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EFFECTS OF ETHIDIUM BROMIDE TREATMENT OF MOUSE CELLS ON EXPRESSION AND ASSEMBLY OF NUCLEAR-CODED SUBUNITS OF COMPLEXES INVOLVED IN THE OXIDATIVE PHOSPHORYLATION

Jun-Ichi Hayashi[1*], Masashi Tanaka[2], Wataru Sato[2], Takayuki Ozawa[2], Hiromichi Yonekawa[3], Yasuo Kagawa[4], and Shigeo Ohta[4]

[1] Department of Biochemistry, Saitama Cancer Center Research Institute, Saitama 362, Japan

[2] Department of Biomedical Chemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan

[3] Department of Laboratory Animal Science, Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan

[4] Department of Biochemistry, Jichi Medical School, Tochigi 329-04, Japan

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SUMMARY: Using mouse cell lines 5P and 5PE^r (ethidium bromide-resistant derivative of 5P), we examined the influence of blocking expression of mitochondrial-gene products with ethidium bromide on the expression and assemblies of nuclear-coded subunits of the complexes involved in the oxidative phosphorylation. The results suggest that in the absence of mitochondrial-coded products, the expressions of subunit VIc of complex IV and β-subunit of F_1-ATPase are not affected, but that most nuclear-coded subunits other than α- and β-subunits of F_1-ATPase cannot be assembled nor inserted into the inner membrane.

The biogenesis of mitochondria is known to be controlled by both nuclear and mitochondrial genomes. All translation products of mitochondrial DNA (mtDNA) are subunits of oligomeric complexes in the oxidative phosphorylation system. As these complexes consist of both nuclear DNA-coded and mtDNA-coded subunits (for review, see ref. 1 and 2), there must be some mechanism that regulates the coordinated expressions of the subunits encoded in the two compartments.

* To whom correspondence should be addressed.

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In yeast cells, respiration-deficient mutants have been used extensively to study this regulation, and results have suggested that mitochondrial-gene products export some signals that regulate nuclear gene expression (2).

Little is known, however, about whether the expression of nuclear DNA-coded subunits of the complexes are affected by the absence of mitochondrial-gene products in mammalian cells. Recently, mtDNA-less mutants of avian (3, 4) and human (5) cells were isolated by ethidium bromide (EB) treatment. As EB is known to block RNA synthesis in mitochondria without affecting nuclear-gene expression (6), we examined the effects of EB treatment of mouse cells on the expressions and assemblies of the subunits of the complexes encoded in the nucleus.

MATERIALS AND METHODS

Cell lines and culture: Mouse fibroblast cell line 5P, derived from carcinogen-treated embryonic cells (7), was grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. The EB-resistant cell line 5PE^r was isolated by treating 5P cells with EB (250 ng/ml) for 10 days and then isolating colonies that grew with EB (500 ng/ml) by the cylinder method.

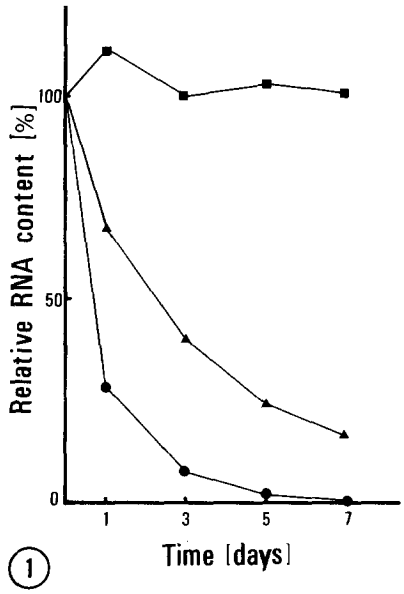
DNA probes: The following probes were used in Northern blot analysis: total mtDNA of a C3H strain mouse inserted into pUC8 at the SpeI site; cDNAs of the human β-subunit of F_1-ATPase (8), human subunit VIc of complex IV (9), and human β-actin (10). DNA probes were labeled with α-[^{32}P]dCTP by random-priming.

