

# Selective and Continuous Elimination of Mitochondria Microinjected Into Mouse Eggs From Spermatids, but Not From Liver Cells, Occurs Throughout Embryogenesis

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## ABSTRACT

Exclusion of paternal mitochondria in fertilized mammalian eggs is very stringent and ensures strictly maternal mtDNA inheritance. In this study, to examine whether elimination was specific to sperm mitochondria, we microinjected spermatid or liver mitochondria into mouse embryos. Congenic B6-mt<sup>sp</sup> strain mice, which are different from C57BL/6J (B6) strain mice (*Mus musculus domesticus*) only in possessing *M. spretus* mtDNA, were used as mitochondrial donors. B6-mt<sup>sp</sup> mice and a quantitative PCR method enabled selective estimation of the amount of *M. spretus* mtDNA introduced even in the presence of host *M. m. domesticus* mtDNA and monitoring subsequent changes of its amount during embryogenesis. Results showed that *M. spretus* mtDNA in spermatid mitochondria was not eliminated by the blastocyst stage, probably due to the introduction of a larger amount of spermatid mtDNA than of sperm mtDNA into embryos on fertilization. However, spermatid-derived *M. spretus* mtDNA was eliminated by the time of birth, whereas liver-derived *M. spretus* mtDNA was still present in most newborn mice, even though its amount introduced was significantly less than that of spermatid mtDNA. These observations suggest that mitochondria from spermatids but not from liver have specific factors that ensure their selective elimination and resultant elimination of mtDNA in them, and that the occurrence of elimination is not limited to early stage embryos, but continues throughout embryogenesis.

**M**ITOCHONDRIAL DNA (mtDNA) has been shown to be inherited strictly maternally in mammalian species using its restriction fragment length polymorphisms observed in different individuals of the same species (for review, see BIRKY 1995). On the other hand, GYLLENSTEN *et al.* (1991) observed the transmission of a very small amount of paternal mtDNA to subsequent generations by application of PCR techniques to mouse congenic strains isolated by successive backcrossing of interspecies female hybrids between *Mus musculus domesticus* and *M. spretus* to male *M. spretus* or *M. m. domesticus*. They proposed that earlier failures in finding paternal mtDNA were due simply to low resolution of the procedures for detecting very small amounts of sperm mtDNA, such as by ethidium bromide staining and Southern blot analysis.

The proposal of sperm mtDNA transmission (GYLLENSTEN *et al.* 1991) is in serious contradiction to other observations on mtDNA heteroplasmy in mammalian species. If the leakage of paternal mtDNA occurs contin-

uously, each individual should eventually possess various kinds of heteroplasmic mtDNAs derived from different ancestral male individuals. However, the polymorphic mtDNA observed in each individual did not coexist within an individual to show heteroplasmy, at least in the rat (HAYASHI *et al.* 1978, 1979), mouse (YONEKAWA *et al.* 1981), and humans (BROWN 1980). Even in the rare case of individuals with mtDNA heteroplasmy reported in a maternal lineage of cows (HAUSWIRTH and LAIPIS 1982; ASHLEY *et al.* 1989) and in a patient with mitochondrial encephalomyopathy (KOBAYASHI *et al.* 1991), no other mutations were observed between the heteroplasmic mtDNA molecules, suggesting that the mtDNA heteroplasmy observed in these individuals was due to a single mutation in mtDNA of their maternal germ lines, not to introduction and transmission of paternal mtDNA. Moreover, nucleotide sequence analysis of human mtDNA showed that mtDNA sequences within single individuals were extremely homogeneous (MONNAT and REAY 1986). Therefore, these observations support the idea that exclusion of sperm mtDNA in fertilized mammalian eggs is very stringent, ensuring strictly maternal mtDNA inheritance (HAYASHI *et al.* 1978).

The apparent discrepancy with respect to transmission of paternal mtDNA was resolved by our recent findings

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that no paternal mtDNA contributes to mtDNA inheritance in intraspecies crossing of *M. m. domesticus* × *M. m. domesticus* mice and that paternal mtDNA leakage is restricted to unusual interspecies crossing of *M. spretus* × *M. m. domesticus* (KANEDA *et al.* 1995). Moreover, the leakage was limited to the first interspecies crossing and did not occur in the subsequent backcrossing (SHITARA *et al.* 1998). Furthermore, in the cross between female *M. m. domesticus* and male congenic strain B6-*mt*<sup>SP</sup> mice, which carry the nuclear genome from *M. m. domesticus* but mtDNA from *M. spretus*, sperm mtDNA of *M. spretus* from the B6-*mt*<sup>SP</sup> male mice was eliminated completely in the fertilized eggs. On the basis of these observations, we propose that mouse egg cytoplasm must have a system for eliminating sperm mitochondria that recognizes nuclear DNA-coded factors in sperm mitochondria of the same species, but not sperm mtDNA or mtDNA-coded factors (KANEDA *et al.* 1995; SHITARA *et al.* 1998).

