Simultaneous transfer of mitochondrial DNA and single chromosomes in somatic cells: a novel approach for the study of defects in nuclear–mitochondrial communication

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The assembly and function of respiratory-competent mitochondria in eukaryotic cells depends on collaboration between the nuclear and mitochondrial genomes, but the molecular mechanisms underlying such cross-talk are poorly understood. Microcell-mediated chromosome transfer has been used to transfer intact chromosomes from one mammalian cell to another, helping to map loci implicated in different diseases and in the senescence process. In the present work, we show that microcells have a significant number of mitochondria which can be transferred to another cell simultaneously with a limited number of chromosomes. By fusing microcells from a colon carcinoma cell line with a mitochondrial DNA (mtDNA)-less osteosarcoma cell line, we were able to isolate transmitochondrial hybrids containing only one of three selectable chromosomes and mtDNA from the donor cell. The proportion of transmitochondrial hybrids containing one chromosomal marker with respect to the total transmitochondrial hybrids and cybrids was ∼1% and no hybrids were isolated containing more than one nuclear marker. The genetic data correlated well with the composition and structure of the microcell preparations, which showed the presence of cytoplast-like structures and microcells containing mitochondria surrounding the micronuclei. Microcell-mediated mtDNA and chromosome transfer can be used to identify nuclear factors implicated in mtDNA maintenance and gene expression, as well as to investigate nuclear factors which modulate clinical phenotypes in mitochondrial disorders.

INTRODUCTION

Approximately 95% of the proteins residing in the mitochondria are nuclear gene products which play key roles in all aspects of mitochondrial function, including mitochondrial DNA (mtDNA) maintenance and gene expression. The molecular mechanisms that underlie cross-talk between the nuclear and mitochondrial genomes in eukaryotic cells, which are necessary to ensure the assembly and function of respiratory-competent mitochondria, are poorly understood. Only a few of the nuclear-encoded proteins involved in mitochondrial gene expression have been cloned and characterized in humans (1–8). A failure in nucleus–mitochondria communication can affect mitochondrial function, ultimately producing alterations in the integrity or maintenance of the mtDNA, such as multiple deletions (9,10) or depletion of mtDNA (11,12). The identification and characterization of unknown nuclear factors which play important roles in mitochondrial regulatory functions is necessary to unveil the mechanisms of mtDNA maintenance and gene expression.

The development of several cellular systems has enhanced the ability to map human genes. Interspecific somatic cell human–rodent hybrids (13,14) and the development of a complete set of human–rodent hybrid clones, each containing a different selectable human chromosome in the rodent cell background, provided a permanent source of specific human chromosomes for chromosome transfer and gene mapping studies (15). Microcell-mediated chromosome transfer (16–20) has been used to transfer intact chromosomes from one mammalian cell to another (21–23). In the last decade, studies of mitochondrial gene expression in mammalian cells were also facilitated by techniques to transfer mitochondria from one human cell to a mtDNA-less ρ− cell (transmitochondrial cybrids; 24) with a complete repopulation of recipient cells with the donor mtDNA.

The present work took advantage of the approaches described above to develop, in a single step, a system which allows the simultaneous transfer of a limited number of chromosomes and mtDNA to a cell depleted of mtDNA. This system can be a powerful tool to study and help identify nuclear genes controlling mtDNA maintenance and gene expression.

RESULTS

Testing the simultaneous transfer of mtDNA and a limited number of chromosomes

To determine if the microcell hybridization approach could be used to simultaneously transfer single chromosomes and mtDNA, we developed cell lines containing three chromosome

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Figure 1. Transmitochondrial hybrids production method. The human cell line SW480, derived from colon carcinoma, was stably transfected with plasmids containing genes for Zeo and Neo resistance (SW480<sup>Neo+/Zeo+</sup> cell line). These cells were used to produce microcells with a microenucleation protocol involving sequential treatment of the donor cells with colcemid and cytochalasin B (19,20). Immunocytochemical staining and transmission electron microscopy studies revealed the presence of four different cellular bodies in the microcell preparation. Microcells were fused with the 143B derivative 206<sup>ρ−</sup> cell line (without mtDNA). The hybrid cells were allowed to grow overnight and were selected for the presence of different markers.

markers, namely zeocin (Zeo) and neomycin (Neo) resistance and bromodeoxyuridine (BrdU) sensitivity. The presence of one of these markers was monitored together with the presence of mtDNA after hybrid preparation. Figure 1 depicts the protocol used. The results below describe the preparation and characterization of cell lines and microcells and the outcome of the hybrid fusion experiments.

