Replicative Advantage and Tissue-Specific Segregation of RR Mitochondrial DNA Between C57BL/6 and RR Heteroplasmic Mice

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ABSTRACT

To investigate the interactions between mtDNA and nuclear genomes, we produced heteroplasmic maternal lineages by transferring the cytoplasts between the embryos of two mouse strains, C57BL/6 (B6) and RR. A total of 43 different nucleotides exist in the displacement-loop (D-loop) region of mtDNA between B6 and RR. Heteroplasmic embryos were reconstructed by electrofusion using a blastomere from a two-cell stage embryo of one strain and an enucleated blastomere from a two-cell stage embryo of the other strain. Equivalent volumes of both types of mtDNAs were detected in blastocyst stage embryos. However, the mtDNA from the RR strain became biased in the progeny, regardless of the source of the nuclear genome. The RR mtDNA population was very high in most of the tissues examined but was relatively low in the brain and the heart. An age-related increase of RR mtDNA was also observed in the blood. The RR mtDNAs in the reconstructed embryos and in the embryos collected from heteroplasmic mice showed a different segregation pattern during early embryonic development. These results suggest that the RR mtDNA has a replicative advantage over B6 mtDNA during embryonic development and differentiation, regardless of the type of nuclear genome.

IN mammals, mitochondria occupy a substantial portion of cell cytoplasm. In general, mitochondrial DNA (mtDNA) is identical in each cell's cytoplasm (homoplasmy), but spontaneous mtDNA mutation has been observed (Brown *et al.* 1982) and may cause a mtDNA sequence variant in an individual (heteroplasmy). MtDNA heteroplasmy is rare, probably because it is unstable and shifts rapidly to a homoplasmic condition (for reviews see Lightowlers *et al.* 1997; Cummins 1998). Pedigree analyses of heteroplasmic displacement loop (D-loop) variants in Holstein cows have demonstrated a return to homoplasmy within one generation or a few generations (Hauswirth and Laipis 1982; Ashley *et al.* 1989; Koehler *et al.* 1991). A proposed mechanism for this phenomenon is the "bottleneck effect" during oogenesis, in which only a small number of mtDNA molecules are randomly distributed to the next generation. Moreover, the inheritance of mtDNA is provided by the maternal line (Gyllensten *et al.* 1985). Fertilization may cause heteroplasmy, although an egg contains ~1000 times more mtDNA molecules than do sperm (Michaels *et al.* 1982; Hecht *et al.* 1984; Hauswirth and Laipis 1985), and the sperm mtDNA is preferentially degraded in the egg (Kaneda *et al.* 1995; Sutovsky *et al.* 1996). Recently, Takeda *et al.* (1999) also showed a reduction of heteroplasmy in bovine embryos reconstructed by nuclear transfer during early embryogenesis.

A common phenomenon in human mitochondrial disease is the coexistence of mutant and wild-type mtDNA molecules (for review, see Wallace 1992). The biased segregation of mutant and wild-type mtDNA molecules has been examined in cultured cells (Yoneda *et al.* 1992; Dunbar *et al.* 1995; Vergani *et al.* 1999). These studies indicated that the nuclear genetic background of the cell could influence the segregation of these mtDNA molecules. However, the segregation rules of different mtDNA molecules in heteroplasmic cells remain unelucidated. Heteroplasmic mice, produced by karyoplast or cytoplast transfer and carrying two different kinds of mtDNA genotypes, have been used as an *in vivo* model to study mtDNA segregation in individual animals (Smith and Alcivar 1993; Jenuth *et al.* 1996, 1997; Meirelles and Smith 1997, 1998). The mtDNAs from BALB/cByJ (BALB) and NZB/BINJ (NZB) heteroplasmic mice segregate rapidly in the female germline due to a genetic bottleneck in early oogenesis (Jenuth *et al.* 1996). A random genetic drift of mtDNAs has been observed in some tissues in heteroplasmic mice, while in other tissues, strong, tissue-specific and age-related selections of either mtDNA genotype have been detected (Jenuth *et al.* 1997). It is unknown how the tissue-specific segregation of mtDNA is accomplished in heteroplasmic mice, though an interaction between the nuclear and mitochondrial genomes may be its cause.