In this study, we used a quantitative PCR method and mitochondria from male B6-*mt*<sup>SP</sup> strain mice to determine whether the species-specific exclusion system of sperm mitochondria present in egg cytoplasm could also exclude mitochondria from spermatids, *i.e.*, immature sperm, and from somatic cells of the same species. We monitored the fate of mtDNA in microinjected mitochondria and obtained two important findings. One is that specific factors ensuring preferential elimination are present in mitochondria from sperm and spermatids but not in liver mitochondria, and the other is that expression of the machinery that excludes spermatid mitochondria is not limited to early stage embryos, but continues effectively throughout embryogenesis.

## MATERIALS AND METHODS

**Preparation of mature oocytes, pronucleus-stage embryos, and round spermatids:** Mature oocytes and pronucleus-stage embryos (zygotes) were obtained from superovulated female mice of C57BL/6J (B6) strain and B6D2F<sub>1</sub>. For collecting pronucleus-stage embryos, female mice of 8–10 wk old were induced to superovulate by consecutive injections of pregnant mare serum gonadotropin (PMSG, 5 IU) and human chorionic gonadotropin (hCG, 5 IU) with an interval of 48 hr between injections. Then, they were caged with fertile male mice overnight. Pronucleus-stage embryos were collected by puncturing the oviducts 15–18 hr after hCG injection. Round spermatids were isolated from testes of congenic strain B6-*mt*<sup>SP</sup> mice (KANEDA *et al.* 1995). The *tunica albuginea* was removed from testes and remaining tissues were placed in cold modified Dulbecco's phosphate buffered medium (PBI) supplemented with 0.01% polyvinylpyrrolidone (PVP, *M*<sub>w</sub> 360,000). The tissue was cut into small pieces and pipetted gently to allow release of spermatogenic cells into the PBI. The cell suspension was filtered through a 50- $\mu$ m nylon mesh and precipitated by centrifugation at 100 × *g* for 5 min at 4°.

**Microinjection of mitochondria into unfertilized oocytes:** For introduction of spermatid mitochondria, *in vitro* fertilization was carried out by microinjection of spermatids into unfertilized oocytes as described previously (OGURA and YANAGIMACHI 1993; KIMURA and YANAGIMACHI 1995). Briefly, oocytes were activated by treating them with Ca<sup>2+</sup>-free Whitten's me-

dium (WM) containing 2.5 mM SrCl<sub>2</sub> for 15–20 min at 37° for microfertilization with spermatids. When the second polar body had been extruded (10–20 min after transfer of Sr<sup>2+</sup>-treated oocytes to regular WM), each oocyte was injected with a round spermatid after mechanical breaking of its plasma membrane in WM with 6% PVP by drawing an individual spermatid into an injection pipette. Microinjection was carried out using a Piezo micro manipulator (Prima Meat Packers, Tsuchiura, Japan).

**Microinjection of mitochondria into pronucleus-stage embryos:** Cytoplasm without a nucleus was prepared by mechanical breaking of the spermatid plasma membrane using an injection pipette followed by selective withdrawal of the cytoplasm into the pipette. Male liver mitochondria were prepared as described (KING and ATTARDI 1988). Cytoplasm of round spermatids or liver mitochondria was injected into pronucleus-stage embryos using a Piezo micro manipulator. Some embryos were incubated in M16 until the blastocyst stage, and others were transferred to the oviducts of ICR females that had been mated with vasectomized males ~15 hr previously.

**DNA preparation:** Embryos at the pronucleus, two-cell, and blastocyst stages were placed in Tyrode solution for removing the zona pellucida and contaminants in perivitelline space. Each embryo was transferred to a 0.2-ml MicroAmp optical tube (ABI, Columbia, MD) containing 5  $\mu$ l PCR buffer/non-ionic detergents and proteinase K solution [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 0.1% gelatin, 0.45% Nonidet P-40, 0.45% Tween-20, and 100  $\mu$ g/ml proteinase K] supplemented with 0.1  $\mu$ g of the sonicated salmon sperm DNA (Wako) as a carrier. After incubation at 55° overnight for digestion of proteins, samples were heated at 95° for 10 min to inactivate proteinase K. Each sample was used directly as template DNA for PCR. In the case of DNA preparations from neonates, each individual was killed by ethyl ether anesthesia and minced with a fresh disposable razor blade in a fresh disposable plastic dish (Falcon, Lincoln Park, NJ). Samples were placed in proteinase K solution and incubated overnight at 55°. Total DNA was purified from the lysate by phenol-chloroform extraction and precipitation in 70% ethanol. Each of the steps was done in a fresh disposable tube (Falcon) to avoid contamination with *M. spretus* mtDNA.

**Estimation of the mtDNA copy number by real-time detection PCR:** Real-time detection PCR (RTD-PCR) was carried out using one set of forward and reverse primers and a TaqMan probe (Figure 1), which were designed for specific amplification of *M. spretus* mtDNA in the presence of *M. m. domesticus* mtDNA. The reporter dye FAM was covalently attached to the 5' end of the TaqMan probe (6-FAM amidite), and the quencher dye TAMRA was joined to its 3' end (3'-TAMRA APG500). To obtain standard curves, standard DNA was constructed to ligate pT7Blue T-vector (Novagen) to PCR products, which was amplified using the primer set mentioned above, and total DNA was prepared from B6-*mt*<sup>SP</sup> liver as a template. Ligation and plasmid purification were performed using a pT7Blue T-vector kit and QIAGEN plasmid kit (Novagen or QIAGEN, Chatsworth, CA). The purified plasmid DNA (3172 bp) was titrated by spectrophotometric measurements and dissolved in TE and serially diluted with DW supplemented with 0.1 mg/ml of sonicated salmon sperm DNA as a carrier in making the standard curves.

