The production of in vitro and in vivo models of mitochondrial DNA (mtDNA) defects is currently limited by a lack of characterized mouse cell mtDNA mutants that may be expected to model human mitochondrial diseases. Here we describe the creation of transmembrane mouse (Mus musculus) cells repopulated with mtDNA from different murid species (xenomitochondrial cybrids). The closely related Mus spretus mtDNA is readily maintained when introduced into Mus musculus mtDNA-less (ρ0) cells, and the resulting cybrids have normal oxidative phosphorylation (OXPHOS). When the more distantly related Rattus norvegicus mtDNA is transferred to the mouse nuclear background the mtDNA is replicated, transcribed, and translated efficiently. However, function of several OXPHOS complexes that depend on the coordinated assembly of nuclear and mtDNA-encoded proteins is impaired. Complex I activity in the Rattus xenocybrid was 46% of the control mean; complex III was 37%, and complex IV was 78%. These defects combined to restrict maximal respiration to 12–31% of the control and the resulting cybrids have normal oxidative phosphorylation (OXPHOS). When the more distantly related Rattus norvegicus mtDNA is transferred to the mouse nuclear background the mtDNA is replicated, transcribed, and translated efficiently. However, function of several OXPHOS complexes that depend on the coordinated assembly of nuclear and mtDNA-encoded proteins is impaired. Complex I activity in the Rattus xenocybrid was 46% of the control mean; complex III was 37%, and complex IV was 78%. These defects combined to restrict maximal respiration to 12–31% of the control and the resulting cybrids have normal oxidative phosphorylation (OXPHOS). When the more distantly related Rattus norvegicus mtDNA is transferred to the mouse nuclear background the mtDNA is replicated, transcribed, and translated efficiently. However, function of several OXPHOS complexes that depend on the coordinated assembly of nuclear and mtDNA-encoded proteins is impaired. Complex I activity in the Rattus xenocybrid was 46% of the control mean; complex III was 37%, and complex IV was 78%. These defects combined to restrict maximal respiration to 12–31% of the control and the resulting cybrids have normal oxidative phosphorylation (OXPHOS). When the more distantly related Rattus norvegicus mtDNA is transferred to the mouse nuclear background the mtDNA is replicated, transcribed, and translated efficiently. However, function of several OXPHOS complexes that depend on the coordinated assembly of nuclear and mtDNA-encoded proteins is impaired. Complex I activity in the Rattus xenocybrid was 46% of the control mean; complex III was 37%, and complex IV was 78%. These defects combined to restrict maximal respiration to 12–31% of the control and the resulting cybrids have normal oxidative phosphorylation (OXPHOS).
NAs and showed defective respiratory chain complex I with preserved function of the other complexes (14).

Here we report the introduction of mtDNAs from different murid species into mouse cells to produce mouse xenomitochondrial hybrids. We have introduced closely related (Mus spretus) and more distantly related (Rattus norvegicus) murid mtDNAs into a Mus musculus ρ0 cell line, which in the latter case results in multiple respiratory chain defects similar to some human mtDNA diseases.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—All cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C and 5% CO2. Primary fibroblast lines were created from a 2-day-old laboratory mouse (M. musculus, CBA/C57B16 cross) and 5-week-old M. spretus (Spr/Ei, a gift from Dr. Simon Foote, Walter & Eliza Hall Institute). Skin from the belly of euthanized mice was washed with phosphate-buffered saline with 2% penicillin/streptomycin (Life Technologies, Inc.) and incubated with 0.25% trypsin (Life Technologies, Inc.) in RPMI medium for 4 h at 4 °C, incubated at 37 °C for 20 min, then passed through a tissue sieve using a glass pestle, and plated in complete medium in 75-cm2 tissue culture flasks (Nunc, Denmark). Media were replaced twice weekly, and after 2–3 weeks the primary cultures began to grow vigorously, and aliquots were viably frozen.

Two independent mouse ρ0 cell clones were used, designated LMBE3 and LMBE5. These were produced from the parental line LMTK by exposure to ethidium bromide and are described in detail elsewhere (15). These mtDNA-less cells are auxotrophic for uridine and pyruvate (see below). The medium was supplemented with glucose to 4.5 mg/ml, uridine, 50 μg/ml, and pyruvate, 1 mM (RPMI/GUP medium).

