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Isolation and Characterization of Mitochondrial DNA-less Lines from Various Mammalian Cell Lines by Application of an Anticancer Drug, Ditercalinium

Kimiko Inoue,* Daisaku Takai,* Hideka Hosaka,* Sayaka Ito,* Hiroshi Shitara,* Kotoyo Isobe,* Jean-Bernard LePecq,† Evelyne Segal-Bendirdjian,‡ and Jun-Ichi Hayashi*,[1]

**Institute of Biological Sciences, University of Tsukuba, Ibaraki 305, Japan; †Rhone-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville 13, Quai Jules Guesde-BP14, 94403 Vitry Sur Seine, France; and ‡Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cédex, France*

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Since ethidium bromide was not effective in mouse cell lines for isolating mitochondrial DNA (mtDNA)-less cells (ρ^0 cells), we examined whether an anticancer drug, ditercalinium (DC), which has been shown to exclude mtDNA from mouse cell lines, could be effective in various mouse and human cell lines. We found that after DC treatment ρ^0 cells could be isolated from all cell lines of mouse or human origin tested. Moreover, these ρ^0 cells maintained ability to receive exogenously imported mtDNA and allow its replication and gene expression. These observations suggest that DC eliminates mtDNA from mouse and human cells without affecting the property to receive exogenous mtDNA. Therefore, DC could be applicable to cell lines expressing various differentiated phenotypes for studying whether mtDNA plays a significant role in expression of phenotypes by manipulating mtDNA elimination and reintroduction.

The coexistence of wild type mitochondrial DNA (mtDNA) and pathogenic mutant mtDNA with large scale deletions including several tRNA genes, and with point mutations in tRNA genes, has frequently been observed in patients with mitochondrial encephalomyopathies (1-3). In addition to pathogeneses, functional consequences of mtDNA mutations in expression of various phenotypes have been suggested. For example, the accumulations of somatic and pathogenic mtDNA mutations, which have been shown to cause various kinds of mitochondrial encephalomyopathies, have been shown to increase with age in normal subjects, suggesting involvement of mtDNA in aging precesses (1- 4). Furthermore, evidence for the presence of the pathogenic mtDNA mutations in patients with diabetes mellitus (DM) has been reported; a point mutation in the $tRNA^{Leu(UUR)}$ gene at 3243, which had been observed in about 90% of patients with MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) (5), was also identified in more than 1% of patients with DM (6-9).

Recently, we showed that mtDNA and mitochondrial respiratory function are necessary for the expression of differentiated phenotypes of glucose-stimulated insulin secretion based on the observations that this property of a mouse insulinoma cell line was lost on depletion of mtDNA, whereas it was restored reversibly by repopulation with foreign mtDNA from a mouse fibroblast cell line, suggesting involvement of mtDNA in expression of differentiated phenotypes (10). Therefore, isolation of mtDNA-less (ρ^0) cell lines from various mammalian cell types seems to be very important for determination of whether mtDNA and its mutations contribute to the expression of differentiated phenotypes. Although ethidium bromide (EB) has been shown to be effective for isolating ρ^0 cell lines from avian (11) and human cells (12,13), it is not effective for every cell line. For example, exposure of mouse cells to EB frequently induces EB-resistant mutant cells containing mtDNA in place of inducing ρ^0 cells, as we reported previously (14).

In recent studies on various chemicals that were expected to decrease the mtDNA contents of mouse cells, we found that one anticancer drug, ditercalinium (DC), is effective for isolating ρ^0 lines from the mouse myoblast line C2C12 (15). In this study, using mouse fibroblast cell lines, B82CAP and NIH3T3, and a human osteosarcoma line, 143BTK-, we examined whether DC treatment was effective for excluding mtDNA in other cell lines. Results showed that treatment of the cells

[1] Corresponding author. Fax: +81-298-53-6614. E-mail: jih45@sakura.cc.tsukuba.ac.jp.

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with this drug progressively eliminated mtDNA, resulting in the isolations of ρ^0 cell lines from all the lines tested.

MATERIALS AND METHODS

Cells and cell culture. Mouse fibroblast lines, B82CAP (16) and NIH3T3 cells, and a human osteosarcoma line, 143BTK- cells, were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo) containing 10 % fetal calf serum, 50 μg/ml uridine and 0.1 mg/ml pyruvate.