For quantification of *M. spretus* mtDNA, RTD-PCR was carried out using a TaqMan PCR reagent kit for the sequence detector system of ABI Prism 7700 under the conditions recommended by the manufacturer (Perkin-Elmer, Norwalk, CT). The RTD-PCR mixture for the 50- $\mu$ l PCR reaction contained 1× TaqMan buffer A, 3 mM MgCl<sub>2</sub>, 200  $\mu$ M dATP/dGTP/dCTP, 400  $\mu$ M dUTP, 200 nM forward primer/reverse primer, 300 nM TaqMan probe, 0.025 units/ $\mu$ l AmpliTaq

Gold, 0.01 units/ $\mu$ l AmpErase uracil-*N*-glycosylase (UNG), and template DNA. Initial activation of AmpErase UNG and AmpliTaq Gold was carried out at 50° for 2 min and at 95° for 10 min, respectively. Subsequent PCR amplification consisted of 50 cycles for denaturation at 95° for 20 sec and annealing and extension at 60° for 1 min in an ABI 7700 sequence detector. After amplification, real-time data acquisition and analysis was performed. Once the threshold was chosen, the point at which the threshold crossed the amplification plot was defined as threshold cycle (Ct). The calculated Ct value is predictive of the quantity of target DNA copies present in the sample. The standard curve for the assay was calculated using a series of 10-fold dilutions of previously titrated synthetic DNA. After the PCR, 10  $\mu$ l of reaction mixture was applied onto a gel of 3% Nusieve agarose/1% agarose for electrophoresis in 1 $\times$  TAE buffer (Figure 2C).

## RESULTS

**Selective and quantitative estimation of amount of *M. spretus* mtDNA:** For examination of whether elimination of exogenous mitochondria by mouse embryos is specific to sperm mitochondria, we microinjected spermatid or liver mitochondria into eggs of B6 strain mice (*M. m. domesticus*) and monitored their fate by estimating the amount of mtDNA in microinjected mitochondria by quantitative PCR. In this case, mitochondria containing mtDNA of different species, such as *M. spretus* mtDNA, had to be used for microinjection for exclusive amplification of the mtDNA in microinjected mitochondria in the presence of excess *M. m. domesticus* mtDNA in the recipient eggs. However, *M. spretus* mtDNA in sperm mitochondria of *M. spretus* could escape from elimination, probably due to species specificity of sperm mitochondria recognition by the rejection machinery in egg cytoplasm (KANEDA *et al.* 1995). This conflict could be resolved by using mitochondria from congenic B6-mt<sup>SPR</sup> strain mice for microinjection into fertilized eggs of B6 strain mice. The congenic B6-mt<sup>SPR</sup> strain

mice possess mtDNA of *M. spretus* but nuclear DNA of *M. m. domesticus*, and we have shown that *M. spretus* mtDNA in sperm mitochondria of the B6-mt<sup>SPR</sup> strain mice is completely eliminated before the two-cell stage (KANEDA *et al.* 1995).

Quantitative estimation of *M. spretus* mtDNA was carried out using the primer set and probe shown in Figure 1. Synthetic vector was produced by ligating pT7 Blue T-vector to *M. spretus* mtDNA-specific PCR products and used for testing the sensitivity and quantitiveness of this PCR procedure (MATERIALS AND METHODS). The results showed that this procedure could detect as few as 10 copies of the synthetic vector (Figure 2). Moreover, the correlation coefficient between DNA quantity and Ct was >0.98 for samples with copy numbers between 10 and 10<sup>5</sup> (Figure 2B) and between 10<sup>2</sup> and 10<sup>6</sup> (data not shown) in the presence of *M. m. domesticus* mtDNA of a B6 strain egg, suggesting that selective and quantitative estimation of exogenously introduced mtDNA could be achieved during early embryogenesis. Then, we examined the copy numbers of mtDNA and found that a single sperm and a single spermatid had only 10 and 150 copies, respectively, while a single oocyte possessed 5  $\times$  10<sup>5</sup> copies. Most of these values corresponded to those obtained previously by Southern blot analysis (HECHT *et al.* 1984; PIKO and TAYLOR 1987) except that a single sperm was reported to possess 50–75 copies (HECHT *et al.* 1984). The difference in the copy number of sperm mtDNA between present and previous estimation may be due at least partly to the differences of the procedures used for DNA preparation and estimation.