Stable transfection of antibiotic resistance genes

SW480 cells were initially transfected with a plasmid containing a Zeo resistance gene. Total DNA extracted from isolated clones was analyzed by Southern blot using a Zeo gene probe (data not shown). One of the clones showing a single band (i.e. likely to have the gene inserted in a single chromosome) was transfected with a plasmid containing a Neo resistance gene. Positively selected clones were also analyzed by Southern blot using a Neo gene probe (see Materials and Methods). Clones showing a single band (clones A1 and A5; data not shown) were chosen for microcell production.

Composition and structure of microcell preparations

A better understanding of the composition of the microcell preparation was important to assess the usefulness of the microcell-mediated transfer of mtDNA. To test for the presence of mitochondria, cells and microcells were incubated with a monoclonal antibody against succinate dehydrogenase (Fp unit; Fig. 2,1) or cytochrome c oxidase (COX IV subunit; data not shown) and co-stained with Hoechst 3342 for nuclei visualization (Fig. 2,2). Cells growing in regular medium showed a typical pattern of staining with mitochondria in red and nuclei in blue (Fig. 2A). After treatment of cells with colcemid, micronucleation was observed (Fig. 2B). The microcell preparation after enucleation of colcemid-treated cells using cytochalasin B and centrifugation showed a heterogeneous composition: cytoplasts containing mitochondria (Fig. 2C right and E), microcells containing mitochondria and a single micronucleus (Fig. 2C left and D) and microcells containing mitochondria and several micronuclei (Fig. 2F). Practically all nuclear-containing microcells observed harbored a perimicronuclear cytoplasmic region with an SDH-positive signal. The vast majority (∼70%) of the cellular bodies showed mitochondrial staining, but no detectable nuclear staining. Rarely, nuclear fragments without mitochondria were also observed.

The structure and composition of the microcell preparation was also analyzed by transmission electron microscopy. After 36 h treatment with colcemid, ∼40% of micronucleated SW480<sup>Neo+/Zeo+</sup> cells were obtained (data not shown). Figure 3A shows a section of a micronucleated cell presenting four micronuclei. The heterogeneous composition of the microcell preparation was confirmed at the ultrastructural level. In ∼80% of the cellular bodies no nuclear material was detected, though most of these had identifiable mitochondria (Fig. 3D). Approximately 20% of the bodies had identifiable nuclei containing a mitochondrial fraction in the cytoplasmic perimicronuclear region (Fig. 3B and C).
Figure 2. Identification of mitochondrial polypeptides in microcells. Immunocytochemical staining of SW480Neo/Zeo cells either (A) non-treated, (B) treated with colcemid or (C–F) microcells (µCells) obtained from this cell line. (1) MITO. To detect the presence of mitochondria, cells and microcells were incubated with a monoclonal antibody against succinate dehydrogenase (Fp subunit) and Texas Red-conjugated AfiniPure donkey anti-mouse IgG. (2) NUCL. Coverslips were incubated with Hoechst 3342 to reveal the nuclei. (3) OVERLAY. Superposition of images 1 and 2. (A) Cells growing in regular medium showed a typical pattern of mitochondrial staining. (B) After treatment with colcemid, micronucleation is produced. (C–F) The microcell preparation after enucleation of colcemid-treated cells using cytochalasin B and ultracentrifugation showed a heterogeneous composition: cytoplasts containing mitochondria (C right and E), microcells containing mitochondria and a single micronucleus (C left and D) and microcells containing mitochondria and more than one micronucleus (F).