The following cell lines were obtained from the ATCC: NRK52E (R. norvegicus kidney epithelial cells), RNIT (R. norvegicus mammary tumor line), Chinese hamster ovary cells (Cricetulus griseus), and Syrian hamster kidney cells (Mesocricetus auratus).

Promitochondrial Cybrids—Cybrids were produced by enucleation of mitochondrial donor cells and fusion of the cytoplasts with mouse ρ0 cells following selection for respiratory-competent transformants as described in detail previously for human cells (17). Cells used as mitochondrial donors included the mouse primary fibroblast line, the M. spretus primary fibroblast line, the Rattus lines NRK52E and RNIT, and the hamster lines Chinese hamster ovary cells and Syrian hamster kidney. Briefly, 5 × 105 cells were treated with 50 μg/ml cytochalasin B (Sigma). The cytoplast/karyoplast mixture was combined with 2 × 106 mouse ρ0 cells and then centrifuged at 20,000 × g for 10 min; the supernatant was aspirated and the pellet overlaid with polyethylene glycol (Sigma) for 1 min. The polyethylene glycol was then removed, and the cells were gently resuspended in complete medium and plated at 105 and 106 cells per 100-mm dish in RPMI-GUP medium. After 24 h the medium was replaced with select medium as follows: RPMI supplemented with 5% dialyzed fetal bovine serum (Life Technologies, Inc.) and 50 μg/ml bromodeoxyuridine (Sigma). In this medium lacking uridine and pyruvate the ρ0 cells cannot grow, and the bromodeoxyuridine kills surviving TK− mitochondrial donor cells so that only LMTK cells can survive. After 7–14 days cybrid clones were isolated using cloning cylinders, expanded, and viably frozen. Three independent clones were frozen from each successful fusion experiment, and cybrids were used in experiments from passage 5 through passage 10.

To confirm the nuclear origin of the cybrids, karyotyping of a control cybrid and a Rattus xenocybrid clone was performed. Cells were grown in RPMI/GUP medium in a 25-cm2 flask. Cells were treated with 50 ng/ml demecolcine (Sigma) for 5 h, harvested, resuspended in 10 ml of 75 mM KCl, and incubated at room temperature for 12 min. Cells were pelleted before being resuspended in 5 ml of fresh methanol/acetic acid (3:1). Cells were fixed overnight at 4 °C, pelleted, washed once in fixative, and then resuspended to give a pale milky solution. Cells were then dropped onto wet, acid-washed slides from a distance of 70 cm, allowed to dry, and fixed with GetGear X2 fixative, and then stained with Giemsa (17).

mtDNA Genotyping by DNA Sequencing—A mtDNA D-loop fragment was generated by PCR from genomic DNA isolated from cybrid clones.

Primers were chosen to enable amplification from both mouse (18) and R. norvegicus (19) mtDNAs. The mouse ampiclon was 461 base pairs in length, whereas the rat ampiclon was 496 base pairs due to intervening sequence insertions compared with the mouse mtDNA (18, 19). The forward primer sequence was 5′-ctc aac atc gta gct gac gc-3′ representing nucleotides 15934–15953 (18), and the reverse primer was 5′-acc aaa ctc tgt tga ttg gtt g-3′ representing nucleotides 59–80. PCR was performed using 1.0 unit of Taq polymerase (Life Technologies, Inc.), 20 ng of DNA, and 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min.

Sequencing was performed using 60 ng of purified ampiclon, with a Thermoc Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech). Sequencing reactions were visualized on an ABI 377 automated sequencer.

Southern Blot Determination of mtDNA—The mouse and rat D-loop PCR amplifiers described above were used to probe mtDNA, whereas a PCR fragment of mouse 18 S rDNA was used as a nuclear probe. This PCR amplimer was 501 base pairs in length; the forward primer sequence was 5′-ctg tga ttc tag atc taa tac atg ccc-3′, representing positions 151–180 of the mouse 18 S rDNA (GenBank accession number X00686). The reverse primer sequence was 5′-tat acg cta tga ctt gct gaa gaa cc-3′, representing nucleotides 626–651. Probes were labeled with [32P]dCTP using a RediPrime II random prime labeling kit (Amersham Pharmacia Biotech). For each lane of a 0.85% agarose gel, 5 μg of genomic DNA was loaded after restriction with ScaI (Life Technologies, Inc.), which has a single restriction site in mouse and rat mtDNA. The transfer, probe annealing, and washing was done using standard conditions.

