or the donor, indicating that the genotypes of the mitochondrial and nuclear genomes as well as their specific interactions play a role in the respiratory competence of a cell.**

MITOCHONDRIAL BIOGENESIS IS under the control of both the nuclear and the mitochondrial genome (*1*). The replication and transcription of mtDNA and the translation of mtDNA-coded mRNAs require components encoded in the nucleus, which must be imported into the organelles. Most of the enzymes of the respiratory chain are oligomeric complexes consisting of both nuclear DNA–coded and mtDNA-coded subunits. Study of these nuclear-mitochondrial interactions would be facilitated if it were possible to manipulate the mtDNA complement of a cell, move mitochondria from one cellular environment to another, or introduce new genes into mitochondria.

We have isolated two derivatives of the human cell line 143B.TK$^-$, which had been entirely depleted of mtDNA by long-term exposure to low concentrations of ethidium bromide (*2*). DNA transfer hybridization analysis of total DNA from these cell lines, designated 143B101 and 143B206 (or $\rho^0$101 and $\rho^0$206), did not reveal the expected mtDNA restriction fragments, under conditions in which much less than one molecule per cell would have been detected (*2*). These $\rho^0$ cells rely exclusively on glycolysis for their energy requirements, and, as previously shown for $\rho^0$ avian cells (*3*), have become pyrimidine auxotrophs because of the deficiency of the respiratory chain–dependent dihydroorotate dehydrogenase (*4*). Unexpectedly, these $\rho^0$ cells have also become pyruvate-dependent (*2*). Complementation of either of these metabolic requirements was used as a selectable marker for the repopulation of $\rho^0$ cells with exogenous functional mtDNA.

The $\rho^0$ cell line 143B206 undergoes less than one population doubling in the absence of uridine, whereas its growth rate closely approaches that of the parental line in the presence of uridine at 50 μg/ml (Fig. 1A). The growth rate of the 143B parental line is not affected by the absence of uridine. When $5 \times 10^6$ 143B206 cells were grown in the absence of uridine, no colony appeared even after 10 weeks of selection in culture. These $\rho^0$ cells have a similar dependence on pyruvate [a normal component of the Dulbecco modified Eagle's medium (DMEM)] for growth, undergoing less than one population doubling in the absence of pyruvate (Fig. 1B). In the presence of pyruvate at 100 μg/ml, the growth rate of this cell line is similar to that of the parental line, whose growth is not affected by the absence of pyruvate. An identical dependence on uridine and pyruvate was observed with the 143B101 cell line (*2*).

We used the two $\rho^0$ cell lines for mitochondrial transformation studies. Initially, mitochondria were transferred by fusion of cytoplasts with $\rho^0$ cells. Cybrids were formed by fusion of cytoplasts from HT1080-6TG human cells (enHT1080) (*5*) with $\rho^0$101 or $\rho^0$206 cells and replated in medium containing bromodeoxyuridine (BrdU) and lacking either pyruvate or uridine. These media permitted only the growth of $\rho^0$ cells that had fused with cytoplasts containing functional mitochondria. Growth of presumptive transformants was observed within 3 to 4 days after cell fusion, and colonies were picked (with a