Isolation of ρ^0 cell lines. Cells were plated at 1×10^2 - 5×10^4 cells/dish, and from 24 hours after plating they were treated with DC (1.5 μg/ml), an antitumor bis-intercalating agent, for two months as described previously (15). The medium containing the drug was changed every 2 days. After two months, colonies growing in the medium were clonally isolated by the cylinder method. The cloned cells were then cultivated in normal medium without the drug.

Introduction of external mtDNA into ρ^0 cells. Mouse synaptosomes and human platelets were used as mtDNA donors of ρ^0 cells. Fusion of mouse ρ^0 cells with mouse synaptosomes was carried out as we described previously (15). Briefly, the brain of B6 strain mice was washed in PBS and homogenized in medium containing 0.25 M sucrose, 50 mM Hepes pH 7.5, and 0.1 mM EDTA in a Teflon-glass potter homogenizer. The homogenate was centrifuged at 1000 $\times$g for 10 min at 4 °C, and the resultant supernatant was centrifuged at 17000 $\times$g for 20 min at 4 °C. The pellet was mixed with 5×10^6 ρ^0 mouse cells and fusion was carried out in the presence of 50 % (w/v) polyethylene glycol 1500 (Boehringer Mannheim, Germany). Human platelets were fused with ρ^0 human cells as described by Chomyn et al. (17). The fusion mixture was cultivated in selection medium RPMI1640 without pyruvate and uridine. On day 60 after fusion, the cybrid clones growing in the selection medium were clonally isolated by the cylinder method. Cybrid clones were cultivated in normal medium.

Southern blot analyses of mtDNA. Total cellular DNA (1-2 μg) extracted from 2×10^5 cells was digested with the restriction enzymes (Nippon Gene, Japan), *Xho*I for mouse samples and *Bam*HI for human samples, and restriction fragments were separated in 0.6% agarose gel, transferred to a nylon membrane and hybridized with [α-^{32}P] dATP-labeled mtDNA. The membrane was washed and exposed to an imaging plate for 2 hours and radioactivities of fragments were measured with a bioimaging analyzer, Fujix BAS 2000 (Fuji Photo Film, Japan).

PCR analyses. For detection of a small amount of mtDNA in mouse ρ^0 cells, total cellular DNA (0.5 μg) extracted from 2×10^5 ρ^0 cells was used as a template for amplification. The nucleotide sequences of mouse mtDNA from position 15495 to 15511 on the light strand and from 15713 to 15697 on the heavy strand (15), and the nucleotide sequences of human mtDNA from position 8196 to 8215 on the light strand and from 8726 to 8707 on the heavy strand (18) were used as oligonucleotide primers. The cycle times were 60 sec denaturation at 94 °C, 60 sec annealing at 45 °C and 120 sec extension at 72 °C for 30 cycles. The products were separated by 4 % agarose gel electrophoresis.

Analysis of mitochondrial translation products. Mitochondrial translation products were labeled with [^{35}S] methionine as described previously (15, 19). Briefly, 2×10^6 cells in a culture dish were incubated in methionine-free medium containing 2 % FCS for 45 min at 37 °C. Then the cells were labeled with [^{35}S] methionine for 2 hours in the presence of emetine to inhibit translation in the cytoplasm. Proteins in the mitochondrial fraction were separated by 0.85% SDS, 6M urea, 12% polyacrylamide gel electrophoresis. For quantitative estimation of [^{35}S] methionine-labeled polypeptides, the dried gel was exposed to an imaging plate for 12 hr and the radioactivities of polypeptides were measured with a bioimaging analyzer.

RESULTS

The influence of DC treatment on the content of mtDNA was examined using mouse cell lines B82CAP and NIH3T3, and a human osteosarcoma line, 143BTK- (Table 1). Mouse B82CAP cells are resistant to bromodeoxyuridine (BrdU^r) and to chloramphenicol (CAP^r) derived from mouse L cells (16). Southern blot analysis showed that the amount of mtDNA in all cell lines decreased significantly on treatment with DC for 24 hrs (data not shown), suggesting the possibility of isolating ρ^0 cell lines by long-term treatment of these cells with DC.