Microcell-mediated mitochondria and chromosome transfer

We performed several experiments to test the ability of microcell-mediated chromosome transfer to simultaneously transfer mtDNA. The results of these experiments are summarized in Figure 4. Initially, we produced microcells from 143B cells and from its derivative 143B/206ρ cell line and fused them to 143B/206ρ cells to assess whether mitochondria could be transferred using the microcell fusion system (Fig. 4 A). Selection for the presence of mtDNA with a medium lacking uridine (Ur–) yielded a high number of hybrid clones (~200 clones) using 143B microcells and no clones using the 143B/206ρ microcells. To address the question of whether or not mitochondria could be transferred together with one or a few chromosomes, microcells produced from SW480Neo/Zeo cells (clones A1 and A5) were fused to 143B/206ρ cells (Fig. 4B–D). Hybrids were independently selected for the presence of one of the three chromosome markers: positively for neomycin resistance with a medium supplemented with BrdU; negatively for thymidine kinase activity in medium supplemented with BrdU. The presence of mtDNA was indicated by growth in medium without uridine. Three different experiments were performed (Fig. 4B–D) which revealed that: (i) the proportion of hybrid clones that received any selected chromosome with respect to the clones selected for the presence of mtDNA was ~1%; (ii) the number of clones selected for the presence of any chromosome marker was essentially the same whether or not the selection system included selection for the presence of mtDNA; (iii) no clones were obtained which contained more than one chromosome marker; (iv) the same number of Neo- and Zeo-resistant clones was obtained whether or not the corresponding antibiotic was added from the beginning of the hybrid selection process, indicating that tagged chromosomes were, for the most part, conserved even when no selection was applied (the segregation pattern of the different markers also indicates that the genes responsible for Neo and Zeo resistance were not integrated in the same chromosome); (v) seven out of eight clones selected only for the presence of the chromosome markers for at least 45 days did not grow in medium lacking uridine, demonstrating that in the majority of these clones repopulation of mtDNA was not achieved if selection was not applied; and (vi) similar results were obtained independently of the original SW480Neo/Zeo clone used.

Hybrid clone characterization

The presence of the Neo and Zeo resistance genes in hybrids was confirmed by PCR using gene-specific primers (see Materials and Methods; Fig. 5A and B). To determine if the mtDNA present in
the hybrids originated from the SW480Neo/Zeo cells and not from a reversion of the 143B/206 ρ+ cells to the parental phenotype (143B), PCR–RFLP studies were performed using a known mtDNA polymorphism. All transmitochondrial hybrids analyzed had the same mtDNA restriction pattern as observed in SW480 cells (Fig. 5C), indicating that mtDNA had been introduced through microcell-mediated transfer together with the chromosomal markers.

KCN-sensitive endogenous cell respiration and the mtDNA/nDNA ratio of the Neo- and Zeo-resistant hybrids selected for the presence of mtDNA was indistinguishable from that of the parental 143B and SW480Neo/Zeo cells (clones A1 and A5; Fig. 6A and B). Under these selection conditions, transfer of mitochondria into 143B/206 ρ+ cells via microcell hybridization allowed for the restoration of normal mitochondrial function. In contrast, seven out of eight hybrids obtained with Neo or Zeo selection but without selection for the presence of mtDNA had oxygen consumption rates and mtDNA relative levels similar to that observed in the 143B/206 ρ+ cells. Only one of the hybrids obtained without selection for respiratory function ([HyA1Z4(Ur+/Zeo−)]) exhibited a respiratory capacity which was similar to the parental cell lines.

**DISCUSSION**

Using the microcell hybridization system, we have been able to simultaneously transfer a limited number of chromosomes and mtDNA to a mtDNA-less human cell, a system that can have wide applicability to the study of nuclear–mitochondrial communication. A detailed description of the structure of mitochondria-containing microcells was one of the goals of this work. After micronucleation by colcemid treatment, cytochalasin B-induced enucleation produced bodies (microcells) which consisted of a single or a few micronuclei and a thin rim of cytoplasm surrounded by an intact plasma membrane (25). Previously, it has been shown that karyoplasts produced by cytochalasin B-induced enucleation contained ∼11% of the mitochondrial volume of whole cells (26). These results suggested that microcells could contain a significant mitochondrial fraction. Using optical and electron microscopy studies, we showed that the cytoplasmic region contains a variable number of mitochondria. However, the composition of microcell preparations was very heterogeneous, presenting small cytoplasts containing mitochondria, microcells containing mitochondria and a single micronucleus, microcells containing mitochondria and a few micronuclei and microcells containing no mitochondria. The proportion of micronuclei-containing microcells was ∼30% by immunocytochemistry and ∼20% by electron microscopy. Ultrastructural estimations are probably less accurate because ultrathin sections may not include the micronucleus. These observations indicate that during the hybridization process many recipient cells receive mtDNA from the donor cells, with or without donor chromosome(s). This aspect should be taken into account when studies on restoration of normal maintenance and expression of mtDNA in defective cells are carried out using the microcell-mediated chromosome transfer system.