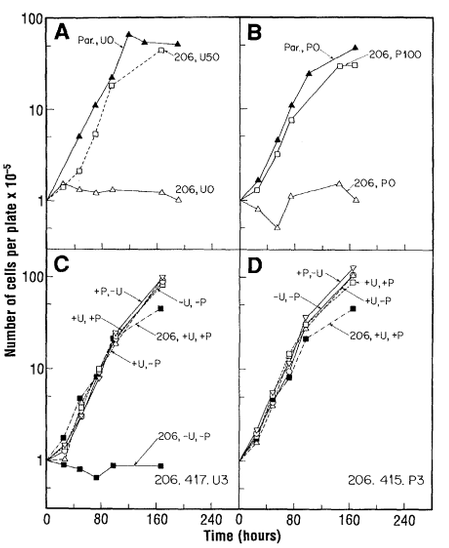


**Fig. 1.** Growth of (**A** and **B**) 143B.TK$^-$ (Par) cells or 143B206 ($\rho^0$206) cells and (**C**) transformants 206.417.U3 and (**D**) transformant 206.415.P3 in the presence or absence of uridine (U) or pyruvate (P). (A and B) Multiple series of 10-cm plastic petri dishes were seeded each with a constant number of cells in (A) 10 ml of normal DMEM supplemented with 5% dialyzed fetal bovine serum (FBS), BrdU at 100 μg/ml, and uridine (U) at 0 or 50 μg/ml, or in (B) 10 ml of pyruvate-deficient DMEM supplemented with 5% dialyzed FBS, BrdU at 100 μg/ml, uridine at 50 μg/ml, and pyruvate (P) at 0 or 100 μg/ml. At various time intervals, cells from individual plates were trypsinized and counted. (C and D) Growth curves of the recipient $\rho^0$206 cells and of the transmitochondrial cell lines were determined as described above. The growth medium was normal DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μg/ml (+P,−U), or pyruvate-deficient DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μg/ml (−U,−P), or the latter medium plus uridine at 50 μg/ml (+U,−P), or the same medium plus uridine at 50 μg/ml and pyruvate at 110 μg/ml (+U,+P). The transformants had been maintained in their original selective media for 5 weeks (206.415) and 7 weeks (206.417) after fusion. The transmitochondrial derivatives were obtained by fusing 143B206 cells with cytoplasts from HT1080-6TG. For preparation of cytoplasts, HT1080-6TG cells were plated on 35-mm dishes at ~$2 \times 10^5$ cells per dish. After ~24 hours, cells were enucleated while attached to the plate (*15*). The cytoplasts were fused as a monolayer with $3 \times 10^5$ to $8 \times 10^5$ 143B206 cells (*16*), and incubated in DMEM supplemented with 5% FBS and uridine at 50 μg/ml. The cells were replated 1 to 3 days after fusion and placed in selective medium. Selective uridine-free medium consisted of DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μg/ml, and selective pyruvate-free medium consisted of DMEM lacking pyruvate supplemented with 5% dialyzed FBS, uridine at 50 μg/ml, and BrdU at 100 μg/ml.

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glass ring) 11 to 15 days after fusion.

It was previously shown that microinjection of a single mitochondrion could replace a resident human mtDNA population in relatively few cell generations (*6*). Hence, isolated mitochondria from HeLa S3 (*7*) or HeLaBU25 10B3R (*8*) or CAP23 cells [a chloramphenicol (CAP)-resistant derivative of the $VA_2B$ cell line (*9*)] were injected into $\rho^0$206 cells, and transformants were selected in the absence of uridine. Colonies were first observed between 10 and 30 days after injection. The efficiency of this transformation was estimated to be $\sim 1 \times 10^{-3}$ to $3 \times 10^{-3}$ per injected cell.

Total DNA was isolated from four independent $\rho^0$101 × enHT1080 cybrids and four independent $\rho^0$206 × enHT1080 cybrids, selected for either uridine- or pyruvate-independent growth. Analysis of the DNA from all transformants revealed both the Hae II and Hae III markers characteristic of HT1080 mtDNA and the pYNH24 variable-number tandem repeat marker characteristic of the 143B nuclear DNA (*6*). Total DNA was also isolated from three $\rho^0$206 transformants injected with HeLa S3 mitochondria, from four $\rho^0$206 transformants injected with CAP23 mitochondria, and from four $\rho^0$206 transformants injected with HeLaBU25 10B3R mitochondria. Analysis of the DNA from these transformants showed both the Hae II and Hae III markers characteristic of the HeLa S3, CAP23, and HeLaBU25 10B3R donor mtDNA and the pYNH24 marker characteristic of the 143B nuclear DNA. The hybridization pattern of representative transmitochondrial cell lines probed with mtDNA-specific probes (Fig. 2, A and B) or with the nuclear DNA–specific probe (Fig. 2, C and D) are shown.