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To investigate the interaction between nuclear and mitochondrial genomes, we produced heteroplasmic maternal lineages by reciprocal cytoplast transfer using blastomeres from embryos at the two-cell stage from two mice strains (C57BL/6 and RR). The resulting heteroplasmic mice showed biased and tissue-specific segregation of RR mtDNA and passed on predominantly the RR mtDNA to their progeny.

MATERIALS AND METHODS

Source of mice and embryos with different mtDNA backgrounds: The RR strain was established as an inbred strain in Japan. This type of mtDNA is identical to that of mice in western China and differs from that of common laboratory inbred strains (Yonekawa *et al.* 1982). Because RR mice show poor reproductive performance, we generated RR/DBA F_1 (female RR × male DBA) mice carrying the RR mtDNA genotype (RR′). The RR′ mice were mated with each other and the embryos recovered from these were used in this study. The C57BL/6 (B6) embryos were obtained from mating B6 males and females.

To obtain embryos at the two-cell stage, females were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotrophin (Teikokuzoki, Tokyo) and 5 IU of human chorionic gonadotrophin (hCG; Mochida, Tokyo) given 48 hr apart. After hCG injection, the females were mated with males. The two-cell stage embryos were recovered from the oviducts 40 hr after hCG injection.

Generation of heteroplasmic mice: Heteroplasmic embryos were reciprocally produced by electrofusion of a blastomere and an enucleated blastomere from the two-cell embryos of RR′ and B6 strains. Each of the blastomeres of the two-cell embryos were incubated in M2 medium (Hogan *et al.* 1986) with 5 μg cytochalasin B ml^{-1} for 15 min and then enucleated with a glass pipette. The enucleated cytoplast was injected into a two-cell-stage embryo, in which one of the two blastomeres was previously destroyed with a fine glass pipette. Electrofusion between the enucleated cytoplast and the blastomere was performed in 0.3 m mannitol solution including 0.1 mm $CaCl_2$ and 0.1 mm $MgSO_4$, by a fusion apparatus (Somatic Hybridizer SSH-2; Simadzu, Kyoto, Japan) at 125 V mm^{-1} direct current pulse for 100 μsec. The reconstructed embryos were then cultured for 2 days at 37°, 5% CO_2 in M16 medium (Hogan *et al.* 1986) supplemented with 0.01 mm EDTA. Embryos that reached the morula stage were transplanted into the uteruses of pseudopregnant ICR strain female mice.

Detection of mtDNA in heteroplasmic embryos and in heteroplasmic mice: The embryos were retrieved from the culture at various stages ranging from just after fusion to the blastocyst stage. The zona pellucida was removed with 0.5% pronase. To separate the blastomeres, embryos were placed in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS^-; Takara Shuzo Co., Tokyo) including 1% (w/v) polyvinylpyrrolidone (PVP; av. M_r 40,000). Each blastomere was then washed nine times in PBS^- including 1% PVP and placed into distilled water in a disposable tube (0.5 ml). After freezing and thawing the tubes three times, the polymorphic region in the D-loop region was amplified by PCR using the following primers: Primer 1, 5′-AACTATTTTCCCCAAGCATATAAGC-3′; Primer 2, 5′-TGACTGTATGGTGTATGTCAGAT-3′ (Figure 1). PCR amplification under "hot start" condition was performed in 20 μl of reaction volume containing 1× PCR buffer (Promega, Madison, WI), 0.2 mm dNTP, 10 pmol of each primer, and 0.9 units of Taq polymerase (Promega) prereacted with 0.23 μg of TaqStart Antibody (Clontech Laboratories, Inc., Palo Alto, CA). The reaction mixture was incubated in a thermal cycler (program temp control system PC-700, Astec Inc., Fukuoka, Japan) under the following conditions: 96° for 1 min for first denaturation, 35 cycles of denaturation, annealing, and extension (95° for 1 min, 57° for 1 min, and extension for 1 min at 72°), and 72° for 4 min for final extension.