The proportion of hybrid clones that received a selectable chromosome to the clones selected only for the presence of mtDNA was ∼1%. This proportion was relatively constant in all experiments, but it could vary depending on the cell type used as the microcell donor. Considering that 70% of the cellular bodies in the microcell preparation are probably cytoplasts and that more than one micronucleus may be present in some microcells, the observed proportion for chromosome transfer was compatible with the theoretical 1/46 for human cells. The number of clones selected for the presence of every chromosome marker was essentially the same whether selection for the presence of mtDNA was included or not, suggesting that microcells containing the selected chromosomes also contained mtDNA. Using microcell-mediated chromosome transfer, hybrids are likely to receive donor chromosomes randomly, but it is possible that some chromosomes are preferentially transferred. Our results indicate that at least the three selectable chromosomes used in this work were transferred at a similar rate. We were not able to detect more than one transchromosome marker in each hybrid, indicating that most microcells contained one single tagged chromosome; nevertheless, the presence of a few plurimicronucleated microcells suggests that clones containing more than one donor chromosome could also be generated during fusion. These results also confirm that no clones were recovered resulting from fusions with intact donor cells contaminating the microcell preparation, attesting to the efficiency of the purification procedure employed.

The hybrid lines selected for the presence of one of the chromosome markers and also for the presence of mtDNA resembled the cell parent in both KCN-sensitive endogenous cell
Figure 4. Hybrid selection conditions and results. The figure summarizes the results of four different experiments examining the transfer of mtDNA and nuclear DNA markers. The number of clones growing under each type of selection is shown in parentheses. n/72 indicates the number of positive clones out of 72 clones analyzed. (A) Microcells (µ) produced from 143B cells and from its derivative 143B/206 p" cell line were fused to 143B/206 p" cells and selected for the presence of mtDNA in medium lacking uridine. (B–D) Microcells (µ) produced from SW480 Neo/Zeo cells (clones A1 and A5) were fused to 143B/206 p" cells. Hybrids were selected for the presence of three chromosome markers: the Neo resistance gene in medium supplemented with G418 (N), the Zeo resistance gene by supplementing the medium with zeocin (Z) and the thymidine kinase gene (TK) in medium with BrdU. The presence of mtDNA was selected for in medium lacking uridine (Ur - ).

respiration and the mtDNA/nDNA ratio, indicating that the transfer of mitochondria into 143B/206 p" cells via microcell hybridization allowed the restoration of normal mitochondrial function under selective conditions. It is intriguing that when hybrids were obtained with Neo or Zeo resistance selection but without selection for the presence of mtDNA, mtDNA repopulation and normal respiration was observed in only one out of eight hybrids obtained. These results indicate that, in most cases, some selective pressure is necessary for repopulation of mtDNA introduced into cells by this system. Although the quantity of mtDNA molecules in a microcell probably influences mtDNA repopulation in hybrids, in most cases (87% in our experiments) microcell hybridization is not expected to generate cell lines with exogenous mtDNA in the absence of selective pressure.

