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# Paternal Mitochondrial DNA Transmission During Nonhuman Primate Nuclear Transfer

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ABSTRACT

Offspring produced by nuclear transfer (NT) have identical nuclear DNA (nDNA). However, mitochondrial DNA (mtDNA) inheritance could vary considerably. In sheep, homoplasmy is maintained since mtDNA is transmitted from the oocyte (recipient) only. In contrast, cattle are heteroplasmic, harboring a predominance of recipient mtDNA along with varying levels of donor mtDNA. We show that the two nonhuman primate *Macaca mulatta* offspring born by NT have mtDNA from three sources: (1) maternal mtDNA from the recipient egg, (2) maternal mtDNA from the egg contributing to the donor blastomere, and (3) paternal mtDNA from the sperm that fertilized the egg from which the donor blastomere was isolated. The introduction of foreign mtDNA into reconstructed recipient eggs has also been demonstrated in mice through pronuclear injection and in humans through cytoplasmic transfer. The mitochondrial triplasmy following *M. mulatta* NT reported here forces concerns regarding the parental origins of mtDNA in clinically reconstructed eggs. In addition, mtDNA heteroplasmy might result in the embryonic stem cell lines generated for experimental and therapeutic purposes ("therapeutic cloning").

THE technique of "cloning," or nuclear transfer (NT), may dramatically alter scientific approaches to the understanding of disease and provide novel therapies. NT is an invasive approach that necessitates the fusion of a donor cell containing the chromosomal genetic material of choice with an enucleated recipient oocyte (Campbell *et al.* 1996). Normally, the recipient oocyte is matured *in vitro* until arrest at metaphase II (MII), as is the case for other assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (Hewitson *et al.* 1999). Since the first report of cloned ovine offspring generated from cultured embryonic cells (Campbell *et al.* 1996), NT has also been achieved using donor genetic material from fetal and adult cell populations in a variety of species. These include sheep (Wilmut *et al.* 1997), cattle (Steinborn *et al.* 1998b), goats (Baguisi *et al.* 1999), pigs (Polejaeva *et al.* 2000), cats (Kitiyanant *et al.* 2003), mice (Wakayama *et al.* 1999), rabbits (Chesne *et al.* 2002), and *Macaca mulatta* (Meng *et al.* 1997).

However, the potential to clone truly genetically identical offspring is negated through the presence of mitochondria in the cytoplasm of the recipient oocytes. This arises because mitochondria possess their own distinct genome, mitochondrial DNA (mtDNA). This extranuclear 16.6-kb circular genome is inherited strictly in a maternal fashion through the oocyte (Giles *et al.* 1980; Birky 1995, 2001). However, those offspring generated through embryo reconstruction techniques can transmit two populations of mtDNA. For example, reconstructed mouse oocytes and zygotes transmit varying amounts of both recipient and donor mtDNA (Jenuth *et al.* 1996; Laipis 1996; Meirelles and Smith 1997), as can humans generated through cytoplasmic transfer (CT; Brenner *et al.* 2000; Barritt *et al.* 2001). Furthermore, varying degrees of heteroplasmic mtDNA transmission have been observed in those offspring generated through both somatic cell nuclear transfer (SCNT; Takeda *et al.* 2003) and embryonic cell nuclear transfer (ECNT; Steinborn *et al.* 1998b, 2000).

Embryonic cell NT offers the opportunity for further aberrant patterns of mtDNA transmission to arise, particularly since the transferred "nucleus" includes the large cytoplasmic volume of the embryonic blastomere and also remnants of the fertilizing sperm. Transmission of sperm mtDNA postfertilization appears to be a phenomenon that is peculiar to interspecific breeding (the crossing of two strains or subspecies; Gyllensten *et al.* 1991; Shitara *et al.* 1998). Consequently, those interspecific offspring will possess both maternal and paternal source mtDNA. However, those offspring generated through intraspecific crossing (crossing between the same strain) eliminate sperm mitochondria prior to the eight-cell stage of embryo development (Kaneda *et al.* 1995; Sutovsky *et al.* 1996; Cummins *et al.* 1997, 1998b, 1999) most likely through the process of ubiquitination

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postfertilization, where the male germ cells are previously labeled during the process of spermatogenesis (Sutovsky *et al.* 1999).

In this study, we have analyzed the only two nonhuman primates generated through ECNT. In this instance, the two donor blastomeres were generated from Indian *M. mulatta* oocytes fertilized with sperm from a Chinese *M. mulatta.* These interspecific blastomeres were subsequently fused with enucleated recipient oocytes derived from Indian *M. mulatta* females (Meng *et al.* 1997).

## MATERIALS AND METHODS

**DNA extraction:** mtDNA was extracted from blood platelets using the QIAamp blood kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Total DNA was extracted from tissue, placenta, blood, and sperm samples using the Puregene DNA isolation kit (Flowgen) according to the manufacturer's protocols. For sperm, the lysate was supplemented with 1.5 μl of 20 mg/ml proteinase K (Sigma, St. Louis) and 12 μl of 1 m dithiothreitol (Sigma) and digested overnight at 55° (see St. John *et al.* 2001). The resultant DNA samples were recovered in 50 μl of autoclaved UltraPure water.