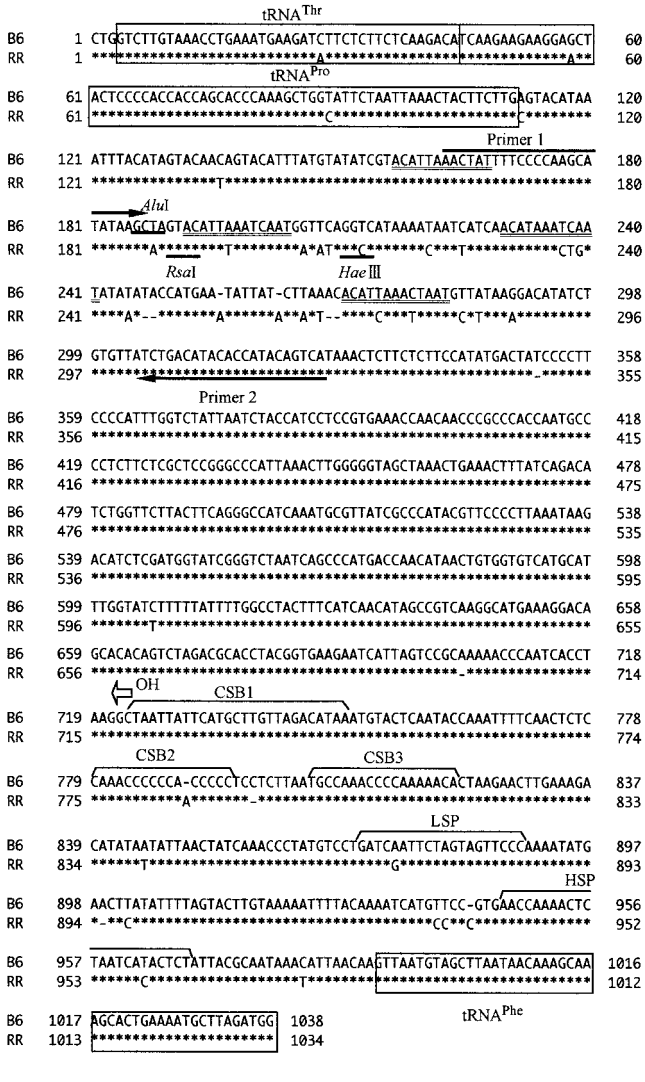

Figure 1.—Mutations of RR mtDNA D-loop region sequences (nucleotide numbers from 112 to 990). There are 33 substitutions and 10 inserted or deleted bases in the RR sequence with respect to B6 strain mice sequence. B6 lines and RR lines show the nucleotide sequence at the mtDNA D-loop region of B6 and RR strain mice, respectively. Primer 1 and Primer 2 are indicated by arrows. Restriction enzyme sites (*Hae*III, *Rsa*I, and *Alu*I) are indicated by boldface lines. The tRNA coding regions are boxed (Bibb *et al.* 1981). The TASs are double-underlined. Ori H, heavy-strand origin of replication; CSBs, conserved sequence blocks; LSP, light-strand promoter; HSP, heavy-strand promoter (Clayton 1982; Chang and Clayton 1986a,b). The same nucleotide base between two mtDNAs is indicated by an asterisk and a lost nucleotide base is indicated by a hyphen. The nucleotide sequence data reported in this figure will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession nos. AB025348 and AB033825.

Total genomic DNAs from the tissues (brain, heart, skeletal

muscle, liver, kidney, spleen, lung, stomach, small intestine, and ovary or testis) were disrupted by 1% SDS solution (10 mm Tris-HCl, pH 8.0, and 5 mm EDTA) with 100 μg proteinase K ml^{-1} and purified by phenol:chloroform extraction and ethanol precipitation. Blood genomic DNA was isolated using a DNA extraction kit for blood (Takara, Shiga, Japan). The extracted DNAs (100 ng) were amplified by PCR as described above.

Analysis of mtDNA heteroplasmy: A *Hae*III site in the amplified region present in RR mtDNA but absent in B6 mtDNA was used to analyze mtDNA heteroplasmy in individual animals and embryos (Figure 1). A *Rsa*I site in the amplified region present in both RR and B6 mtDNAs was also used to exclude nondigested PCR fragments. The PCR fragments were cut with *Hae*III and *Rsa*I, producing a nonspecific 29-bp fragment and either 112-bp and 21-bp RR-specific fragments or a 135-bp B6-specific fragment. The digested fragments were run on an 8% polyacrylamide gel and stained with ethidium bromide. The distribution ratios of RR and B6 mtDNAs were determined by densitometry of the respective strain-specific restriction fragments using computer software (NIH Image version 1.57, National Institutes of Health) and were corrected for fragment size and heteroduplexes.