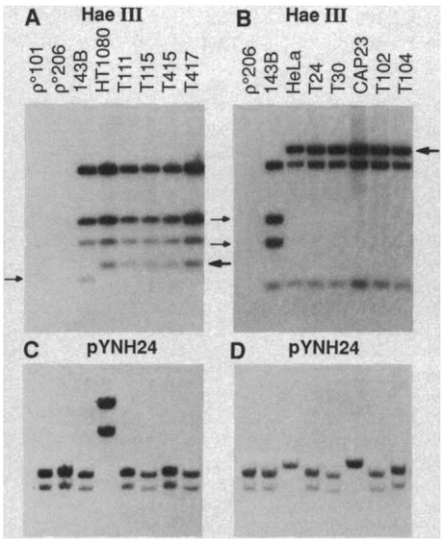


**Fig. 2.** Mitochondrial transformants contain the exogenous mtDNA and 143B.TK$^-$ nuclear DNA. Autoradiograms of blots of restriction digests of total DNA from the 143B.TK$^-$ parental cell line, its derivatives 143B101 and 143B206, the mitochondrial donor cell lines HeLa S3, CAP23, and HT1080-6TG, and various transformants of 143B101 and 143B206, hybridized with (A and B) mtDNA and (C and D) nuclear DNA probes. (**A** and **C**) The transmitochondrial derivatives of 143B101 and 143B206 analyzed here were obtained by fusing the $\rho^0$ cells with cytoplasts from HT1080-6TG as described in the legend of Fig. 1. Transformants 101.111.P2 (T111) and 206.415.P3 (T415) selected in pyruvate-free medium and transformants 101.115.U3 (T115) and 206.417.U3 (T417) were selected in uridine-free medium. (**B** and **D**) The 143B206 transformants analyzed here were obtained by injection of mitochondria from HeLa S3 cells [206.24 (T24) and 206.30 (T30)] or CAP23 cells [206.102 (T102) and 206.104 (T104)] into 143B206 cells, followed by selection in uridine-free medium. Mitochondria were isolated and injected as described (*6*). Transformants obtained by injection of HeLa S3 or CAP23 mitochondria were subcloned 4 to 5 weeks after injection. Samples of total DNA from the transformants and from the parental cell lines were digested (A and B) with Hae III and (C and D) with MsP I and, after electrophoresis and electroblotting, were probed (A and B) with mp8.M9 and mp8.M6 (*6*) or (C and D) with the nuclear variable number tandem repeat marker pYNH24 (*17*).