Mitochondrial Protein Translation—Mitochondrial translation products were labeled with [35S]methionine as described previously (20). Approximately 105 cells in single wells of a 24-well tissue culture dish (Nunc) were washed with methionine-free RPMI before adding 0.5 ml of the same medium containing 0.1 mg/ml cycloheximide and incubating the cells at 37 °C, 5% CO2 for 15 min. After this preincubation 50 μCi of [35S]methionine (ICN) was added, and the cells returned to the incubator for 2 h. Cold methionine (0.1 mM, Sigma) was then added, and the cells were incubated a further 30 min. Cells were immediately harvested, centrifuged, and frozen at −80 °C.

Cell pellets were prepared for electrophoresis by thawing and dilution in 50 nM Tris, pH 6.8, containing 0.1% SDS and 1 mM β-mercaptoethanol. The cell lysates were sonicated with 3 pulses at setting 5 using a Tosco sonicator (MSE, Melbourne, Australia). Electrophoresis of labeled proteins was performed using 15% acrylamide (1.36 bisacylamide) mini-gels (CBS Scientific, Del Mar, CA) with 10 μg of cellular protein per lane. Gels were electrophoresed at 100 V for 30 min, fixed in methanol/acetic acid, treated with "Amplify" (Amersham Pharmacia Biotech), and dried for autoradiography.

Lactate Measurement—Lactate was measured in media using a commercial lactate kit (Sigma). Cells were grown to confluence in 24-well culture dishes, and the media were replaced with 0.5 ml of fresh media after two rinses with 1 ml of media, and the cells were incubated for 10 h. Samples of media were removed at 2 h intervals and tested immediately for lactate concentration.

Mitochondrial Isolation and Polargraphic Analysis—Mitochondrial isolation and polargraphy using freshly isolated mitochondria was performed in standard conditions (16). Details for preparation of bovine mitochondria (17). Control and xenomitochondrial cybrids (xenocybrids) were grown in paired cultures so that a control was always processed alongside the experimental xenocybrid culture. Cultures were expanded to around 106 cells by seeding 2 × 105 cells into roller bottles (Corning) in 200 ml of complete medium, expanded after 4 or 5 days to 1000 ml, and then harvested after another 4 days.

Mitochondria were isolated by digitonin lysis and homogenization, followed by differential centrifugation (17). The mitochondria were resuspended to around 20 mg/ml protein in isolation buffer and used immediately for polargraphic analysis. Protein was measured according to the Lowry method. Aliquots were also frozen at −80 °C for enzymological analysis.

Polargraphy was performed using an Instech (Plymouth Meeting, PA) microelectrode and chamber coupled with a magnetic stirrer and an Instech model 203 dual oxygen electrode amplifier with output to a chart recorder. Each mitochondrial isolate was tested for respiratory capacity using pyruvate plus malate, glutamate plus malate, and succinate as substrates. Two runs were performed with each substrate, and in each run two additions of ADP were made. Conditions and experiments were as described previously (17).

OXPHOS Enzymology—Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3), complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1), complex II + III (succinate:cytochrome c oxidoreductase), and complex IV (ferrocytochrome-c:oxygen oxidoreductase, or cytochrome oxidase, EC 1.9.3.1) activities were measured spectrophotometrically, essentially as described previously (17), with the following modific
**Mouse Xenomitochondrial Cybrids**

**Fig. 1. Brief phylogeny of the Muridae showing divergence times estimated from molecular studies (22–24).** Divergence times are indicated by the scale below the phenogram, in millions of years before present (m.y.b.p.). Although *M. spreptus* and *R. norvegicus* (subfamily Murinae) mtDNA could readily be maintained in *M. musculus* cells, *Cricetulus* and *Mesocricetus* (subfamily Cricetinae) mtDNA could not.