Cloning and sequencing of RR and B6 mtDNA at the D-loop region: The *Bst*UI digested fragments (1.9 kb), including the D-loop region of B6 and RR mtDNAs, were ligated into the plasmid vector pBluescript II (Stratagene, La Jolla, CA). The cloned DNAs (pBSB61.9 and pBSRR1.9) were sequenced with the ABI PRISM™ dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster, CA).

RESULTS

The nucleotide sequences of the B6 and RR mtDNA D-loop regions are shown in Figure 1. There are 43 mutations in the D-loop region when these sequences are compared to each other.

An initial experiment was undertaken to determine the ratio of different mtDNA molecules in our protocol. To do this, pBSB61.9 and pBSRR1.9 mixed at weight ratios 1:9 to 19:1 were used as PCR templates. Errors due to amplification, such as the heteroduplex formation hybridizing between the B6 and the RR fragments, could be corrected by using regression analysis. The true proportion of RR genotypes obtained from the regression curve was $Y = -0.0059X^2 + 1.5827X + 0.809$, while the coefficient of determination (R^2) was 0.9962. To estimate the proportion of heteroduplex molecules, the PCR products were digested at two strain-specific restriction sites, *Hae*III and *Alu*I. The maximal proportion of heteroduplex structures was 5.0% in equal amounts (1:1) of mixed mtDNA.

Segregation of mtDNA in heteroplasmic mice: We produced 349 heteroplasmic embryos by electrofusion, of which 278 (79.7%) developed beyond the two-cell stage. The RR mtDNAs were detected in the embryos immediately after fusion ($n = 11$, 66%) and in the blastocyst stage embryos ($n = 15$, 70%). Of the embryos that reached the morula stage, 174 were transplanted into pseudopregnant females, and 27 (15.5%) developed into progeny (Table 1). The progeny consisted of 17 mice with a RR/DBA nuclear background (10 males, R-1∼10 and 7 females, R-a∼g) and 9 mice with a B6 nuclear background (6 males, B-1∼6 and 3 females, B-a∼c) and one mouse whose sex could not be determined. Twenty-four of the progeny were identified as heteroplasmic mice (R-1∼4,7∼10, R-a∼g, B-1∼6, and B-a∼c) with two types of mtDNAs determined by genotype analysis from ear or blood biopsies. The proportions of the mtDNA genotypes were determined in the various tissue samples from 20 heteroplasmic mice (R-1∼3,5,8∼10, R-a,b,d,e, B-1∼6, and B-a∼c) (Figures 2a and 3a). The average proportions of RR mtDNA in the heteroplasmic mice were 95% in the RR/DBA nuclear background mice and 94% in the B6 nuclear background mice. The RR mtDNA ratio was very high in most tissues, but was significantly lower in the heart (89% on average) and the brain (88% on average) than in the small intestine (98%, $P < 0.01$) or ovary and liver (97%, $P < 0.02$).

A total of 189 progeny of the first to third generations was obtained from the eight maternal lineages of heteroplasmic females (six lineages with of RR/DBA nuclear background, R-a,b,d∼g, and two lineages with the B6 nuclear background, B-a,c). All of the mice passed on the RR mtDNA genotype to their offspring. The average proportion of the RR mtDNA genotype in these progeny was very high (98%). We also determined the proportions of the RR mtDNA genotype in various tissue samples including heart, skeletal muscle, and ovary for 19 heteroplasmic progeny from the four maternal lineages (Figures 2b and 3b). The average proportion of the RR mtDNA in the tissues was 96%. The values were

TABLE 1

Development of reconstructed embryos and progeny derived from them

Mouse strain		No. of embryos				No. of progeny			
Blastomere	Cytoplast	Electrofused	Developed	%[a]	Transplanted	Male	Female	Total	%[b]
RR′	B6	199	146	73.4	85	10	7	18[c]	15.1
B6	RR′	150	131	87.3	89	6	3	9	10.1

[a] No. of embryos developed/no. of embryos electrofused × 100.
[b] No. of progeny/no. of embryos transplanted × 100.
[c] The sex of one of them could not be determined.