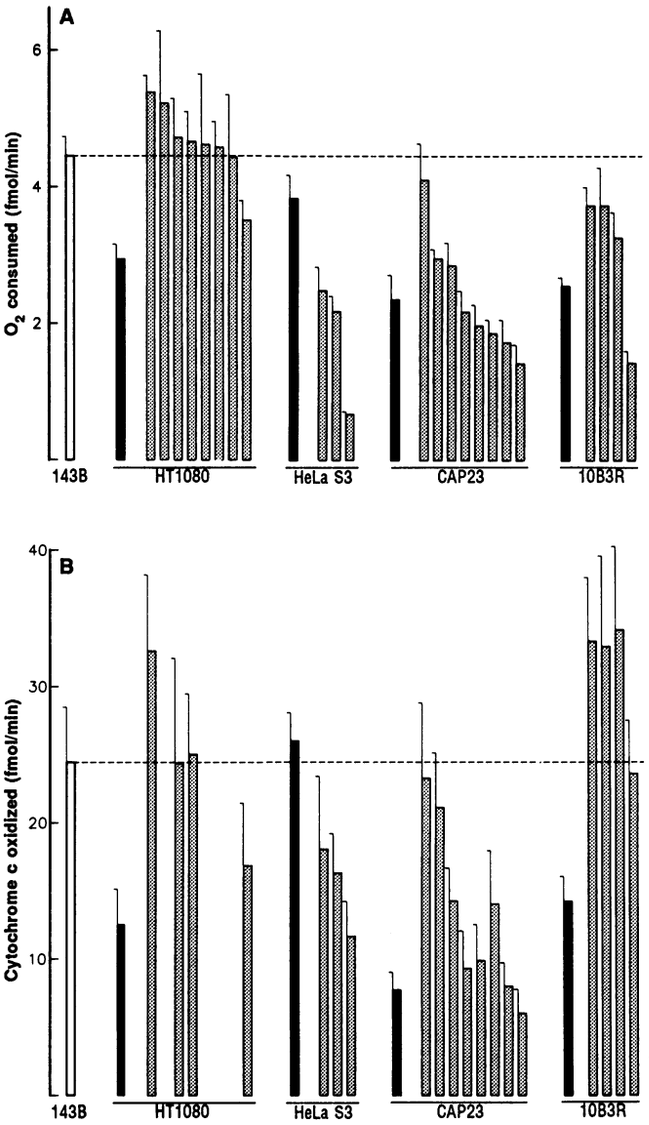


**Fig. 3.** Histogram of (**A**) the rates of oxygen consumption per cell and (**B**) the levels of cytochrome c oxidase activity per $10^4$ mtDNA molecules of 143B.TK$^-$ (open bars), the mitochondrial donors (solid bars) HT1080-6TG, HeLa S3, CAP23, and HeLaBU25 10B3R, and the transmitochondrial cell lines (shaded bars). The rates of oxygen consumption per cell and levels of cytochrome c oxidase per $10^4$ mtDNA molecules are shown with error bars representing two standard errors. The cell lines are listed below in the order in which they appear in the histogram. After each cell line in parentheses is the number of determinations of oxygen consumption, the number of weeks after fusion or after injection that this was determined, then the number of determinations of cytochrome c oxidase activity, and the number of weeks after fusion or after injection that this was determined. 143B.TK$^-$ (13, -; 10, -), HT1080-6TG (13, -; 9, -), 206.417.U3 (6, 8; 5, 6), 206.412.P4 (2, 8; ND), 101.111.P2 (5, 7; 5, 6), 206.415.P3 (5, 8; 5, 6), 206.416.U3 (2, 8; ND), 101.116.U3 (2, 7; ND), 101.121.P2 (3, 7; ND), 101.115.U3 (5, 7; 5, 6), HeLa S3 (8, -; 8, -), 206.24.2 (7, 15–20; 4, 18), 206.30.1 (7, 15–20; 5, 18), 206.7.1 (4, 9; 3, 10), CAP23 (7, -; 9, -), 206.105.3 (4, 12; 2, 12), 206.113.4 (4, 11; 2, 11), 4.22.66 (5, 7; 2, 7), 4.21.57 (7, 9; 2, 9), 4.21.63 (4, 7; 3, 7), 4.29.104 (4, 7; 3, 7), 206.102.3 (4, 15–19; 5, 18), 206.104.1 (4, 15–19; 5, 18), HeLaBU25 10B3R (6, -; 10, -), 206-227 (7, 10; 2, 10), 206-230 (4, 10; 5, 10), 206-211 (5, 13; 5, 13), 206-217 (6, 13; 2, 13). Consumption of $O_2$ was determined on samples of $5 \times 10^6$ cells in 1.85 ml of DMEM lacking glucose, supplemented with 5% dialyzed FBS at 37°C (Gilson 5/6 oxygraph). Cytochrome c oxidase activity was determined as described (*2*). The mtDNA concentration of the relevant samples was determined by dot blot analyses as described in the legend of Table 1, and standards were prepared with closed circular HeLa mtDNA mixed with 143B206 mitochondria. All CAP-resistant cell lines were grown for at least 1 week in the absence of CAP before analyses.

The kinetics of mtDNA repopulation of $\rho^0$206 cells transformed by injection of mitochondria from HeLa S3 or CAP23 cells was analyzed by dot blot hybridization of total DNA isolated from six transformants at various times after injection. As previously observed (*6*), injection of single mitochondria into $\rho^0$ cells resulted in the restoration of a full complement of mtDNA in fewer than 35 generations. Thus the normal constraints limiting the rate of mtDNA replication to an average of one replication cycle per mtDNA molecule per cell generation were not operating in the microinjected $\rho^0$ cells, a phenomenon presumably linked to their reduced mtDNA complements.