**Quantitative analysis of the fate of spermatid or sperm mtDNA introduced into oocytes during embryogenesis:** First, *in vitro* fertilization was carried out using spermatids or sperm from B6-mt<sup>SPR</sup> mice and oocytes from B6 mice for comparison of the fate of exoge-

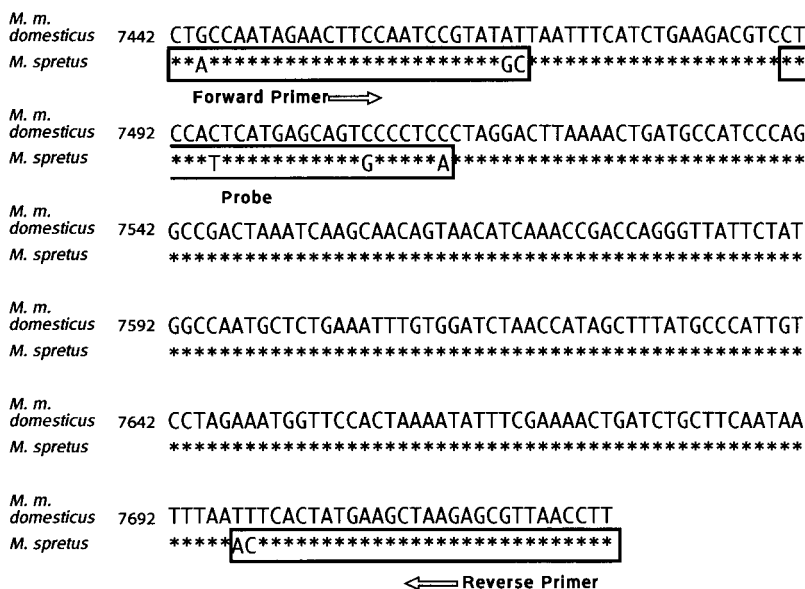


FIGURE 1.—Comparison of mtDNA sequences from nucleotide positions 7442 to 7726 between *M. m. domesticus* (top lines; BIBB *et al.* 1981) and *M. spretus* (bottom lines; H. KANEDA, unpublished results). This region corresponds to parts of the *COII* and *tRNA<sup>Lys</sup>* genes. Asterisks indicate identity with the *M. m. domesticus* sequence. A primer set and a probe used are marked by long boxes.



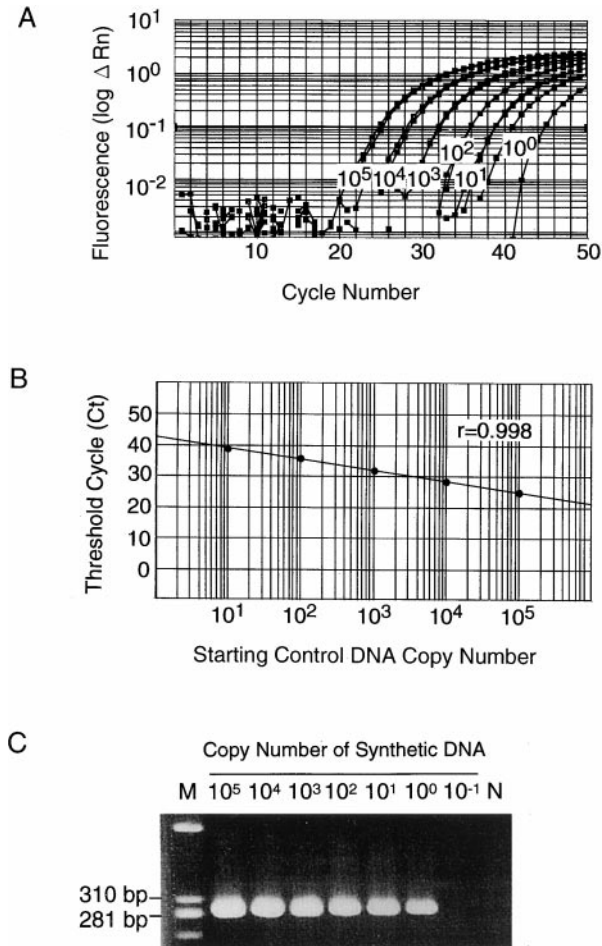


FIGURE 2.—Amplification profiles of standard DNA. Plasmid DNA including the *M. spretus* mtDNA sequence was constructed by ligation of the pT7Blue T vector and PCR products, which was amplified using the primer set shown in Figure 1 and total DNA prepared from B6-mt<sup>SPT</sup> as a template. Standard DNA samples were prepared in duplicate by 10-fold serial dilution of purified plasmid DNA. (A) Amplification curves. At each dilution,  $\Delta$  normalized reporter (Rn) is plotted against each cycle number. (B) Threshold cycle (Ct) values are plotted against the relative starting copy number to make the standard curve. (C) Detection of 285-bp PCR products by 3% Nusieve agarose/1% agarose gel electrophoresis. M, molecular weight standards ( $\phi$ X174, *Hae*II digests); N, negative control (salmon sperm DNA).