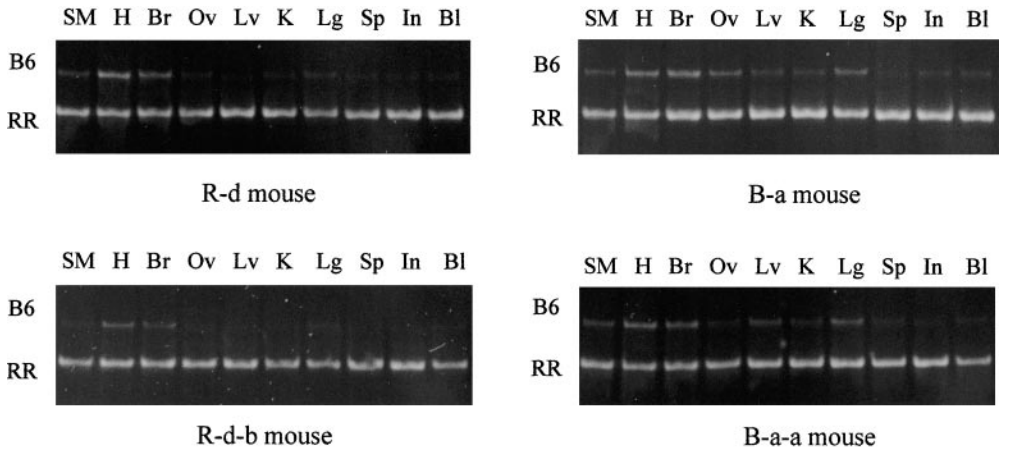

Figure 2.—PCR-restriction fragment length polymorphism patterns of mtDNA in various tissues of heteroplasmic female mice. Top and bottom show B6- and RR-specific bands after *Hae*III and *Rs*aI digestion of the PCR product. The R-d mouse with a RR/DBA nuclear background and the B-a mouse with a B6 nuclear background originated from reconstructed embryos. The R-d-b mouse is progeny of a R-d female, and the B-a-a mouse is progeny of a B-a female. SM, skeletal muscle; H, heart; Br, brain; Ov, ovary; Lv, liver; K, kidney; Lg, lung; Sp, spleen; In, small intestine; and Bl, blood.

significantly lower in the heart (92%) and the brain (92%) than in the small intestine, spleen, kidney, lung, stomach, and ovary ($P < 0.01$).

Age-related changes of heterogeneous mtDNA population in blood: Five heteroplasmic mice with the B6 nuclear background (B-c, 3∼6) were used to determine the age-related changes of the mtDNA genotype. For each mouse, blood samples were collected from the tail at 1, 2, 3, 4, 5.5, 8, 10, and 13 weeks after birth. An increased proportion of the RR mtDNA genotype was observed in all animals as their ages increased (Figure 4).

Differences in mtDNA ratios among blastomeres: The segregation pattern of RR mtDNA during early embryonic development was determined from the two-cell stage to the morula stage in each blastomere isolated from the reconstructed embryos (Figure 5a) and in a blastomere from heteroplasmic mice (Figure 5b). The coefficient of variation (CV) of the RR mtDNA ratio was used as an index for estimating the mtDNA segregation pattern in the blastomere. The CV in the reconstructed embryos (CV = 23.8%) was significantly higher ($P < 0.01$) than that in the embryos derived from heteroplasmic mice (CV = 2.3%).

DISCUSSION

Our results clearly demonstrate that RR mtDNA has a replicative advantage over B6 mtDNA during embryonic development and differentiation, despite the differences in the nuclear genome. The reconstructed embryos showed almost equivalent populations of both RR and B6 types of mtDNAs at the blastocyst stage. However, the proportion of RR mtDNA genotype increased significantly or became dominant in all of the progenies derived from the reconstructed embryos. Moreover, an age-dependent increase of RR mtDNA in the heteroplasmic mice was observed. We did not rule out the possibility of the recombination of the mtDNA D-loop in heteroplasmic mice (Thyagarajan *et al.* 1996). However, similar RR biased and tissue-specific segregation was revealed by Southern blot analysis of the whole mtDNAs extracted from three of the heteroplasmic mice (data not shown). Meirelles and Smith (1997) also observed a decrease in B6 mtDNA, in their case relative to NZB mtDNA. On the other hand, from *in vitro* analysis using human cell lines containing mixtures of pathological mutant and wild-type mtDNAs, mutant mtDNA showed a selective advantage in one cell line, whereas segregation bias was reversed in another cell line (Dunbar *et al.* 1995; Vergani *et al.* 1999), suggesting that nuclear genetic background can influence the segregation of mutant mtDNA in human heteroplasmic cells. However, our results showed a strong segregation bias toward RR mtDNA irrespective of nuclear background.