The mtDNA content per cell was determined by dot blot hybridization for each of the transmitochondrial cell lines, as well as for the four donor cell lines and 143B (Table 1) (*10*). We included four cell lines (4.21.57, 4.21.63, 4.22.66, and 4.29.104) that were obtained by injection of CAP23 mitochondria into CAP-sensitive 143B cells and selection for CAP resistance (*6*). The ranges of values for mtDNA observed for the various sets of transformants were not significantly different from each other or from the parental mtDNA complements. The cell size of the transmitochondrial lines was quite uniform (Table 1); nearly all the transformants had cell sizes averaging 5.0 to 7.0 pl, values quite similar to those of the $\rho^0$ cells.

Comparison of the growth properties of two representative cybrids formed between $\rho^0$206 cells and HT1080 cytoplasts with the $\rho^0$206 cell line shows that transformant 206.417.U3, selected for uridine independence, could grow not only in medium lacking uridine but also in medium lacking pyruvate, or both, in contrast to the $\rho^0$206 cell line (Fig. 1C). Similarly, transformant 206.415.P3, selected for pyruvate independence, could grow in medium lacking pyruvate or uridine, or both (Fig. 1D).

The population doubling times of $\rho^0$101 × enHT1080 or $\rho^0$206 × enHT1080 cybrids were significantly shorter than that of the corresponding $\rho^0$ parent (Table 1), being comparable to the doubling times of those of the 143B cells and of the HT1080 mitochondrial donor cells. The doubling times of the transformants obtained by injection of HeLa S3 or CAP23 mitochondria into $\rho^0$206 cells were similar to those of the $\rho^0$206 parent and of CAP23 cells, but longer than that of HeLa S3 cells (Table 1).

The respiratory competence of the transmitochondrial and parental cell lines was assessed by measuring the $O_2$ consumption by intact cells and by the activity of cytochrome c oxidase (COX) in mitochondria isolated from cells. The $O_2$ consumption of the two $\rho^0$ cell lines was less than 5% of that of the parental 143B line. No COX activity was detected in the $\rho^0$ mitochondria. The four cell lines used as mitochondrial donors and the 143B cell line exhibited considerable differences in respiratory capacity (Fig. 3).

The various transmitochondrial cell lines also displayed a comparable broad range of respiratory capacities. Cell lines derived from the same mitochondrial donor exhibited similar levels of respiratory competence, although within each group certain individual lines deviated significantly from the average (Fig. 3). However, groups of transformants obtained with different mitochondrial donors differed in their respiratory phenotype. The respiratory capacities of the transformants did not simply reflect those of the corresponding mitochondrial donors or of the parent of the $\rho^0$ cells. Whereas HT1080 cells had a much lower respiratory capacity than 143B cells, transformants obtained by fusion of $\rho^0$ cells with HT1080 cytoplasts had respiratory rates significantly higher than for HT1080, but similar to that for 143B. The converse occurred with the HeLa S3 transformants. Although HeLa S3 and 143B cells had similar respiratory capacities, the rates of $O_2$ consumption and the COX activities of the three transmitochondrial lines obtained by injection of HeLa S3 mitochondria into $\rho^0$ cells were considerably lower than those of either parental line.

Although HeLa S3 and HeLaBU25 10B3R are derivatives of the same cell line (*8*), it was not surprising because of the large number of years in culture that the respiratory capacities of these two lines differed, with 10B3R having a lower rate of $O_2$ consumption and lower COX activity. Three of the four transmitochondrial lines obtained by injection of 10B3R mitochondria into $\rho^0$ cells showed a higher rate of $O_2$ consumption than 10B3R cells, but lower than 143B cells. More striking was the COX activity; in the three transformants, the COX activity was more than twice that of 10B3R and higher than those of either 143B or HeLa S3. In the fourth transformant, 206-217, although it was markedly deficient in its rate of $O_2$ consumption, the COX activity was higher than that of 10B3R.