nous mtDNA in mouse embryos. Fertilization and introduction of spermatid mitochondria could be attained simultaneously by microinjection of single spermatids with ruptured cell membranes into oocytes. On the other hand, simple mixing of sperm and oocytes resulted in fertilization and introduction of sperm mitochondria into oocytes. Then, the average number of spermatid- or sperm-derived mtDNA introduced into oocytes was monitored by quantitative estimation during *in vitro* embryogenesis (Figure 3A). As expected, sperm-derived mtDNA, which was first detectable at  $\sim 10$  copies in the pronucleus-stage embryos, disappeared completely before the two-cell stage, while the average num-

ber of mtDNA in microinjected spermatid mitochondria unexpectedly increased more than twofold to  $\sim 350$  copies in the two-cell stage, although we could not explain why a significant divergence in estimated number of spermatid mtDNA was observed. Then, it decreased gradually to 110 copies by the blastocyst stage (Figure 3A). On the other hand, the average number of mtDNA in recipient eggs was  $5 \times 10^5$  and decreased to  $3 \times 10^5$  at the blastocyst stage (Figure 3B). The increased copy number of spermatid mtDNA suggested the occurrence of mtDNA replication in microinjected spermatid mitochondria. The subsequent decrease may have been due to selective but incomplete destruction of spermatid-derived mitochondria by a mechanism similar to that working for complete elimination of sperm-derived mitochondria (KANEDA *et al.* 1995; SHITARA *et al.* 1998). The decrease in the number of maternal mtDNA (Figure 3B) and spermatid mtDNA (Figure 3, A and C) would not be due to a decrease of the efficiency of our RTD-PCR by accumulation of nuclear DNA during early embryogenesis, because the amplification efficiency of *M. spretus* mtDNA did not change in the presence of extra salmon sperm DNA, or two-cell-stage, blastocyst-stage embryos of B6 strain mice with *M. m. domesticus* mtDNA. Escape of spermatid-derived mtDNA from complete elimination during *in vitro* culture may have been due at least partly to introduction of more mtDNA than sperm mtDNA into oocytes on fertilization (Figure 3A).

Then we examined whether the introduced spermatid mtDNA remaining during early embryogenesis could be detected in newborn mice. As mouse embryos could not be cultured *in vitro* beyond the blastocyst stage, pronucleus-stage embryos with microinjected spermatid mitochondria were transferred to the oviduct of foster mothers. After birth, total DNA was prepared from whole bodies of newborn mice and used for PCR analysis. No spermatid-derived mtDNA was observed in any of the nine newborn individuals examined (Table 1). Thus, most spermatid mtDNA that was not eliminated during early embryogenesis was eventually excluded from the embryos before birth. These observations suggest that the exclusion system in oocyte cytoplasm is effective for exogenously introduced mitochondria irrespective of whether they were derived from sperm or spermatids and that its efficiency is not restricted to before the two-cell stage but continues throughout embryogenesis.

**Quantitative analysis of the fate of liver or spermatid mtDNA microinjected into fertilized eggs during embryogenesis:** For examination of whether the rejection is specific to mitochondria from the male germ line, or is also effective for mitochondria from somatic cells, we microinjected male liver mitochondria of B6-mt<sup>SPT</sup> strain mice into fertilized eggs of B6 mice and examined the fate of the liver mtDNA quantitatively by PCR (Figure 3C). Since fertilized eggs had to be used as recipients of liver mitochondria, microinjection of spermatid mito-

chondria into fertilized eggs was also carried out as a control.

Quantitative PCR analysis showed that microinjection of male liver and spermatid mitochondria of B6-*mt*<sup>SP</sup>

strain mice into fertilized eggs (pronucleus-stage embryos) resulted in introduction of  $\sim 30$  copies of liver and 138 copies of spermatid mtDNA (Figure 3C). The copy numbers of microinjected liver and spermatid mtDNAs increased from 30 to 67 and from 138 to 436, respectively, in 15–20 hr to the two-cell stage (Figure 3C). Therefore, the copy number of liver-derived mtDNA was significantly lower than that of spermatid-derived mtDNA. But progressive decrease of the mtDNA copy number was observed only in spermatid-derived mtDNA, suggesting the possibility of its selective elimination, although neither exogenous mtDNA was completely eliminated by the blastocyst stage (Figure 3C).

Then, the fates of liver- and spermatid-derived mtDNA, which had been present during *in vitro* cultivation, were examined using newborn mice. Pronucleus-stage embryos with microinjected mitochondria were transferred to the oviduct of foster mothers. Total DNA was prepared from whole bodies of newborn mice and used to detect the exogenous mtDNA by PCR. Only 1 of 30 newborn mice possessed spermatid-derived mtDNA, while 7 of 16 newborn mice possessed liver-derived mtDNA (Table 1), in spite of the fact that the amount of microinjected liver mtDNA was less than a quarter of that of spermatid mtDNA (Figure 3C).