Sequence analysis revealed 43 differences in the D-loop region when these sequences are compared to each other. The major polymorphic region lies near the tRNA-proline gene, downstream of the heavy strand origin. It has been reported this is the most highly variable base site in the D-loop in other strains of mice (Prager *et al.* 1996), in humans (Horai and Hayasaka 1990), and in cattle (Loftus *et al.* 1994). The termination-associated sequences (TASs) relative to D-loop strand synthesis are located in this region (Clayton 1982, 1984). NZB D-loop sequence (accession no. U47448 in GenBank by Prager *et al.* 1996) also has some base differences in comparison with B6. However, many more base differences between RR and B6 were observed in this region (Figure 1). It may be considered that the sequence differences in the polymorphic region effect replication ability.

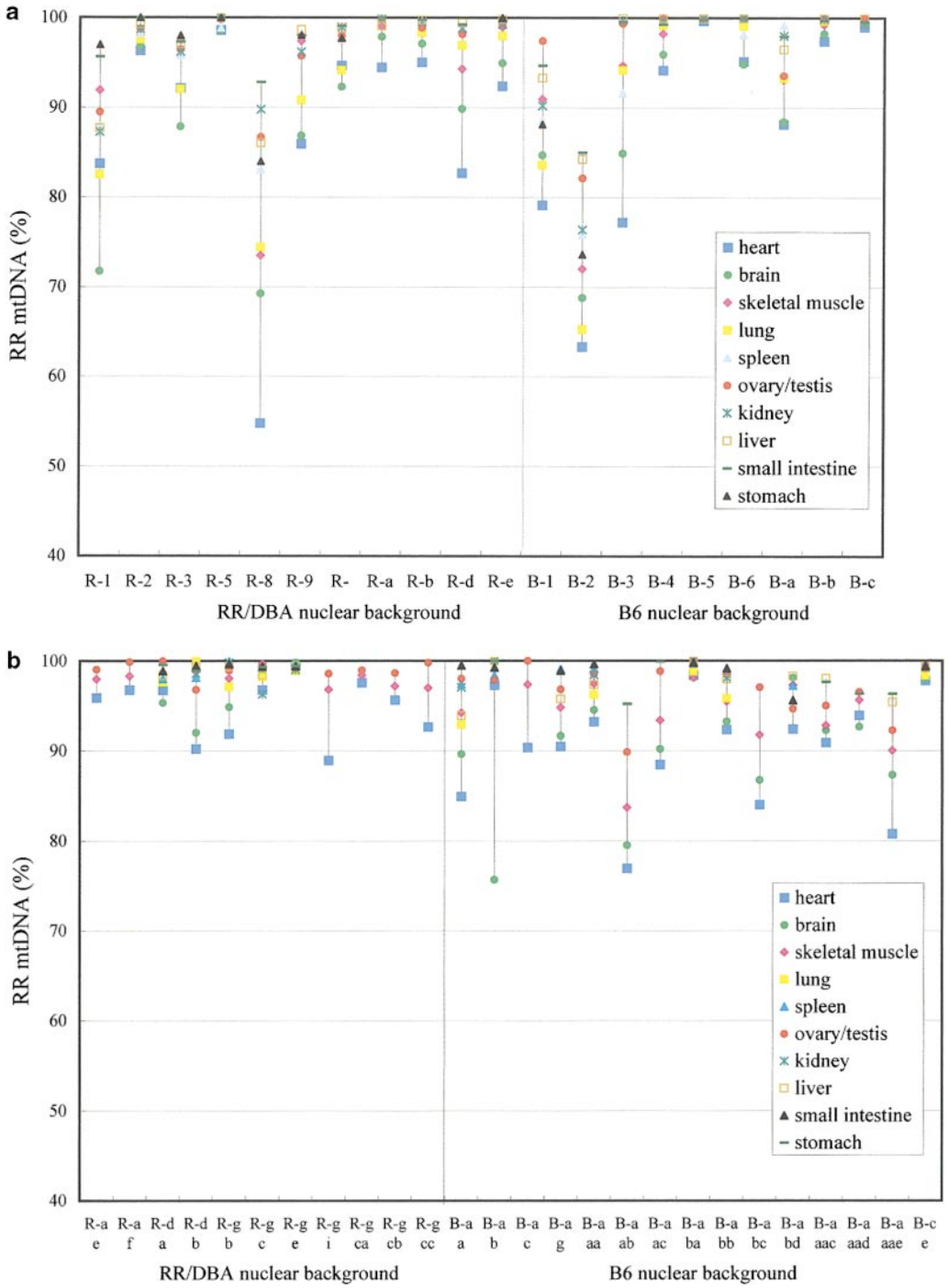

Figure 3.—Segregation patterns of RR mtDNA genotypes in tissues of individual animals. (a) Proportions of RR mtDNA genotypes in tissues of heteroplasmic mice derived from reconstructed embryos. R-1∼3, 5, and 8∼10 are males and R-a, b, d, and e are females with a RR/DBA nuclear background. B-1∼6 are males, and B-a∼c are females with a B6 nuclear background. (b) Proportions of RR mtDNA genotypes in tissues of female progeny of heteroplasmic mice. R-a-e, f, progeny of R-a mouse; R-d-a, b, progeny of R-d mouse; R-g-b∼e, i, progeny of R-g mouse; R-g-ca∼c, progeny of R-g-c mouse; B-a-a∼c, g, progeny of B-a mouse; B-a-aa∼c, progeny of B-a-a mouse; B-a-ba∼c, progeny of B-a-b mouse; B-a-aac∼e, progeny of B-a-aa mouse; B-c-e, progeny of B-c mouse.