**TABLE I**

| mt gene product | Identity | No. of amino acids | MtDNA encoded |
|-----------------|----------|-------------------|---------------|
| NAD1            | 90       | 318 315 312       | 32            |
| NAD2            | 74       | 345 345           | 88            |
| COX I           | 97       | 514 514           | 14            |
| COX II          | 99       | 227 227           | 3             |
| ATP8            | 79       | 67 67             | 14            |
| ATP6            | 95       | 226 226           | 12            |
| COX III         | 97       | 261 261           | 9             |
| NAD3            | 87       | 115 114           | 15            |
| NAD4L           | 86       | 98 97             | 14            |
| NAD4            | 87       | 459 459           | 59            |
| NAD5            | 78       | 610 607           | 133           |
| NAD6            | 80       | 172 172           | 34            |
| Cytochrome b    | 93       | 380 381           | 26            |

**Comparison of *M. musculus* and *R. norvegicus* mitochondrial translation products**

The cybrids made from RN1T cells showed a single nucleotide difference to the *R. norvegicus* sequence, an insertion of a C in a 5-C tract at nucleotide pair 16075. This cell line is listed by ATCC as *Rattus rattus*, but this almost perfect identity with the *R. norvegicus* sequence indicates it is also derived from *R. norvegicus*, as *R. rattus* would be expected to show many more sequence differences in this highly polymorphic D-loop region, based on molecular studies of these two species (25).

**RESULTS**

**Production of Transplantidional Cybrids—**Control cybrids produced by fusion of the ρ₀ cell clone LMEB3 with enucleated *M. musculus* primary fibroblasts were obtained at a frequency of around one per 5 × 10⁶ ρ₀ cells used. Similar cybrid frequencies were obtained in a fusion of LMEB3 with enucleated *M. spreptus* cells (diverged from *M. musculus* 2–3 million years ago, see Refs. 22–24, and see Fig. 1). Both the control cybrids and *M. spreptus* xenomitochondrial cybrids were similar in appearance and growth characteristics to each other and to the parental LMTK⁺ cell line. Fusions using enucleated *Rattus* NRK52E cells with LMEB5 and enucleated *Rattus* RN1T cells with LMEB3 also produced cybrids at high frequencies, around one per 10⁵ ρ₀ cells used. However both these sets of cybrid clones grew more slowly, showed a morphology intermediate between the control cybrids and the ρ₀ cells, and acidified the media much more quickly than the control cybrids. The *Mus/Rattus* divergence is dated at around 10 million years ago (Fig. 1).

Table I summarizes the amino acid sequence differences between the *M. musculus* and *R. norvegicus* mtDNA-encoded proteins.

Fusions of enucleated hamster cells with LMEB3 failed to produce any cybrids in two experiments, one using Chinese hamster (*C. grigaeus*) cells and the other using Syrian golden hamster (*M. aureus*) cells. The Muridae subfamily Cricetinae, which includes the hamsters, diverged from the Murinae subfamily around 16 million years ago (Fig. 1).

Karyotyping of a control (*M. musculus*) cybrid and a *Rattus* xenocybrid revealed the same number of chromosomes, with a mean of 40 ± 2.2 (n = 4 counts) for the control, and 40 ± 1.5 (n = 6 counts) for the xenocybrid.

**mtDNA Sequencing of Cybrids by DNA Sequencing.—**For the *M. spreptus* and *R. norvegicus* cybrids, mitochondrial transfer was verified by direct sequencing of a PCR fragment of the mtDNA D-loop region. The *M. spreptus* xenocybrid showed 81% identity with the published *M. musculus* sequence (18) for the region sequenced (GenBank™ accession number AF287305). The *Rattus* xenocybrids produced using NRK52E cells as mitochondrial donors showed only 62% identity to *M. musculus* for the region sequenced and in agreement with the published *R. norvegicus* sequence (19). The cybrids made from RN1T cells showed a single nucleotide difference to the *R. norvegicus* sequence, an insertion of a C in a 5-C tract at nucleotide pair 16075. This cell line is listed by ATCC as *Rattus rattus*, but this almost perfect identity with the *R. norvegicus* sequence indicates it is also derived from *R. norvegicus*, as *R. rattus* would be expected to show many more sequence differences in this highly polymorphic D-loop region, based on molecular studies of these two species (25).