The establishment and characterization of transmitochondrial hybrids could be helpful in the elucidation of different aspects of nuclear–mitochondrial cross-talk. (i) To help identify nuclear factors implicated in species-specific mtDNA maintenance. Recently, we showed that mtDNA from common and pigmy chimpanzee and gorilla were able to restore oxidative phosphorylation in the context of a human nuclear background, whereas mtDNA from orangutan and other primates were not (27). We are presently attempting to produce xenomitochondrial hybrids to identify the factor(s) responsible for the acceptance of exogenous mtDNA from other species. A similar approach can be used in studies of hybrid systems of cells from two different species or strains, where one of the mitochondrial populations is preferentially selected for (28–33). By introducing mtDNA and a chromosome(s) from the ‘non-dominant’ species, one may allow for the co-existence of the two mtDNA populations or a change in mtDNA ‘preference’.(ii) To study nuclear factors that modulate mtDNA expression. This can be exemplified by the case of patients harboring Leber hereditary optic neuropathy (LHON) mutations, in which expression of mtDNA mutations seems to be modulated by nuclear factors. LHON is primarily caused by a mutation(s) of mtDNA which produces a mitochon-
Mitochondrial DNA (mtDNA) quantification and mitochondrial function studies were performed on total DNA purified from parental cell lines 143B, SW480, SW480<sup>Neo</sup>, SW480<sup>Zeo</sup> and SW480<sup>Neo/Zeo</sup> (clones A1 and A5) and hybrids selected for the presence of mtDNA and one of the chromosome markers [HyA1N1(Ur<sup>−</sup>/Neo<sup>+</sup>), HyA1Z1(Ur<sup>−</sup>/Zeo<sup>+</sup>), HyA5N1(Ur<sup>−</sup>/Neo<sup>+</sup>) and HyA5Z1(Ur<sup>−</sup>/Zeo<sup>+</sup>)]. (A) The presence of the Neo (NEO) and Zeo (ZEO) resistance genes was monitored by PCR using specific primers (see Materials and Methods). MW, 100 bp molecular weight marker. (B) ΔDeI restriction digest of a 3.1 kb PCR-amplified mtDNA region (nt 7407–10542). An additional ΔDeI site was found in mtDNA from 143B cells that was not present in mtDNA from SW480 cells. All transmitochondrial hybrids analyzed had the same mtDNA restriction pattern as SW480 cells, indicating that mtDNA was introduced through microcell-mediated transfer, together with the chromosomal markers.

Figure 5. Genetic characterization of the transmitochondrial hybrids. Studies were performed on total DNA purified from parental cell lines 143B, SW480, SW480<sup>Neo</sup>, SW480<sup>Zeo</sup> and SW480<sup>Neo/Zeo</sup> (clones A1 and A5) and hybrids selected for the presence of mtDNA and one of the chromosome markers [HyA1N1(Ur<sup>−</sup>/Neo<sup>+</sup>), HyA1Z1(Ur<sup>−</sup>/Zeo<sup>+</sup>), HyA5N1(Ur<sup>−</sup>/Neo<sup>+</sup>) and HyA5Z1(Ur<sup>−</sup>/Zeo<sup>+</sup>)]. (A) The presence of the Neo (NEO) and Zeo (ZEO) resistance genes was monitored by PCR using specific primers (see Materials and Methods). MW, 100 bp molecular weight marker. (B) ΔDeI restriction digest of a 3.1 kb PCR-amplified mtDNA region (nt 7407–10542). An additional ΔDeI site was found in mtDNA from 143B cells that was not present in mtDNA from SW480 cells. All transmitochondrial hybrids analyzed had the same mtDNA restriction pattern as SW480 cells, indicating that mtDNA was introduced through microcell-mediated transfer, together with the chromosomal markers.

Figure 6. Mitochondrial DNA (mtDNA) quantification and mitochondrial function in transmitochondrial hybrids. Studies were performed on parental cell lines 143B, 143B/206<sup>ρ</sup> and SW480<sup>Neo/Zeo</sup> (clones A1 and A5) and hybrids selected for the presence of mtDNA and one of the chromosome markers [clones HyA1N1(Ur<sup>−</sup>/Neo<sup>+</sup>) and HyA5Z1(Ur<sup>−</sup>/Zeo<sup>+</sup>)] and hybrids selected for the presence of one of the chromosome markers but not for the presence of mtDNA. At least 45 days after selection, clones from the latter group were selected for the presence of mtDNA. Seven did not survive [clones HyA1N3(Ur<sup>−</sup>/Neo<sup>+</sup>) and HyA1N4(Ur<sup>−</sup>/Neo<sup>+</sup>) represented in the figure] but one did (HyA1Z2(Ur<sup>−</sup>/Zeo<sup>+</sup>)). Results are expressed as percentages of the values obtained for cell line 143B. (A) KCN-sensitive endogenous cell respiration. Oxygen utilization was measured polarographically with a Clark oxygen electrode. (B) Quantification of mtDNA relative to the nuclear DNA (nDNA) was performed as described in Materials and Methods.