The change in genotype frequency in the offspring of a given mother can be used to calculate the relative replicative advantage of the RR genotype in the germline and during fetal life. It was considered that the frequency of RR mtDNA never decreased relative to the mother in any of the offspring from founder females. However, further offspring from heteroplasmic females showed different results from this expectation (see B-a lineage in Figure 3b). It may be that selection was not always more important than drift in the germline of these mice.

A tissue-specific distribution of RR mtDNA was also observed in heteroplasmic mice derived from the reconstructed embryos and their progenies. However, the segregation pattern of the RR mtDNA genotypes in the reconstructed embryos differed from that of the embryos collected from heteroplasmic mice. These results suggest that the RR mtDNA genotype increased in all tissues during development, while the increase tended to be faster in the small intestine, and slower in the heart and the brain. The proportional differences among blastomeres in reconstructed embryos seemed

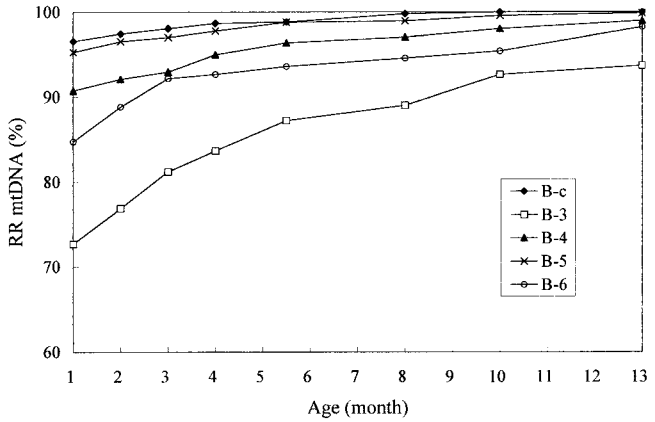

Figure 4.—Age-related proportional changes of RR mtDNA in blood of heteroplasmic mice with a B6 nuclear background.

to be independent on the tissue-specific segregation pattern in heteroplasmic mice (Figure 3a and 5a).