CAP23 cells have a mutation in the mitochondrial large ribosomal RNA gene that confers resistance to CAP, an inhibitor of mitochondrial translation (*6, 9*). This mutation also reduces the rate of mitochondrial protein synthesis and causes defective assembly of the ribosomal subunits in CAP23 cells (*11*). Thus, the low respiratory capacity of these cells was not unexpected. Most of the

**Table 1.** Amount of mtDNA, cell size, and doubling time of transmitochondrial and parental cell lines. The mtDNA content of cells was determined as described (*10*). Cell size was determined by measuring the volume (typically, ~1 ml) of a known number of cells that had been suspended in 130 m*M* NaCl, 5 m*M* KCl, and 7.5 m*M* $MgSO_4$, and centrifuged for 7 min at 1000*g*. Population doubling time was determined as described (*9*). All CAP-resistant lines were grown for at least 1 week in the absence of CAP before analyses, except for the determination of the doubling times of 4.21.63 and 4.22.66 (*6*). The mitochondrial donor for transformants 101.111.P2 to 206.417.U3 was HT1080-6TG; that for transformants 206.7.1 to 206.30.1 was HeLa S3, that for transformants 206.102.3 to 4.29.104 was CAP23, and that for transformants 206-211 to 206-230 was HeLaBU25 10B3R.

| Cell line | mtDNA* (molecules per cell) | Cell size† (pl) | Population doubling time‡ (hours) |
|---|---|---|---|
| 143B.TK$^-$ | 9,100 ± 1,600 (13) | 7.0 | 17 |
| 143B101 | 0 | 6.2 | 20 |
| 143B206 | 0 | 6.8 | 19.8 |
| HT1080-6TG | 9,700 ± 1,800 (8) | 6.1 | 17 |
| HeLa S3 | 7,900 ± 1,700 (8) | 4.9 | 16 |
| CAP23 | 10,300 ± 2,100§ (18) | 4.1 | 19 |
| HeLaBU25 10B3R | 8,700 ± 2,100 (8) | 3.5 | 33 |
| 101.111.P2 | 6,700 ± 1,800 (4, 7) | 5.4 (6) | 16.1 (6) |
| 101.115.U3 | 10,200 ± 1,500 (4, 7) | 7.0 (6) | 16.8 (6) |
| 101.116.U3 | 8,000 ± 800 (4, 7) | ND‖ | ND |
| 101.121.P2 | 6,600 ± 500 (4, 7) | ND | ND |
| 206.412.P4 | 7,300 ± 2,700 (4, 8) | ND | ND |
| 206.415.P3 | 8,200 ± 900 (4, 8) | 5.3 (6) | 17.8 (5) |
| 206.416.U3 | 12,600 ± 1,900 (4, 8) | ND | ND |
| 206.417.U3 | 13,100 ± 3,800 (6, 8) | 5.5 (6) | 17.9 (7) |
| 206.7.1 | 8,200 ± 2,000 (8, 10) | 6.4 (10) | 20.2 (15) |
| 206.24.2 | 11,800 ± 1,300 (8, 12–18) | 5.4 (18) | 18.8 (12) |
| 206.30.1 | 8,000 ± 1,300 (8, 12–18) | 5.8 (18) | 20.7 (12) |
| 206.102.3 | 12,300 ± 2,200 (8, 12–15) | 5.4 (18) | 19.5 (13) |
| 206.104.1 | 13,900 ± 4,400 (3, 14–15) | 5.0 (18) | 20.2 (14) |
| 206.105.3 | 12,800 ± 2,600 (5, 12) | 9.0 (12) | 22.9 (14) |
| 206.113.4 | 8,200 ± 1,300 (8, 11) | 6.3 (11) | 20.3 (13) |
| 4.21.57 | 10,900 ± 3,300 (8, 9) | 5.5 (9) | ND |
| 4.21.63 | 6,800 ± 1,000 (8, 7) | 6.0 (7) | 17 (7) |
| 4.22.66 | 8,600 ± 2,400 (5, 7) | 6.6 (7) | 17 (7) |
| 4.29.104 | 7,300 ± 1,000 (8, 7) | 6.2 (7) | ND |
| 206-211 | 8,400 ± 600 (6, 13) | 6.1 (13) | ND |
| 206-217 | 11,400 ± 900 (6, 13) | 5.8 (13) | ND |
| 206-227 | 7,200 ± 1,300 (8, 10) | 5.4 (10) | ND |
| 206-230 | 8,600 ± 1,600 (6, 10) | 5.6 (10) | ND |

*The results are expressed as the mean ± two standard errors. The first figure in parentheses is the number of determinations; for the transmitochondrial cell lines, the second figure or range of figures represents the number of weeks passed after fusion or after injection when the determinations were made. †For 143B.TK$^-$, HeLa S3, CAP23, HeLaBU25 10B3R, 206-211, and 206-230, these values are the averages of two determinations; the other values are based on a single determination. The figure in parentheses is the number of weeks after fusion or after injection when the determinations were made. ‡The figure in parentheses is the number of weeks after fusion or after injection when the determination was made. §The mtDNA content of CAP23 was determined in a separate experiment. ‖ND, not determined.

transmitochondrial lines obtained by injection of CAP23 mitochondria into either $\rho^0$206 or 143B had low respiratory competence.