For further examination of transmission of leaked liver-derived mtDNA to following generations, we analyzed punched ear fragments of female newborn mice, which had been microinjected with liver mitochondria at the pronucleus stage. Six of nine *F*<sub>0</sub> females possessed liver-derived mtDNA in their ears (Table 1) and were used for further examination of its transmission to the following generation. Of six females, four were pregnant and gave 34 *F*<sub>1</sub> newborn mice (Table 1). PCR analysis showed that liver-derived mtDNA had been transmitted to only one *F*<sub>1</sub> individual (Table 1), suggesting that microinjected liver mtDNA could be transmitted to a

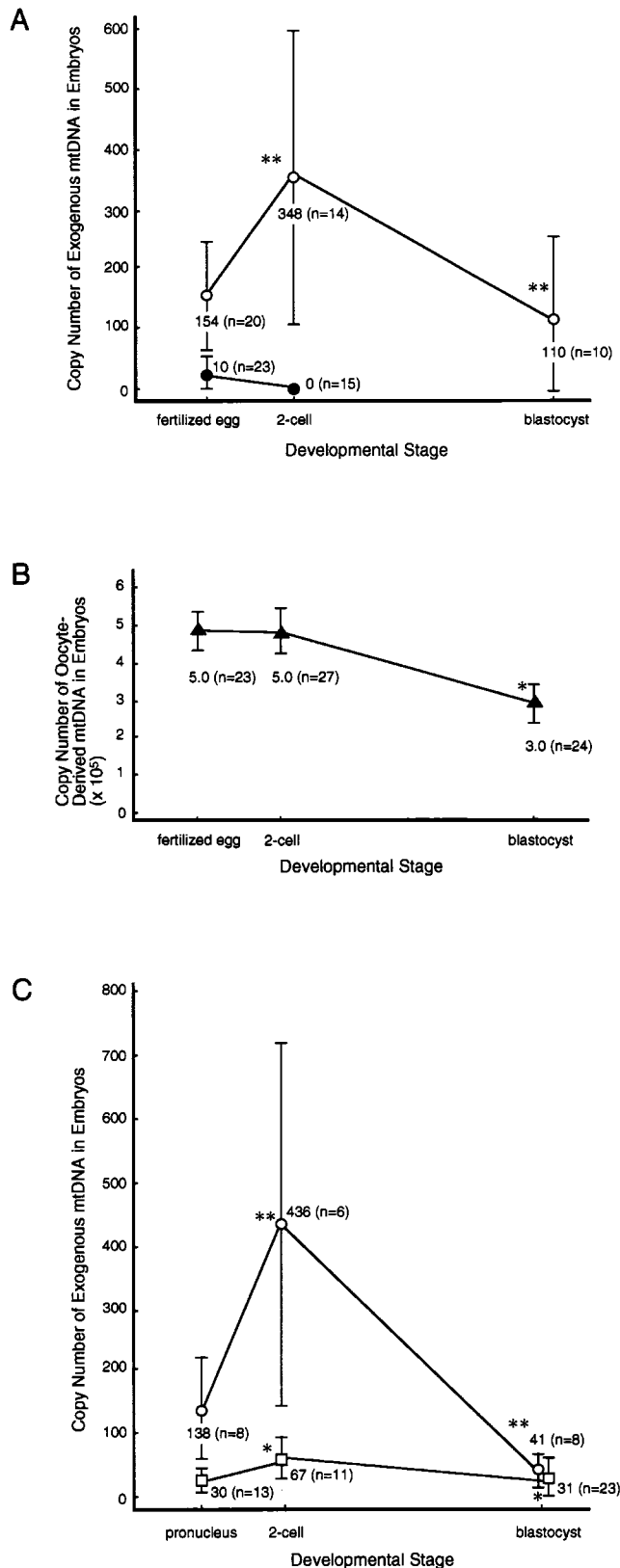


FIGURE 3.—Changes of copy number of mtDNA in mitochondria derived from sperm, spermatids, oocytes, and liver during early embryogenesis. All measurements of copy number were shown as mean  $\pm$  SD. The statistical test (*t*-test) was used to estimate the significance of the differences of mtDNA copy numbers between a stage *vs.* a previous stage. \**P* < 0.01; \*\**P* < 0.05. (A) Copy number of *M. spretus* mtDNA in sperm (●) or spermatid (○) mitochondria introduced into oocytes. *In vitro* fertilization was carried out using sperm or spermatid from B6-*mt*<sup>SP</sup> mice and oocytes from B6 strain mice. (B) Copy number of *M. spretus* mtDNA present in oocyte mitochondria (▲). *In vitro* fertilization was carried out using sperm and oocytes of B6-*mt*<sup>SP</sup> strain mice. (C) Spermatid or liver mitochondria from B6-*mt*<sup>SP</sup> male mice were used for microinjection into fertilized eggs. *In vitro* fertilization was carried out using oocytes and sperm derived from B6 strain mice. (○) *M. spretus* mtDNA in microinjected spermatid mitochondria from B6-*mt*<sup>SP</sup> males; (□) *M. spretus* mtDNA in microinjected liver mitochondria from B6-*mt*<sup>SP</sup> males. *n*, number of embryos examined.