The segregation patterns of mtDNA in the heart and the brain were more heteroplasmic than they were in the other tissues examined. A similar tissue-specific segregation pattern was observed in heteroplasmic mice produced by karyoplast or cytoplast transfer between zygotes of *Mus musculus molossinus* and *M. m. domesticus* (Smith and Alcivar 1993). The *M. m. molossinus* mtDNA was dominant in most of the organs and tissues examined, but *M. m. domesticus* mtDNA was detected only in the brain, heart, and spleen. On the other hand, Jenuth *et al.* (1997) reported a different mtDNA segregation pattern in heteroplasmic mice with BALB and NZB mtDNAs. The proportion of BALB mtDNA genotype increased in blood and in the spleen, while the proportion of NZB mtDNA increased in the liver and kidney. These different tissue-specific segregation patterns in heteroplasmic mice may be due to a complicated interaction between mtDNA and a nuclear genome derived from a different mouse strain. The restriction fragment length polymorphism of RR mtDNA shows a pattern similar to that of *M. m. molossinus* captured in China, but different from NZB which is similar to *M. m. brevirostris* (Yonekawa *et al.* 1982). Therefore, the tissue-specific mtDNA segregation pattern in heteroplasmic mice observed in this study and by Smith and Alcivar (1993) may be due to a similarity of mtDNA sequences between RR mice and *M. m. molossinus.*

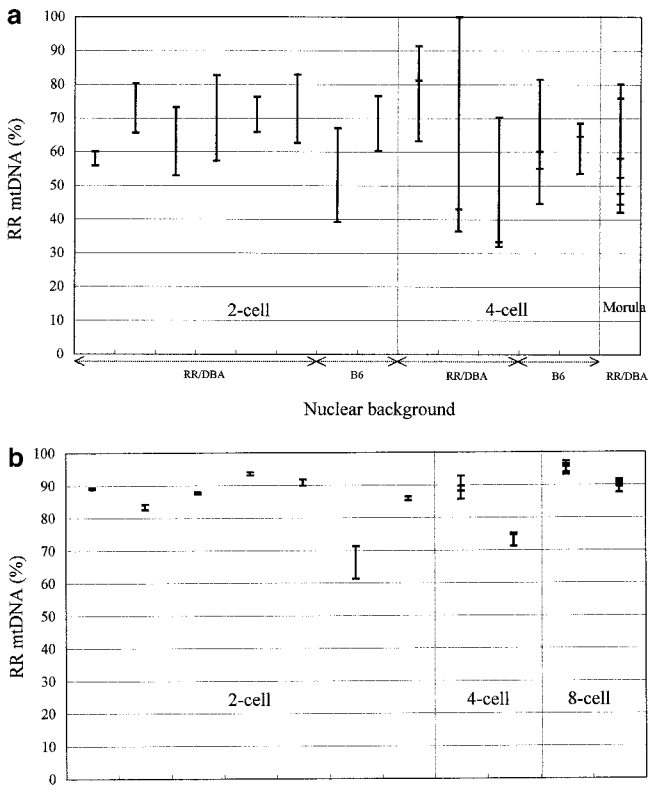

Figure 5.—Segregation pattern of RR mtDNA of individual embryos at early stage. (a) Segregation pattern of RR mtDNA in reconstructed embryos at the two-cell, four-cell, and morula stage. The nuclear background of each embryo is indicated below. (b) Segregation pattern of RR mtDNA in heteroplasmic embryos derived from heteroplasmic mice with a B6 nuclear background at the two-cell, four-cell, and eight-cell stage. Bars indicate the difference between maximum and minimum ratios of RR mtDNA in an individual embryo.

Mikami *et al.* (1989) reported that the mitochondrial activity of cytochrome c oxidase in the liver was lower in the RR strain than in the B6 strains. Qualitative or quantitative differences in respiratory chain activity between these mice may cause the biased distribution of RR mtDNA in heteroplasmic mice. However, in spite of the reciprocal exchange of nuclear genomes between these strains, similar tissue-specific RR mtDNA distributions were observed in the heteroplasmic mice. Therefore, it may be difficult to explain the distribution bias of RR mtDNA only in terms of an interaction between the nuclear and mitochondrial genomes. The interaction between different types of mtDNAs and differences in their replication abilities may be important keys for understanding the mechanisms of the intracellular selection or inheritance of mtDNA.

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