The variation in respiratory competence observed among sets of transformants obtained with different mitochondrial donors did not correlate with the number of mtDNA molecules per cell in the transformants (Table 1), nor did the striking deviation in respiratory capacity of an occasional cell line within each group. This intragroup variability in respiratory competence probably reflects variation among the individual recipient cells. Yet, the possibility that heterogeneity in the exogenous mtDNA population may have contributed to the observed variability in respiratory phenotype among transformants obtained by injection of individual organelles cannot be excluded.

Our results show that $\rho^0$ human cells can be repopulated with exogenous human mitochondria and restored to respiratory competence. Cell lines differing only in their mtDNA genotype—that is, the parental 143B.TK$^-$ cell line and the various groups of transformants containing mitochondria from HT1080, HeLa S3, HeLaBU25 10B3R, or CAP23 cells—exhibited respiratory differences. The sequence diversity of human mtDNA and of the mtDNA of these cell lines (*12*) is probably responsible for differences in respiratory capacity. A comparison of the respiratory capacities of the various sets of transmitochondrial cell lines with those of the mtDNA donors indicates that nuclear background also affects the respiratory competence of a cell.

The genetic analysis of mitochondrial biogenesis applied to *Saccharomyces cerevisiae* (*1*) can now be extended to mammalian cells. Thus, it should be possible to select for nuclear suppressors of mtDNA mutations affecting mitochondrial function, or conversely, for mtDNA suppressors of mutations of nuclear genes controlling mitochondrial biogenesis.

Our work is also relevant to the study of mitochondrial neuromuscular diseases (*13*). In several cases, specific mtDNA mutations have been associated with these diseases (*14*). Our results suggest that one possible mechanism for the observed different degrees of penetrance of these diseases is the existence of specific interactions of the variable complements of nuclear genes present in different individuals with a mitochondrial genome containing a mutation. Introduction of mitochondria derived from patients affected with these disorders into $\rho^0$ cells may help distinguish whether a defect is mtDNA- or nuclear DNA–encoded. Furthermore, repopulation of $\rho^0$ cells by microinjection of single organelles could be used to clone the mutant mitochondrial genome from a heteroplasmic population.