**Southern Blot Determination of mtDNA in the Rattus Xenocybrid—**Southern blot of control cybrid and *Rattus* xenocrybrid DNA after restriction with *SacI* and probing with a mouse and rat mtDNA D-loop PCR amplicon showed similar mtDNA hybridization signals. *SacI* has a single restriction site in both mouse and rat mtDNA, resulting in a single band seen at 16.5 kilobase pairs. The blot showed that similar levels of mtDNA are maintained in the xenocybrid compared with the control.

**Mitochondrial Protein Translation in the Rattus Xenocybrid—**Mitochondrial translation products labeled in the presence of cycloheximide are shown after electrophoresis in Fig. 2. Most of the 13 proteins are identifiable by mobility together with characteristic intensity of labeling, as shown at left in Fig. 2. The profiles of the control cybrid and *Rattus* xenocrybrid are similar overall. One mobility polymorphism between the *M. musculus* and *Rattus* products is evident, possibly the ND2 gene product NAD2, which was also seen in the parental NRK52E cells (not shown). Two proteins appear to be produced in increased amounts, possibly NAD4 and ATP6, while two others, likely NAD5 and cytochrome b, appear to be decreased. The same pattern was observed in two independent labeling experiments. The similarity of the profiles shows that transcription and translation of the foreign mtDNA is not markedly impaired.

**Lactate Measurement—**Fig. 3 shows the lactate production from the control and *M. spreptus* cybrids, the *Rattus* cybrid, and the ρ₀ cell line. A respiratory defect is signaled in the *Rattus* xenocrybrid by the 10-fold greater lactate production compared with the control cybrid, whereas the *M. spreptus* xenocrybrid shows similar low lactate production to the control (Fig. 3). The ρ₀ cell line produced 2-fold greater amounts of lactate than the *Rattus* xenocrybrid, indicating that the cybrid was able to produce some ATP oxidatively.

**Polarographic Measurement of OXPHOS in Xenocybrids—**Fig. 4 shows typical polarographic traces of site I (glutamate + malate) and site II (succinate) oxidation in the control and *Rattus* xenocrybrid mitochondria. The control traces (A and B) show good coupling to ADP stimulation and high respiration rates and ADP/O ratios. In the *Rattus* xenocrybrid (C and D),...
products are identified at trol cybrid; X, R. norvegicus h) labeling experiment, as indicated by the xenocybrid, although ratios of some appear to differ in this long pulse (2
cytochrome cells in the presence of chloramphenicol in addition to cycloheximide.

Table II). These results are consistent with defects in either or both complex III or IV limiting respiratory chain electron flow, as indicated by the inability of the mouse cell mutants to maintain hamster mtDNA. Divergence of hamsters (Muridae subfamily Cricetidae) from the Murinae subfamily including mice and rats was around 16 million years ago (Fig. 1 (22)). The Mus/Rattus divergence was around 10 million years ago (Fig. 1 (23)) and may approximate the limit of compatibility in this system. The resulting xenocybrids exhibited a severe OXPHOS impairment (Table II and III and Figs. 3 and 4) despite showing preserved mtDNA replication and translation (Fig. 3). This shows the defects are consequent to the mismatches between the mouse nuclear OXPHOS subunits and the Rattus mtDNA subunits (Table I; see also accompanying article (33)). A marked defect of complex I was seen in the Rattus xenocybrids (Table III) but unlike the human/primate xenomitocondrial cybrids exhibiting defects in respiratory chain complex I (14) and an increased sensitivity to programmed cell death when treated with complex I inhibitors (31).

The finding by Moraes et al. (32) that human cells preferentially replicate even defective human mtDNA over introduced normal primate mtDNAs showed that mtDNA determinants for trans-acting factors may be more important than OXPHOS functionality.