Northern blot analysis: Total RNA was isolated from 2 x 10^7 cells as described (11). Total RNA (10 μg) separated by electrophoresis in 1.2% agarose/formaldehyde gel was transferred to nitrocellulose filters and hybridized with labeled DNA probes. The filters were washed in high stringency conditions and exposed to X-ray film at -80°C. For quantitative measurement of the mitochondrial RNA (mtRNA) content, the regions of the filter corresponding to the radioactive bands were cut out and their radioactivities were measured in a Beckman scintillation counter.

Immunoblot analysis: Antibodies against complexes I, III, IV, and F_1-ATPase of beef heart mitochondria were raised in rabbits as described (12). Mitochondrial proteins of mouse 5P and 5PE^r cells were separated by SDS polyacrylamide gel electrophoresis and transferred to Durapore filters. Binding of antibodies to the subunits was detected by the peroxidase-anti-peroxidase method (12).

RESULTS AND DISCUSSION

The cell line 5P was treated with EB for 1-7 days, and total RNA was extracted from the cells. For determination of the levels of mtRNAs, total RNA separated by agarose gel electrophoresis was blotted onto a nitrocellulose membrane and hybridized with labeled mtDNA probes. Figure 1 shows that after



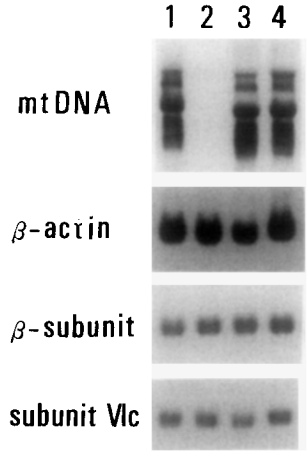


Fig. 1. Effects of EB treatment on the mtRNA and β-actin mRNA contents of 5P cells. 5P cells were treated for the indicated times with 50 ng/ml (▲) or 250 ng/ml (■, ●) of EB. Total RNA (10 μg) extracted from the cells was separated by agarose gel electrophoresis, blotted onto a filter, and hybridized with labeled mtDNA (▲, ●) or β-actin cDNA (■). The RNA content was measured as described in MATERIALS AND METHODS.

Fig. 2. Northern blot analysis of RNA from 5P and $5PE^r$ cells incubated with or without EB. Total RNA (10 μg) extracted from the cells was separated on agarose/formaldehyde gels, blotted onto filters, and hybridized with four different probes, mtDNA, and cDNAs of β-subunit of F_1-ATPase, subunit VIc of complex IV, and β-actin. Lane 1, 5P cells; lane 2, 5P cells treated with EB; lane 3, $5PE^r$ cells; lane 4, $5PE^r$ cells treated with EB.

7 days incubation with EB (250 ng/ml), no mtRNA was detectable in 5P cells, but the contents of β-actin mRNA were not affected.

Inhibition of mtRNA synthesis impairs the formation of subunits encoded by mtDNA. Thus, cells treated with EB for 7 days are good systems for study of whether the inhibition of expression of mtDNA-coded subunits of complexes affects the transcription of their nuclear DNA-coded subunits, such as the β-subunit of F_1-ATPase and subunit VIc of complex IV. To monitor the side effects of EB, we established EB-resistant cell line $5PE^r$ from 5P cells and used it as a control. We examined the amounts of mRNAs of these subunits in EB-treated cells by Northern blot hybridization. Total RNA extracted from 5P and $5PE^r$ cells incubated with and without EB for 7 days were hybridized with labeled probes (Fig. 2). The results showed that the complete loss of mitochondrial-gene products did not affect the mRNA levels of nuclear DNA-coded

subunits, β-subunit and subunit VIc. This means that there is no mitochondrial feedback or compensation system that directly affects the expressions of at least these nuclear DNA-coded subunits.