drial complex I deficiency (34; reviewed in ref. 35), but several aspects of its inheritance (i.e. male predominance, reduced penetrance and later age of onset in females) cannot be explained by mitochondrial inheritance alone. A model has been proposed in which a pathogenic mtDNA mutation interacts with an X-linked visual loss susceptibility locus (36,37), but pedigree and linkage analyses have given controversial results (38,39). If this modulatory phenotype could be measured in cultured cells (unfortunately this is not yet possible), the system described here could be useful to study hypothetical nuclear factors modulating mtDNA expression. Likewise, some dominant Mendelian disorders can have differential penetrance depending on mtDNA haplotypes. Strategies could be devised to study this concerted effect of nuclear and mtDNA in cultured cells using this system.

In conclusion, microcell-mediated mtDNA and chromosome transfer allows the generation of cell lines that could help to elucidate different aspects of nucleus–mitochondria cross-talk, such as providing the chromosomal location of nuclear factors implicated in mtDNA maintenance and gene expression, as well as of nuclear factors which modulate clinical phenotypes in mitochondrial diseases.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

The human cell line SW480, derived from a colon carcinoma, and the line SW480<sup>Zeo</sup>, stably transfected with plasmid pVgXR (Invitrogen, San Diego, CA) containing a gene for Zeo resistance, were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 mg/ml sodium pyruvate. The SW480<sup>Neo</sup> medium was additionally supplemented with 300 µg/ml Zeo (Invitrogen).

The human osteosarcoma-derived cell line 143B(TK<sup>−</sup>) and its derivatives, i.e. donor cells, by sequential treatment with 50 ng/ml colcemid (Gibco BRL) (48 h for SW480 derivatives or 36 h for SW480<sup>Neo</sup> and SW480<sup>Zeo</sup>). Stable transfections were performed using a Lipofectamine kit (Gibco BRL, Grand Island, NY) following the manufacturer’s instructions, selecting cells in a medium containing 450 µg/ml G418 (Gibco BRL). Clones were isolated by ring cloning and after 20 days total DNA was extracted using standard procedures. To estimate the number of insertion sites, Southern blot analyses were performed after digesting the total DNA with SpeI (Boehringer Mannheim, Indianapolis, IN), an endonuclease that does not digest the transfected DNAs, and using an [α-<sup>32</sup>P]dCTP-labeled 287 bp Neo gene probe or a 366 bp Zeo gene probe. The probes were obtained by PCR amplification using the primer pairs Neo47-F (nt 47–65)/Neo334-B (nt 334–315) and Zeo6-F (nt 6–26)/Zeo372-B (nt 372–354) and purification of the products with the Qiaquick Spin PCR purification kit (Qiagen, Chatsworth, CA).

**Microcell isolation**

Microcells were isolated essentially as described (19,20). Briefly, microcells were generated from SW480<sup>Neo/Zeo</sup> or 143B derivatives, i.e. donor cells, by sequential treatment with 50 ng/ml colcemid (Gibco BRL) (48 h for SW480 derivatives or 36 h for 143B derivatives), which arrests the cells in mitosis and results in the formation of micronuclei, and 10 µg/ml cytochalasin B.
Purified microcells were centrifuged at 2000 r.p.m. for 10 min at 34°C. The microcell pellet was placed into 100 ml DMEM and filtered sequentially through 12, 8, 5 and 5 µm Nucleopore filters (Costar, Cambridge, MA). The purified microcells were used for cytochemical staining, electron microscopy and hybridization experiments.

Immunocytochemical staining

SW480NeoZeo cells, both treated and non-treated with colcemid, and microcells obtained from this cell line were seeded on glass coverslips, washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin. Cells were incubated for 4 h with monoclonal antibodies against COX IV subunit or the succinate dehydrogenase flavoprotein (Fp) subunit in PBS with 10% FBS. After washing with PBS, coverslips were incubated for 2 h with Texas Red-conjugated AfiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 10% FBS and washed again with PBS. Coverslips were incubated for 30 min with 10 µg/ml bis-benzimide Hoechst 3342 (Sigma) in PBS to reveal the nuclei, washed with PBS and mounted on glass slides with a ProLong™ Antifade kit (Molecular Probes). Fluorescence was inspected with a Wild Leitz photomicroscope (Fluovert Heerbrugg, Switzerland).

