IDENTIFICATION OF MITOCHONDRIAL DNA SPECIES IN INTERSPECIFIC CYBRIDS AND RECONSTITUTED CELLS USING RESTRICTION ENDONUCLEASE

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1. Introduction

Genes for mitochondrial proteins are known to be encoded in both nuclear DNA and mitochondrial DNAs (mtDNAs). So, it is important to elucidate whether the nucleus and mitochondria can function in harmony in cytoplasmic hybrids (cybrids) and reconstituted cells in which the nucleus and mitochondria are of different species. Transfer of chloramphenicol-resistant (CAP^r) cytoplasm to whole cells of different species was extremely difficult [1]; this may be due to the incomplete cooperation of the products of nuclear DNA and mtDNAs of different species [1]. However, viable interspecific reconstituted cells could be isolated by the fusion of human karyoplasts to CAP^r mouse cytoplasts [2]. mtDNA species of the cells were identified by a difference in buoyant densities of the mtDNAs of the parent cells [2]. However, this identification technique is applicable only to mtDNA species having buoyant densities sufficiently different to be clearly distinguished.

We developed another procedure for identification of mtDNA species in culture cells using restriction endonuclease. This procedure has many advantages:

(i) It requires <0.1 ml packed cells ($\sim 2 \times 10^7$ cells) without labelling;
(ii) The cleavage patterns of the mtDNAs are species-specific [3], and even intraspecifically different mtDNAs can be clearly distinguished [4–6];
(iii) Sequence identification of mtDNAs is unambiguous.

Using this procedure we examined the mtDNA sequences of CAP^r interspecific cybrids, or reconstituted cells, obtained by fusion of rat CAP^r cytoplasts to hypoxanthine–aminopterin–thymidine-resistant (HAT^r) mouse whole cells or karyoplasts after cultivation in selective medium for 5 months. Unexpectedly, the *Eco*RI cleavage patterns of their mtDNAs were the same as those of mouse mtDNAs.

2. Materials and methods

2.1. *Isolation of interspecific cybrids and reconstituted cells*

Mouse melanoma B16 cells were used as whole cells or karyoplast-donors. They are HAT-resistant and CAP-sensitive cells. As cytoplasmic donors, rat L6TGCAP^r cells (HAT^s, CAP^r) were used. They were derived from L6TG cells after mutagenesis by ethylmethane sulfonate (200 μg/ml for 18 h) and stepwise selection in medium with CAP. L6TG cells were provided by Dr M. R. Ringertz, Karolinska Institute, Sweden. Their karyotypes have been described [7].

Interspecific cybrids and reconstituted cells were isolated as in [8] employing both nuclear marker HAT-resistancy and cytoplasmic marker CAP-resistancy to exclude contaminating parent cells. Cells were enucleated by treatment with cytochalasin B as in [9]. Karyoplasts were purified by 'stayput' purification using a sterile linear gradient of 1–3% bovine serum albumin [10]. The proportion of enucleated L6TGCAP^r cells was >98%. Each Petri dish received 4 discs with $\sim 2 \times 10^5$ cytoplasts/disc. Ultraviolet-inactivated HVJ suspended in 0.1 ml Okada's buffer was added to each disc. After 15 min at 4°C, $\sim 10^5$ mouse B16 whole cells or karyoplasts were added to each disc. After cold treatment for 15 min, the discs were incubated at 37°C for 45 min, then rinsed 3 times with PBS and cultured in Eagle's MEM containing 10% fetal calf serum. After overnight incubation, the discs were trypsinized and plated in medium con-

taining HAT and CAP (100 μg/ml). They were then incubated under a 5% CO_2 atmosphere for 3 weeks. The colonies that appeared were cloned by the cylinder method and maintained in selective medium.

2.2. *Identification of mtDNA species*

The cybrids and reconstituted cells were used after clonal isolation for 5 months (100–150th subdivision). Mitochondria were prepared as in [11] from ~0.1 ml packed cells in medium consisting of 220 mM mannitol, 70 mM sucrose, and 2 mM Hepes buffer (pH 7.4). The mitochondrial pellet was suspended in 3.5 ml of 0.1 M Tris–EDTA (pH 8.0) supplemented with 0.6% sarkosyl and stood for 1 h at room temperature for lysis. MtDNAs were then isolated by ethidium bromide (EtBr)–CsCl centrifugation (100 000 × *g*, 40 h at 20°C) as in [12] omitting the second CsCl centrifugation [13]. The upper band was discarded and the lower band containing closed circular mtDNAs was collected. The dye was removed by isopropanol extraction and after dialysis against 0.1 mM EDTA, mtDNAs were digested completely with restriction endonuclease *Eco*RI under the conditions described in [14]. Agarose gel electrophoresis of the digested mtDNA fragments was performed in glass tubing (6 × 135 mm), because as little as 0.02 μg *Eco*RI fragments of rat type-A mtDNAs could be detected in disc-type agarose gels [15], but could not be detected in slab-type agarose gels. After electrophoresis, gels were stained with EtBr (0.1 μg/ml) and the DNA bands were located under short-wave UV light and photographed through an orange filter (Nikon CP-3) using Tri-X film.

3. Results and discussion

On *Eco*RI digestion, mtDNAs of mouse B16 cells gave two fragments, Ma and Mb, with mol. wt 8.6 and 1.2 (× 10^6) (fig.1.1). mtDNAs of rat L6TG and L6TGCAP^r cells gave 6 *Eco*RI fragments, Ra-Rf, with mol. wt 3.8, 2.4, 1.85, 1.14, 0.44 and 0.3 (× 10^6), respectively (fig.1.2,3). So, the *Eco*RI cleavage pattern of mtDNAs from B16 cells was the same as that of laboratory mice [16,17] and those of mtDNAs from L6TG and L6TGCAP^r cells were the same as that of rat type-A mtDNAs [13].