REFERENCES AND NOTES

1. G. Attardi and G. Schatz, *Annu. Rev. Cell Biol.* **4**, 289 (1988).
2. M. P. King and G. Attardi, in preparation.
3. P. Desjardins *et al.*, *Mol. Cell. Biol.* **5**, 1163 (1985); P. Desjardins *et al.*, *Somatic Cell Mol. Genet.* **12**, 133 (1986); R. Morais *et al.*, *In Vitro Cell. Dev. Biol.* **24**, 649 (1988).
4. M. Grégoire *et al.*, *Eur. J. Biochem.* **142**, 49 (1984).
5. C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3248 (1976).
6. M. P. King and G. Attardi, *Cell* **52**, 811 (1988).
7. T. T. Puck *et al.*, *J. Exp. Med.* **103**, 273 (1956).
8. J. Masters, B. Keeley, H. Gay, G. Attardi, *Mol. Cell. Biol.* **2**, 498 (1982). The 10B3R, originally a methotrexate-resistant derivative of HeLa BU25, has been grown for an extended time in the absence of methotrexate and has lost the amplified copies of the dihydrofolate reductase gene.
9. C. H. Mitchell, J. M. England, G. Attardi, *Somatic Cell Genet.* **1**, 215 (1975).
10. Dot blot analyses were carried out on 50- to 100-μl samples of cell suspensions containing $5 \times 10^4$ cells, which were brought to 1 ml with 50 m*M* tris, *p*H 8 (25°C), 10 m*M* EDTA, 100 m*M* NaCl, digested overnight at 50°C with 250 μg of proteinase K, and incubated with 0.5*M* NaOH at 37°C for 16 hours to hydrolyze RNA [G. Attardi, H. Parnas, M.-I. H. Hwang, B. Attardi, *J. Mol. Biol.* **20**, 145 (1966)]. A standard curve was constructed with known amounts of closed circular or Bam HI–digested HeLa mtDNA mixed with samples of $5 \times 10^4$ 143B206 cells digested and alkali-treated as described above. The mtDNA clone mp18.XH5.2, containing the 1237-bp Xba I–Hind III fragment of HeLa mtDNA [2204-7441; S. Anderson *et al.*, *Nature* **290**, 457 (1981)] cloned into Xba I + Hind III double-digested M13mp18, was labeled by extension of the universal primer and used as probe [H. M. Sucov *et al.*, *Dev. Biol.* **44**, 47 (1987)]. Our values for mtDNA for the parental cell lines were lower than those previously reported (*6*), because of the more complete removal of RNA by alkali digestion in our experiments.
11. A. Wiseman and G. Attardi, *Mol. & Gen. Genet.* **186**, 364 (1982).
12. Cann *et al.* [R. L. Cann, M. Stoneking, A. C. Wilson, *Nature* **325**, 31 (1987)] estimated that the mtDNA sequence diversity between random individuals is 0.32%. From a survey of ~7% of the mitochondrial genome with ten restriction enzymes, we have found an approximately 0.3% difference in the mtDNA nucleotide sequence between HeLa S3 and 143B.TK$^-$ and between 143B.TK$^-$ and HT1080 and a 0.5% difference between HeLa S3 and HT1080, in substantial agreement with the above estimate. The mtDNAs of HeLa S3, HeLaBU25 10B3R, and CAP23 are closely related.
13. S. DiMauro *et al.*, *Ann. Neurol.* **17**, 521 (1985); J. A. Morgan-Hughes, *Trends Neurosci.* **2**, 15 (1986); R. A. Capaldi, *Trends Biochem. Sci.* **13**, 144 (1988); D. C. Wallace, *Trends Genet.* **5**, 9 (1989).
14. I. J. Holt *et al.*, *Nature* **331**, 717 (1988); M. Zeviani *et al.*, *Neurology* **38**, 1339 (1988); D. C. Wallace *et al.*, *Science* **242**, 1427 (1988).
15. G. E. Veomett, in *Techniques in Somatic Cell Genetics*, J. W. Shay, Ed. (Plenum, New York, 1982), pp. 67–79.
16. T. H. Norwood and C. J. Zeigler, *ibid.*, pp. 35–45.
17. Y. Nakamura *et al.*, *Science* **235**, 1616 (1987); Y. Nakamura *et al.*, *Nucleic Acids Res.* **15**, 10073 (1987).


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# Myristoylated and Nonmyristoylated Forms of a Protein Are Phosphorylated by Protein Kinase C


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**Activation of protein kinase C is thought to require association of the kinase with the cell membrane. It has been assumed that cellular substrates for the kinase must likewise be associated with membranes, and previous studies with membrane-associated myristoylated proteins have supported this view. It is now shown that a mutation that prevents the normal amino-terminal myristoylation of a prominent cellular substrate of protein kinase C, and appears to prevent its membrane association, does not prevent the normal phosphorylation of this protein in intact cells in response to phorbol esters. Thus, membrane association may not be required in order for protein kinase C substrates to undergo phosphorylation.**


PROTEIN KINASE C (PKC), THE DIacylglycerol-activated, $Ca^{2+}$- and phospholipid-dependent protein kinase, has been implicated in the regulation of many cellular processes (*1*). Because the kinase requires lipid cofactors for activation, it is thought to exist in an active form only in close proximity to the cell membrane (*2, 3*). In addition, most cellular substrates for PKC are thought to be either integral membrane proteins or associated with the membrane in other ways (*3*). One example of such a substrate is pp60$^{v\text{-}src}$, in which $NH_2$-terminal myristoylation promotes membrane association (*4–6*); a mutation that


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