**TABLE 1**  
**Detection of exogenously introduced *M. spretus* mtDNA in F<sub>0</sub> and F<sub>1</sub> mice**

Mitochondria recipients	Mitochondria donors	Tissue tested	No. of PCR-positive/no. of newborn individuals tested
F <sub>0</sub> generation			
Oocytes	Spermatid	Whole bodies	0/9
Fertilized eggs	Spermatid	Whole bodies	1/30
Fertilized eggs	Liver	Whole bodies	7/16
Fertilized eggs	Liver	Ear fragments	6 <sup>a</sup> /9
F <sub>1</sub> generation			
Fertilized eggs	Liver	Whole bodies	1/34

<sup>a</sup> Six F<sub>0</sub> females that were found to possess microinjected liver-derived mtDNA in their ear fragments were used as female parents and four females were pregnant, giving rise to 34 F<sub>1</sub> mice.

following generation through the female germ line, but was segregated rapidly. Since we could not provide precise information on the quantity of liver-derived mtDNA relative to the host mtDNA in whole bodies of the parental F<sub>0</sub> females, it is very difficult to determine whether segregation of the donor mtDNA in F<sub>1</sub> progenies is merely by chance or due to some selective mechanisms during its transmission.

#### DISCUSSION

We showed in this study that introduced mitochondria from spermatid into mouse eggs were present during early embryogenesis, but were eliminated from embryos by the time of birth, while mitochondria from male liver were still present in newborn mice. The quantitative PCR method excluded the possibility that leakage of liver mitochondria was due to the introduction of too many mitochondria into eggs for their complete exclusion. In these experiments, introduction of liver and spermatid mitochondria was carried out by the use of mitochondrial fraction and cytoplasm, respectively. However, difference of the procedures for mitochondrial preparation would not be responsible for difference of the transmission profiles, since escape of mitochondria from elimination was also observed when cytoplasm from fibroblasts was introduced into fertilized eggs (INOUE *et al.* 2000). These observations suggest that specific factors that ensure strictly maternal inheritance of mtDNA must be present exclusively in mitochondria in male germ cells before sperm maturation and that the exclusion mechanisms in fertilized eggs are effective throughout embryogenesis.

After microinjection of spermatid mitochondria, 15 times more mtDNA than sperm mtDNA was introduced into oocytes on *in vitro* fertilization, and the copy number of spermatid mtDNA unexpectedly increased by the two-cell stage, while sperm mtDNA was completely eliminated by this stage (Figure 3A). Since the copy number of mtDNA in recipient oocytes did not increase

during *in vitro* cultivation (Figure 3B), nuclear factors required for mtDNA replication are not expressed during early embryogenesis. Thus, an increase in the amount of mtDNA in microinjected spermatid mitochondria probably occurred by mtDNA replication machinery already present in spermatid mitochondria before the microinjection. On the contrary, the absence of increase in sperm-derived mtDNA may indicate that mitochondria of matured sperm have already lost the function required for mtDNA replication (HECHT and LIEM 1984; ALCIVAR *et al.* 1989; LARRSON *et al.* 1996). Therefore, there seem to be three reasons for preferential exclusion of sperm mtDNA, but not spermatid mtDNA, before the two-cell stage: first, the copy number of introduced sperm mtDNA was 15 times less; second, sperm mitochondria lost the mtDNA replication function; and third, eggs may have an exclusion machinery that does not recognize spermatid-derived mitochondria. However, the last possibility can be excluded, because most spermatid mtDNA that had escaped from exclusion during early embryogenesis was eventually eliminated from the embryos before birth (Figure 3 and Table 1). Thus, the exclusion mechanism, which could rapidly and completely eliminate sperm-derived mtDNA before the two-cell stage, remains effective throughout embryogenesis, resulting in elimination of a large amount of spermatid mtDNA from embryos by the time of birth.

We showed previously that sperm mtDNA from *M. spretus* escaped from exclusion, but sperm mtDNA from *M. m. molossinus* was completely excluded from eggs of *M. m. domesticus* (KANEDA *et al.* 1995). Complete exclusion was also observed in sperm mtDNA from congenic B6-mt<sup>SPR</sup> mice possessing mtDNA of *M. spretus* and the nuclear genome of *M. m. domesticus* (KANEDA *et al.* 1995). Our previous work suggested that the exclusion system in egg cytoplasm could not recognize sperm mtDNA or its transcripts, but could recognize sperm mitochondrial factors, which are nuclear DNA coded, species specific, and probably located in outer membranes (KANEDA *et al.* 1995). In this study, we showed that the rejection of

exogenously introduced mitochondria by mouse embryos was specific to mitochondria from the male germ line, since newborn mice that had received a microinjection of mitochondria from male liver at the pronucleus stage still possessed liver-derived mtDNA (Table 1), although the amount of microinjected liver mtDNA was significantly less than that of spermatid mtDNA (Figure 3C). These observations suggest the occurrence of sperm-specific modification in mitochondria before spermatid formation, modification of which is responsible for their stringent recognition by egg cytoplasm followed by selective elimination from embryos. To prove this idea, transmission of mitochondria from female germ line and other somatic tissues must be examined. Recently, we found maternal transmission of mouse mtDNA with a somatic deletion mutation, which was introduced from cultured fibroblast lines into mouse embryos (INOUE *et al.* 2000).