Then, we examined whether these nuclear DNA-coded subunits of the complexes could be assembled or inserted into the inner membrane in the absence of mitochondrial-gene products. For this, mitochondria were isolated from 5P and $5PE^r$ cells incubated with or without EB for 7 days, and the contents of the subunits of the complexes were analysed by immunoblotting using antibodies against complexes I, III, IV and F_1-ATPase purified from beef heart mitochondria (12). Figure 3 shows that the α- and β-subunits of F_1-ATPase were present in EB-treated 5P cells, but that the contents of all detectable subunits of other complexes, i.e., the 30-kDa subunit of complex I, core protein and iron-sulfur protein of complex III, and subunit IV of complex IV, were much lower than in EB-treated $5PE^r$ cells. These results suggest that none of these complexes except the F_1-ATPase can be assembled or inserted into the inner membrane in the absence of mtDNA-coded subunits.

These findings seem to be slightly different from those in yeast cells, respiration-deficient mutants of which are available. Parikh et al. (13) reported that respiratory-deficient derivatives of yeast, such as the mit^-, ρ^-, and ρ^0 petite strains, accumulated much higher levels of the mRNA of the

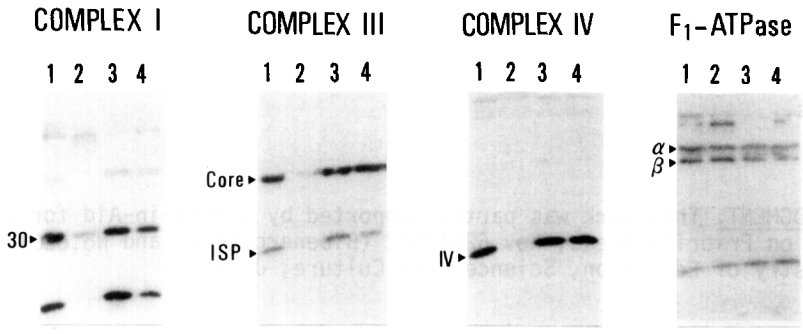


Fig. 3. Immunoblotting of mitochondrial proteins from 5P and $5PE^r$ cells incubated with or without EB. Antibodies against beef heart complexes I, III, and IV, and F_1-ATPase were used to detect subunits of each complex of mouse mitochondria. Lane 1, 5P cells; lane 2, 5P cells treated with EB; lane 3, $5PE^r$ cells; lane 4, $5PE^r$ cells treated with EB. Core, core protein; ISP, iron-sulfur protein.

nuclear DNA-coded subunit VI of complex IV than isonuclear, respiratory-competent strains, whereas their mRNA levels of nuclear DNA-coded cytochrome c and α- and β-subunits of F_1-ATPase were the same as in the latter strains. Reduced levels of mRNAs of the α- and β-subunit of F_1-ATPase (14) and three subunits of complex III (15) were, however, observed in some *pet* mutants of yeast which is deficient in mitochondrial protein synthesis. Thus, the expressions of some nuclear DNA-coded subunits of the complexes may respond to the absence of mitochondrial-gene products, even though the response patterns are not the same in different mutants (13-15). It is also generally thought that in yeast cells, nuclear DNA-coded components continue to be synthesized, imported into mitochondria, and inserted into the inner membrane, even when the synthesis of mtDNA-coded subunits is blocked (2).

The apparent difference between mouse cells and yeast cells in expression of the subunits of complex IV might be attributable to short-term block in the synthesis of mitochondrial products in the mouse cells (Fig. 1). Long-term exposure to EB frequently induced "EB-resistant mutants" of mouse cells that expressed mtRNA in the presence of a high concentration of EB (500 ng/ml), instead of inducing the "mtDNA-less mutants" observed with avian cells (4). Recently, King and Attardi (5) isolated human cell lines without mtDNA by EB treatment. This procedure should allow study of the influence of long-term loss of mtDNA on the phenotypes of mammalian cells. We are investigating whether long-term defects of mitochondrial products in mouse cells export some signals that affect expression and assembly of nuclear DNA-coded subunits of complexes.

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