For isolation of ρ^0 cell lines, cells were cultivated in the presence of DC for two months and growing colonies were isolated clonally. These clones showed no detectable mtDNA on Southern blot analysis. Subsequently, one clone from each cell line was picked up randomly and cultivated in the absence of DC for 3 months. Southern blot analysis of these clones again showed that they did not recover mtDNA during cultivation without DC (Fig. 1A), suggesting that they were ρ^0 clones. Then, using the PCR technique, we examined whether a small amount of mtDNA, that was not detectable by Southern blot analysis, still remained in the presumptive ρ^0 clones. Figure 1B shows that mtDNA was not amplified from DNA samples prepared from these clones. As these clones were entirely devoid of mtDNA, we isolated ρ^0 cell lines from B82CAP, NIH3T3, and 143BTK- cells. Moreover, these ρ^0 lines, named ρ^0 B82CAP, ρ^0 NIH3T3, and ρ^0 143BTK-, respectively (Table 1), required pyruvate and uridine in the medium for growth like other ρ^0 lines (11, 12, 15).

The absence of mitochondrial translation activity in these ρ^0 lines was confirmed by the absence of [^{35}S] methionine incorporation into polypeptides synthesized in the mitochondria (Fig. 2), suggesting that complete depletion of mtDNA resulted in complete absence of mitochondrial translation. Next, we examined cytoplasmic transfer of foreign mtDNA to each ρ^0 line to determine whether these ρ^0 lines maintained the abilities to receive and allow replication of exogenously imported mtDNA. We reported previously that mtDNA cannot be transferred to cells of different species due to incompatibilities of mitochondrial and nuclear genomes of different species (20). Therefore, mouse brain synaptosomes and human platelets were used as mtDNA donors to mouse ρ^0 and human ρ^0 cells, respectively.

After fusion of the synaptosomes or platelets with mouse ρ^0 or human ρ^0 cells in the presence of polyethylene glycol, colonies growing in selective medium without pyruvate and uridine were isolated as cybrid clones (Table 1). No colonies were obtained from simple mix-

TABLE 1

Genetic Characteristics of Parental Cell Lines, ρ^0 Lines, and Their Cybrids

Cell lines	Species	Drug resistance	mtDNA (origin)
Parent cell lines			
B82CAP (fibroblasts)	mouse	$BrdU^r$, CAP^r	+
NIH3T3 (fibroblasts)	mouse		+
143BTK- (osteosarcoma)	human	$BrdU^r$	+
ρ^0 cell lines			
ρ^0 B82CAP			−
ρ^0NIH3T3			−
ρ^0 143BTK-			−
Cybrids			
CyB82CAP (ρ^0 B82CAP × synaptosomes)			+ (mouse synaptosomes)
CyNIH3T3 (ρ^0 NIH3T3 × synaptosomes)			+ (mouse synaptosomes)
Cy143BTK- (ρ^0 143BTK-x platelets)			+ (human platelets)

tures of ρ^0 cells and synaptosomes or platelets in the absence of polyethylene glycol. Southern blot analysis showed that all the cybrid clones contained comparable levels of mtDNA to those of the normal parent cells (data not shown). Restoration of normal mitochondrial translation activity, indicated by [^{35}S] methionine labeling of polypeptides synthesized in the mitochondria, was observed in all cybrid clones (Fig. 2). Therefore, all ρ^0 lines from parental B82CAP, NIH3T3, and 143BTK- cells maintained the abilities to receive exogenous mtDNA and allow its replication, gene expression, and translation in mitochondria.

DISCUSSION

In this study, we examined the effect of DC treatment on the abilities of various mouse and human cell lines to maintain their mtDNA contents, and found that the drug decreased their mtDNA contents, so that ρ^0 cell lines could be isolated from all lines tested.