There have been many reports on the introduction of exogenous mitochondria into mouse embryos to test their transmission. Rapid mtDNA segregation was observed in mtDNA heteroplasmy created by cell fusion of embryos  $\times$  embryonic cytoplasts (JENUTH *et al.* 1996) and embryonic karyoplasts  $\times$  embryonic cytoplasts (MEIRELLES and SMITH 1997). For identification of exogenous mtDNA, these studies used a combination of conventional strains and NZB strain mice, which belong to the same species as *M. m. domesticus*, but have different mtDNA polymorphisms as we reported (YONEKAWA *et al.* 1982). Using these mtDNA polymorphisms, CUMMINS *et al.* (1999) reported that spermatocyte mitochondria of NZB strain mice microinjected into pronucleus-stage embryos of B6D2F1 mice were not detectable in newborn mice by Southern blot analysis or ethidium bromide staining of restriction fragments of PCR products for detection of NZB mtDNA. However, they did not confirm the introduction and presence of NZB mtDNA just after microinjection of spermatocyte mitochondria into mouse embryos. But, considering that oocytes and spermatocytes possess  $10^5$  and  $10^3$  copies of mtDNA, respectively, it is possible that these procedures could not detect mtDNA of spermatocyte mitochondria after microinjection, even when it was not rejected by embryos. Failure to find exogenous sheep mtDNA of somatic nuclear donors in nuclear transfer-derived cloned sheep was also reported (EVANS *et al.* 1999). In these cases, the PCR technique could not be used for selective amplification of spermatocyte- or somatic cell-derived mtDNA in the presence of excess recipient oocyte-derived mtDNA, since sequence divergence between these polymorphic mtDNAs of the same species is not sufficient for selective amplification of exogenous mtDNA. These cases show why we did not use mitochondria with mtDNA of the same mouse species for microinjection in this study.

Mice of B6 strain (*M. m. domesticus*) and *M. spretus* belong to different species, and thus sequence diver-

gence of their mtDNAs is sufficient for selective amplification of a single sperm mtDNA of *M. spretus* even in the presence of egg mtDNA of *M. m. domesticus*. By use of nested PCR, we previously demonstrated leakage of *M. spretus* sperm mtDNA in interspecies F<sub>1</sub> hybrids between male *M. spretus* and female *M. m. domesticus* (KANEDA *et al.* 1995). Using the same combination of mouse species and the same primer sets as we used, PINKERT *et al.* (1997) reported that microinjected liver mitochondria from *M. spretus* into fertilized eggs of *M. m. domesticus* could also escape elimination. Therefore, introduced mitochondria from *M. spretus* could not be eliminated from embryos, irrespective of whether they were derived from sperm (KANEDA *et al.* 1995) or liver (PINKERT *et al.* 1997). In this study, however, we used liver mitochondria from a congenic strain B6-mt<sup>SPR</sup> for microinjection and showed the escape of their mtDNA from elimination, although *M. spretus* mtDNA from B6-mt<sup>SPR</sup> sperm was completely excluded from fertilized eggs of female *M. m. domesticus* mated with male B6-mt<sup>SPR</sup>. Sperm mtDNA rejection would be because the species of nuclear genome of B6-mt<sup>SPR</sup> mice was the same as that of *M. m. domesticus*. Moreover, the results of quantitative PCR analysis in Figure 3C clearly show that leakage of liver mitochondria is not because their amount was too large for complete exclusion from embryos, but because liver mitochondria were not recognized by egg cytoplasm, resulting in their absence of elimination. These observations suggest that sperm or spermatid mitochondria but not liver mitochondria have factors that are responsible for their selective elimination from embryos.

Success of introduction of somatic cell mitochondria into embryos has practical implications for introduction of exogenous mtDNA into progenies. IRWIN *et al.* (1999) reported the transmission of very little microinjected liver mtDNA of *M. spretus* to the following generation and stated that microinjection of mitochondria is valuable for creation of animal models of mitochondrial diseases. However, microinjection does not have to be used for generation of mice with a predominant amount of *M. spretus* mtDNA, because complete replacement of mouse mtDNA by *M. spretus* mtDNA has been attained in B6-mt<sup>SPR</sup> mice, which did not show any mitochondrial dysfunction (YAMAOKA *et al.* 2000). Moreover, we obtained mtDNA-repopulated cybrids by the fusion of mtDNA-less mouse cells with platelets from different species for finding the mtDNA species that could induce mitochondrial abnormalities and showed that rat mtDNA but not *M. spretus* mtDNA induced mitochondrial dysfunction in mouse cells (YAMAOKA *et al.* 2000). Therefore, mice with predominant rat mtDNA have to be isolated for use as models of mitochondrial diseases. However, since rat mtDNA could not be introduced into mouse cells in the presence of mouse mtDNA (HAYASHI *et al.* 1980), it would be difficult to generate respiration-deficient mice by introduction of mtDNA from dif-



ferent species. On the other hand, microinjection of exogenous mitochondria with a somatic mutant mtDNA into mouse embryos was successfully used for generation of mice with mitochondrial dysfunction (INOUE *et al.* 2000), which should be useful for studying the precise mechanisms of transmission and expression of the pathogenic mutant mtDNA in various tissues.

Recently, the possible involvement of ubiquitin in destruction of sperm mitochondria in fertilized cow and monkey eggs was suggested (SUTOVSKY *et al.* 1999). We are now investigating mitochondrial factors that are nuclear DNA coded and involved in species-specific and male germ line-specific recognition by the mitochondrial rejection machinery in mouse eggs.

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