Electron microscopy

Colcemid-treated and non-treated SW480NeoZeo cells and microcells obtained from this cell line were prepared for transmission electron microscopy as previously described (26). The sections were examined in a JEM-IOO CXII electron microscope (Jeol, Tokyo, Japan) at 60 kV.

Microcell-mediated mitochondrial and chromosome transfer

Purified microcells were centrifuged at 2000 r.p.m. for 10 min at 4°C and resuspended in 6 ml lecit solution (Sigma) to promote microcell adherence to recipient cells. The 143B derivative 206 p+ cell line, used as recipient cells, were grown in six 60 mm tissue culture dishes. The recipient cells were washed in DMEM and incubated with the microcell solution (1 ml/dish) for 30 min at 37°C. Cell fusion was performed by treatment with freshly prepared 46% polyethyleneglycol 1450 (Boehringer Mannheim) solution (pH 7.5) for 1 min. The hybrid cells were allowed to grow overnight and selected for the presence of the different markers. Microcells were also produced from 143B and 143B/206 p− cells, fused independently from 143B/206 p+ and selected for the presence of mtDNA in medium lacking uridine.

Hybrid clone selection

Hybrids were selected for the presence of the Neo or the Zeo resistance genes by supplementing the growth medium with 450 µg/ml G418 or 300 µg/ml zeocin, respectively. Because the 143B/206 p− cells have a mutation in the thymidine kinase gene (TK− cells), the presence of chromosome 17 (the TK locus) could be also selected for by growth in the presence of BrdU. Cell hybrids were maintained in adequate selection media. Selection for the presence of functional mitochondria (presence of mtDNA) was performed using a medium without uridine (U−).

Hybrid clone genetic characterization

Total DNA was extracted from cells following standard procedures, after at least 30 days under selection. The presence of the Neo and Zeo genes was monitored by PCR using primers Neo47-F/Neo 334−B and Zeo6−F/Zeo372−B, respectively. To ensure that the mtDNA present in the hybrids came from SW480Neo cells and not from a reversion of 143B/206 p+ cells to the parental phenotype, we amplified a 3.1 kb mtDNA region using primers Asu7416−F (nt 7407−7427) and Spe10526−B (nt 10542−10522) and searched for differences in the restriction pattern using several endonucleases. An additional DdeI site was found in the mtDNA from 143B cells which differed from SW480 cells.

Cell respiration studies and mtDNA quantification

Exponentially growing cells were collected by trypsinization, pelleted, resuspended in cold PBS and used for the different studies. The protein content in the cell samples was determined by the Bradford method (41). Parental cell lines 143B, 143B/206 p+ and SW480Neo clones A1 and A5, hybrids doubly selected for the presence of mtDNA and for one chromosome marker [HyA1N1(Ur−/Neo+), HyA1Z1(Ur−/Zeo+), HyA5N1 (Ur+/Neo+)] and HyA5Z1(Ur+/Zeo+) and hybrids selected for the presence of one of the chromosome markers but not for the presence of mtDNA [HyA1N3(Ur+/Neo+), HyA1N4 (Ur+/ Neo+), HyA1Z3(Ur+/Zeo+) and HyA1Z4(Ur+/Zeo+)] were used for these studies. Oxygen utilization was measured polarographically in 0.3 ml standard medium (0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, 1 mg/ml BSA, 10 mM KH2PO4, pH 7.4) with a Clark oxygen electrode in a micro water-jacketed cell, magnetically stirred, at 37°C (Hansatech Instruments, Norfolk, UK). After measurement of intact cell-coupled endogenous respiration, the reaction was inhibited with KCN (700 µM).

To determine if clones selected for the presence of the chromosome markers were repopulated by the donor’s mitochondria, the mtDNA content relative to the nDNA was quantified by a slot blot experiment, using 956 bp human mtDNA (nt 3305−4261) and 5.8 kb nuclear 18S rDNA (42) [or32P]dCTP-labeled probes. For each probe we used three slots containing 100, 200 and 300 ng total DNA. After scanning the autoradiograms, band signals were quantified using NIH Image 1.62 b7 software. The mtDNA:nDNA ratio was considered as the division of the arbitrary densitometric values of the signals using each probe.

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