Recently, we reported evidence for the involvement of mtDNA in the expressions of differentiated phenotypes of glucose-stimulated insulin secretion based on the observations that in the mouse insulinoma cell line MIN6 this activity was lost on depletion of mtDNA, but reversibly restored on repopulation of the cells with foreign mtDNA from a mouse fibroblast cell line (10). Since DC treatment was effective for isolating ρ^0 lines

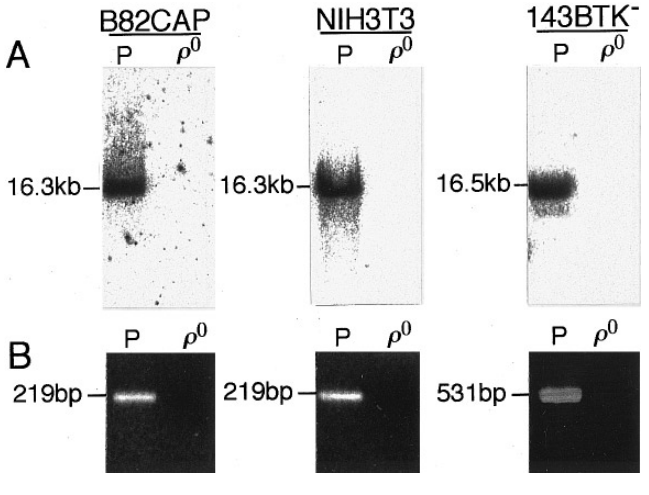

FIG. 1. Analyses of mtDNA contents of clones grown in medium containing DC. P, parent cell lines with mtDNA; ρ^0, ρ^0 mutant lines. A, Southern blot analysis of XhoI fragments of mouse cell lines and PvuII fragments of human cell lines; B, PCR analysis.

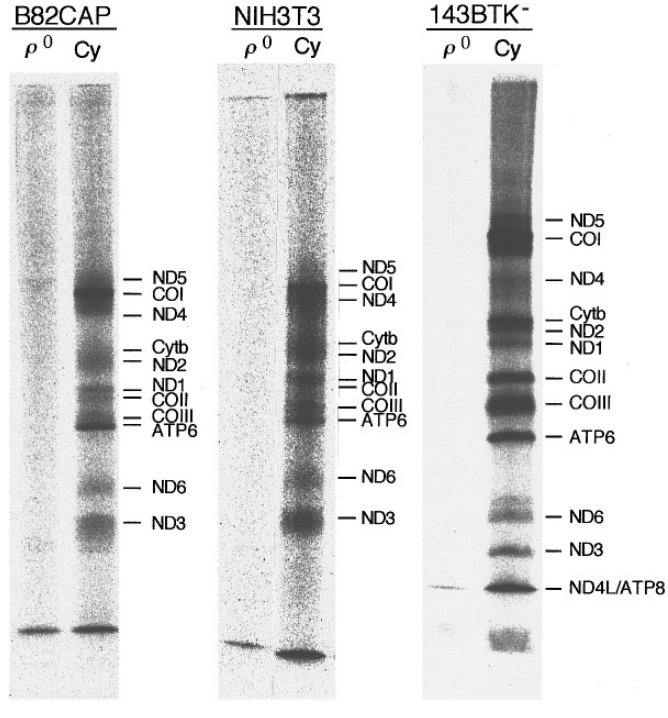

FIG. 2. Characterization of ρ^0 lines with respect to restoration of mitochondrial translation by exogenously transferred mtDNA. ρ^0, ρ^0 lines; Cy, cybrid clones with exogenously repopulated mtDNA; ND5, COI, ND4, Cytb ND2, ND1, COII, COIII, ATP6, ND6, ND3, ATP8, and ND4L are polypeptides assigned to mtDNA genes.

from all cell lines tested without affecting the property to receive exogenous mtDNA, it could be used to study the roles of mtDNA in expression of differentiated phenotypes by testing whether the expressions of these phenotypes are affected by depletion and subsequent readdition of mtDNA.

Furthermore, ρ^0 lines could be used to study how the complete absence of mtDNA and its products affect the expression of mitochondria-related proteins encoded by nuclear DNA. The biogenesis of mitochondria is known to be controlled by both nuclear and mitochondrial genomes (1-3). All translation products of mtDNA are subunits of oligomeric complexes of the oxidative phosphorylation system. As these complexes consist of both nuclear DNA-coded and mtDNA-coded subunits, there must be some mechanism that regulates the coordinated expressions of the subunits encoded in the two compartments. Furthermore, mtDNA replication, transcription, and translation in mitochondria are mainly controlled by nuclear factors. Accordingly, a deficiency of mtDNA and its products may generate some feedback signals that affect the expression of nuclear DNA-coded factors. Using mouse cell lines, we showed previously that short-term block of mtDNA transcription by EB treatment did not affect the expressions of nuclear DNA-coded subunits, but blocked their assembly into complexes (14). However, we could not examine the influence of long-term block, because we could not isolate mouse ρ^0 lines by EB treatment (14).