In the present studies we found that there is also a limit on mtDNA compatibility as indicated by the inability of the mouse ρ0 cells to maintain hamster mtDNA. Divergence of hamsters (Muridae subfamily Cricetidae) from the Murinae subfamily including mice and rats was around 16 million years ago (Fig. 1 (22)). The Mus/Rattus divergence was around 10 million years ago (Fig. 1 (23)) and may approximate the limit of compatibility in this system. The resulting xenocybrids exhibited a severe OXPHOS impairment (Table II and III and Figs. 3 and 4) despite showing preserved mtDNA replication and translation (Fig. 3). This shows the defects are consequent to the mismatches between the mouse nuclear OXPHOS subunits and the Rattus mtDNA subunits (Table I; see also accompanying article (33)). A marked defect of complex I was seen in the Rattus xenocybrids (Table III) but unlike the human/primate xenomitocondrial cybrids there was also a striking defect of complex III together with a partial defect of complex IV (Table III). This shows that predictions from the human/primate system do not translate directly into the murid system.

The number of amino acid substitutions range from only 3 in the highly conserved COX-2 protein, to 133 in NAD5 (Table I), making it difficult to predict which changes are functionally important. The suggestion of a severe complex III defect is particularly interesting since cytochrome b is the only mtDNA-encoded subunit of this complex. Cytochrome b shows 26 amino acid differences between M. musculus and R. norvegicus (Table I). The polarographic results (Table II and Fig. 4) show that succinate-linked respiration is more severely impaired than site 1-linked respiration, despite the latter requiring the same electron transfer pathway through complexes III and IV. This

**DISCUSSION**

By introducing R. norvegicus mtDNA into a mouse mtDNA-less cell line, we have produced a new mouse in vitro model of a severe mtDNA OXPHOS defect. Only a handful of mtDNA mouse cell mutants have been described, the best characterized being a point mutation in the 16 S rRNA gene resulting in resistance of the mitochondrial ribosome to chloramphenicol (CAPβ) (27) which results in multiple respiratory chain defects (10, 28).

The lack of methods to produce targeted mtDNA knockouts has limited attempts to produce mtDNA mutant mice. A transfection approach using a DNA construct linked to a mitochondrial target peptide leader sequence is promising but has not yet succeeded in transforming mitochondria in cultured cells (29, 30). Kenyon and Moraes (13) pioneered the approach of xenomitocondrial cybrids by showing that only the most closely related primate mtDNAs could be maintained in human ρ0 cells. These human/primate xenomitocondrial cybrids exhibited defects in respiratory chain complex I (14) and an increased sensitivity to programmed cell death when treated with complex I inhibitors (31).

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protein, and the numbers to the measurable with succinate in the Rattus 1 respiration. ADP/O ratio indicates the number of ADP molecules phosphorylated per oxygen atom used.

Differences in OXPHOS capacity for the million years ago (Fig. 1). Although the present studies show no complex II defect, as there will be a greater steady state ubiquinol concentration during succinate oxidation compared with site linked substrate oxidation due to the higher specific activity concentration during succinate oxidation compared with site

could result from substrate inhibition exacerbating the complex III defect, as there will be a greater steady state ubiquinol concentration during succinate oxidation compared with site 1-linked substrate oxidation due to the higher specific activity of complex II over complex I.

M. spretus and M. musculus diverged approximately 2–3 million years ago (Fig. 1). Although the present studies show no differences in OXPHOS capacity for the M. spretus xenocybrids, further studies may show subtle but important defects in this model. The levels of M. spretus mtDNA were too low in the mice produced by Irwin et al. (9) to conclude there were no pathogenic effects, and creation of this mouse would be an important proof of the principle from which to proceed to make other xenomitochondrial mice using the ES cell approach.

By making further xenomitochondrial constructs with species intermediate between M. spretus and Rattus, it should be possible to produce cybrids with intermediate OXPHOS phenotypes. By comparative studies of OXPHOS and mtDNA sequences from the species used, it should be possible to gain important new insights into the nuclear-mtDNA-encoded subunit interactions, in addition to the potential value of this system in producing mouse models of human mtDNA diseases. Evolution has provided a rich source of options. The Muridae is the most successful family of the order Rodentia, having the greatest number of extant species of any mammalian family (22, 24). There are literally dozens of potential constructs that can be produced. The Gerbillinae subfamily of Muridae, the gerbils, are intermediate in divergence to Rattus and the hamsters (Fig. 1) and may produce viable xenocybrids. We would predict these constructs to produce severe OXPHOS defects if the mtDNAs could be replicated.

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