After fusion of cytoplasts of rat L6TGCAP^r to mouse B16 karyoplasts or whole cells (HAT^r) and selective cultivation in HAT and CAP, several colonies

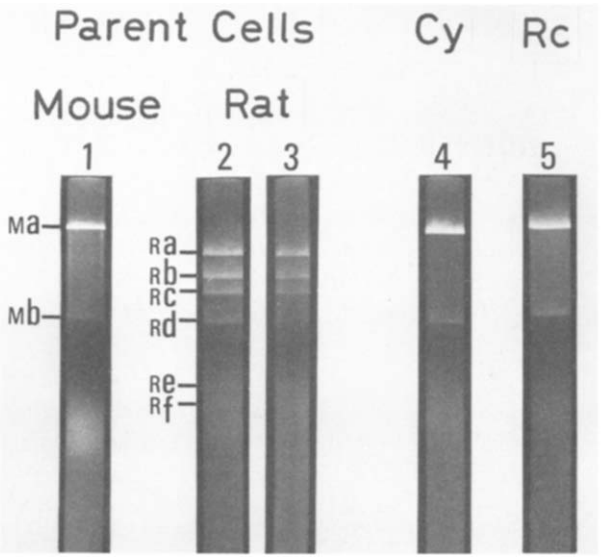

Fig.1. *Eco*RI cleavage patterns of mtDNAs from cybrids, reconstituted cells, and the parent cells. Sources of mtDNAs: (1) mouse B16 cells; (2) rat L6TG cells; (3) rat L6TGCAP^r cells; (4) cybrids (Cy); (5) reconstituted cells (Rc). Ma and Mb, *Eco*RI fragments of mouse B16 cells; (Ra–Rf) *Eco*RI fragments of rat L6TG and L6TGCAP^r cells.

that appeared were cloned and maintained in the selective medium. No parent cells among 2 × 10^7 cells in the control survived in this medium. These clonal cells showed a distinct morphology and the same karyotype as that of mouse B16 cells, and possessed surface antigens specific to rat cells in an early stage of clonal isolation [18]. Thus, they were thought to be either CAP^r interspecific reconstituted cells or cybrids, and were expected to contain mainly mtDNAs of L6TGCAP^r cell origin, i.e., rat type-A mtDNA. Unexpectedly, however, at 5 months after clonal isolation (100–150th subdivision), the *Eco*RI cleavage patterns of the mtDNAs from both the cybrids and the reconstituted cells were the same as those of mouse mtDNAs and no faint band with the same mobility as the *Eco*RI bands of rat type-A mtDNA was detected (fig.1.4,5). Thus, contrary to expectation, the CAP^r cybrids and reconstituted cells contained predominantly mouse mtDNA species that must have originated from CAP^s B16 cells. Similar results have been observed in CAP^r mouse–human cybrids [19].

In the reconsituted cells, mouse mtDNAs probably arose from B16 karyoplasts that contained small amounts of mitochondria. The mtDNA species of

L6TGCAP^r cytoplasts (type-A) probably largely disappeared during prolonged culivation (100–150th subdivision) of the cells.

Why can these cybrids and reconstituted cells, whose mtDNA species are the same as that of mouse CAP^s B16 cells, proliferate in the selective medium containing CAP, in which B16 cells cannot survive? At least 4 possibilities can be imagined.

(1) CAP^r mutants may be formed spontaneously in B16 cytoplasm during isolation of the clonal cells. However, since no mouse B16 cells among 2×10^7 cells survived in the selective medium, it may be unlikely that cytoplasm of B16 cells acquired CAP-resistancy by chance by spontaneous mutation in both the cybrids and reconstituted cells.
(2) There was a small amount of CAP^r rat mtDNAs that could not be detected by our procedure in these cells, and thus that the phenotype was resistant to CAP.
(3) A small region of rat mtDNAs that was responsible for the CAP-resistant phenotype was integrated into mouse mtDNAs by recombination; if rat mitochondria cannot survive in cells with a mouse nucleus because of incomplete cooperation between the products of the mtDNAs and the nuclear DNA, only the recombinant mouse mtDNAs can replicate.
(4) CAP^r genes were encoded in selfreplicative cytoplasmic DNAs other than mtDNAs; in fact, there is no direct evidence that in mammalian cells CAP^r genes are encoded in mtDNAs.

We cannot decide yet whether any of these 4 possibilities or another explanation is the true explanation.

Our results show that by fusing rat CAP^r cytoplasts to either mouse HAT^r and CAP^s whole cells or karyoplasts and cultivating the resulting cells for 5 months in selective medium with CAP and HAT, only some CAP^r cell lines containing predominantly mouse mtDNAs could be observed. Thus, although the apparently discrepant results reported here may not always be observed, they indicate that it cannot be taken for granted that when drug-resistancy is employed as a cytoplasmic genetic marker, the mtDNA species in the drug-resistant interspecific cybrids or reconstituted cells must be the same as that of the drug-resistant parent cells. Thus the mtDNA species should be checked whenever cybrids, hybrids, or reconstituted cells are isolated, and especially after they have been cultivated for long time. The procedure described here may be widely applicable, since mtDNA species can be identified with only 0.1 ml packed cultured cells without labelling, and since even mtDNA types of intraspecific cybrids, hybrids, or reconstituted cells can be identified when parent cells are chosen that have mtDNAs showing different restriction endonuclease cleavage patterns.

We are examining the stage during prolonged cultivation at which cybrids and reconstituted cells lose most of the mtDNAs of L6TGCAP^r cell origin.

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