Now, we can investigate whether the long-term absence of mtDNA and its products in the mouse ρ^0 lines isolated in this work export some signals that affect the expressions of nuclear DNA-coded mitochondrial factors involved in oxidative phosphorylation and/or mtDNA replication and expression. We are also investigating how and why DC treatment is effective for isolating ρ^0 lines from all cell lines examined.

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REFERENCES

1. Attardi, G., and Schatz, G. (1988) *Ann. Rev. Cell Biol.* **4,** 289–333.
2. Wallace, D. C. (1992) *Ann. Rev. Biochem.* **61,** 1175–1212.
3. Larson, N. G., and Clayton, D. A. (1995) *Ann. Rev. Genetics* **29,** 151–178.
4. Linnane, A. W., Marzuki, S., Ozawa, T., and Tanaka, M. (1989) *Lancet* i, 642–645.
5. Goto, Y., Nonaka, I., and Horai, S. (1990) *Nature* **348,** 651–653.
6. van den Ouweland, J. M. W., Lemkes, H. H., Ruitenbeek, W., Sandkuijl, L. A., de Vijlder, M. F., Struyvenberg, P. A., van den Kamp, J. J., and Maassen, J. A. (1992) *Nat. Genet.* **1,** 368–371.
7. Reardon, W., Ross, R. J. M., Sweeney, M. G., Luxon, L. M., Pembrey, M. E., Harding, A. E., and Trembath, R. C. (1992) *Lancet* **340,** 1376–1379.
8. Oka, Y., Katagiri, H., Yazaki, Y., Murase, T., and Kobayashi, T. (1993) *Lancet* **342,** 527–528.
9. Kadowaki, T., Kadowaki, H., Mori, Y., Tobe, K., Sakuta, R., Suzuki, Y., Tanabe, Y., Sakura, H., Awata, T., Goto, Y.-i., Hayakawa, T., Matuoka, K., Kawamori, R., Kamada, T., Horai, S., Nonaka, I., Hagura, R., Akanuma, Y., and Yazaki, Y. (1994) *N. Engl. J. Med.* **330,** 962–968.
10. Soejima, A., Inoue, K., Takai, D., Kaneko, M., Ishihara, H., Oka, Y., and Hayashi, J.-I. (1996) *J. Biol. Chem.* **271,** 26194–26199.
11. Desjardins, P., de Muys, J.-M., and Morais, R. (1986) *Somat. Cell Mol. Genet.* **12,** 133–139.
12. King, M. P., and Attardi, G. (1989) *Science* **246,** 500–503.
13. Hayashi, J.-I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y., and Nonaka, I. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10614–10618.
14. Hayashi, J.-I., Tanaka, M., Sato, W., Ozawa, T., Yonekawa, H., Kagawa, Y., and Ohta, S. (1990) *Biochem. Biophys. Res. Commun.* **167,** 216–221.
15. Inoue, K., Ito, S., Takai, D., Soejima, A., Shisa, H., LePecq, J.-B., Segal-Bendirdjian, E., Kagawa, K., and Hayashi, J.-I. (1997) *J. Biol. Chem.* **272,** 15510–15515.
16. Hayashi, J.-I., Gotoh, O., Tagashira, Y., Tosu, M., Sekiguchi, T., and Yoshida, M. C. (1982) *Somac. Cell Genet.* **8,** 67–81.
17. Chomyn, A., Lai, S. T., Shakeley, R., Bresolin, N., Scarlato, G., and Attardi, G. (1994) *Am. J. Hum. Genet.* **54,** 966–974.
18. Hayashi, J.-I., Ohta, S., Kagawa, Y., Kondo, H., Kaneda, H., Yonekawa, H., Takai, D., and Miyabayashi, S. (1994) *J. Biol. Chem.* **269,** 6878–6883.
19. Takai, D., Inoue, K., Goto, Y.-i, Nonaka, I., and Hayashi, J.-I. (1997) *J. Biol. Chem.* **272,** 6028–6033.
20. Hayashi, J.-I., Tagashira, Y., Yoshida, M. C., Ajiro, K., and Sekiguchi, T. (1983) *Exp. Cell Res.* **147,** 51–61.