**Determination of *M. mulatta* D-loop:** The DNA sequence for the *M. mulatta* D-loop from nucleotide (nt) 16386 to nt 00950, relevant to the human mitochondrial genome, was determined by amplifying tissue samples from four individuals using primers RhDF (5′ tag gag tcc ctt act cac c 3′) and RhDR (5′ tta aac acc ctc tac gcc g 3′). PCR using 2 units Taq 2000 polymerase (Stratagene, La Jolla, CA) was performed in 50 μl volumes in 1× PCR buffer with 0.5 μm each primer and 200 μm dNTP mix (Bioline, London). Reaction conditions were initial denaturation at 95° for 5 min and then 30 cycles of denaturation at 94° for 2 min, annealing at 57° for 1 min, and extension at 72° for 5 min, generating sequence data of ~1020 bp. A second PCR using Rhe 2/F (5′ taa cat atc cga tca gag cc 3′) with RhDR generated ~450 bp of sequence. Reagents and cycling conditions were as described above, except annealing was at 55°. The sequence was verified using combinations of forward [RhDF, Rhe 2/F, 4F (5′ ggt cta tca ccc tat taa cc 3′), and 4F/2 (5′ tcc tgt atg cgc ctg tct tt 3′)] and reverse [RhDR, 4R (5′ ggc tct gat cgg ata tgt ta 3′), and 4R/2 (5′ ggc agt tgg agt tgt gta ca 3′)] primers. Reaction conditions were: initial denaturation at 95° for 2 min; 30 cycles of denaturation at 94° for 30 sec, annealing at 55° for 30 sec, and extension at 72° for 45 sec; and a final extension at 72° for 10 min. mtDNA primate specificity was confirmed by amplifying platelet DNA. Position of primers is indicated in Figure 1.

**PCR and mtDNA analysis of cloned offspring:** mtDNA analysis was performed on white blood cells ($n = 3$ trials) for the female that produced the recipient eggs (16426), its mother (8090), its daughter (19486), an abortus (16426/FT), and the two resultant offspring (19235 and 19255). For the donor lineage, white blood cells ($n = 3$ trials) were analyzed from the Oregon National Primate Research Center (ONPRC) serum bank, including the nuclear donor (14893) and its mother (13487) and daughter (17286). Purified sperm samples ($n = 3$ trials) and white blood cells ($n = 3$ trials) from a Chinese *M. mulatta* male used to generate donor blastomeres (14609) were also analyzed. HV2 was amplified using combinations of RhDF, 4F, and 4F/2 and RhDR, 4R, and 4R/2, using the reaction conditions described above. Products from these reactions were then sequenced to determine polymorphic variation in the HV2 region by direct sequencing.

**Subcloning of PCR products:** PCR products were subcloned into the pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and were amplified and sequenced with forward and reverse sequencing primers M13F (5′ tgt aaa acg acg gcc agt 3′) and M13R (5′ cag gaa aca gct atg acc 3′) and combinations of RhDF, 4F, and 4F/2 and RhDR, 4R, and 4R/2.

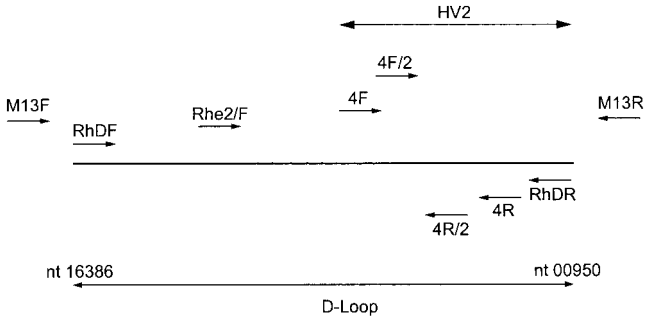


Figure 1.—Location of PCR primers. Combinations of primers located in the D-loop and specifically in HV2 were utilized for PCR amplification to determine polymorphic variation and restriction enzyme digest sites. M13F and M13R are common sequencing primers also utilized following subcloning of PCR products.

**DNA sequencing:** PCR products using combinations of M13F, RhDF, 4F, and 4F/2 and M13R, RhDR, 4R, and 4R/2 were resolved on 2% agarose electrophoretic gels at 100 V for 1 hr and the bands were excised and purified using the QIAGEN gel extraction kit. The purified product was then sequenced using the automated direct sequencing protocol as described by Hopgood *et al.* (1991). Sequencing was performed on an ABI (Foster City, CA) Prism 373 stretch gene sequencer.

**Restriction enzyme digest:** Restriction enzyme digest was performed on both PCR products amplified with primer set RhDF and 4R and subcloned PCR products generated with M13F and M13R. Reactions consisted of 5–10 units of one of *Dde*I, *Hin*fI, or *Alu*I (New England Biolabs, Beverly, MA) and 1× buffer. Restriction sites are described in Figure 4A. Products were resolved on 3% agarose electrophoretic gels.

**Allele-specific PCR:** Allele-specific PCR (AS-PCR) was performed in 50-μl volumes using Biolase Diamond polymerase (Bioline) with 1× PCR buffer, 2.2 mm $MgCl_2$, 0.5 μm each primer, and 200 μm dNTP mix (Bioline) to confirm each of the three lineages and the specificity of the primers to their intended targets through mismatch assays (oocyte donor, 5′ cac acc aca cca caa cca t 3′; recipient, 5′ tac caa taa tca tcc cag ccg 3′; and sperm, 5′ tac caa taa cca gtt ccg cg 3′ lineages). PCR products were resolved on 2% agarose electrophoretic gels.

## RESULTS

**Heteroplasmic mtDNA transmission during primate embryonic cell NT:** The control region or D-loop of mtDNA has two hypervariable regions (HV1 and HV2) that are susceptible to polymorphic variation and are decisive for determining inheritance patterns (Ivanov *et al.* 1996). We sequenced the majority of the previously unsequenced *M. mulatta* D-loop and identified a region of ~590 bp corresponding to HV2 of human mtDNA. Amplification of platelet DNA confirmed the specificity

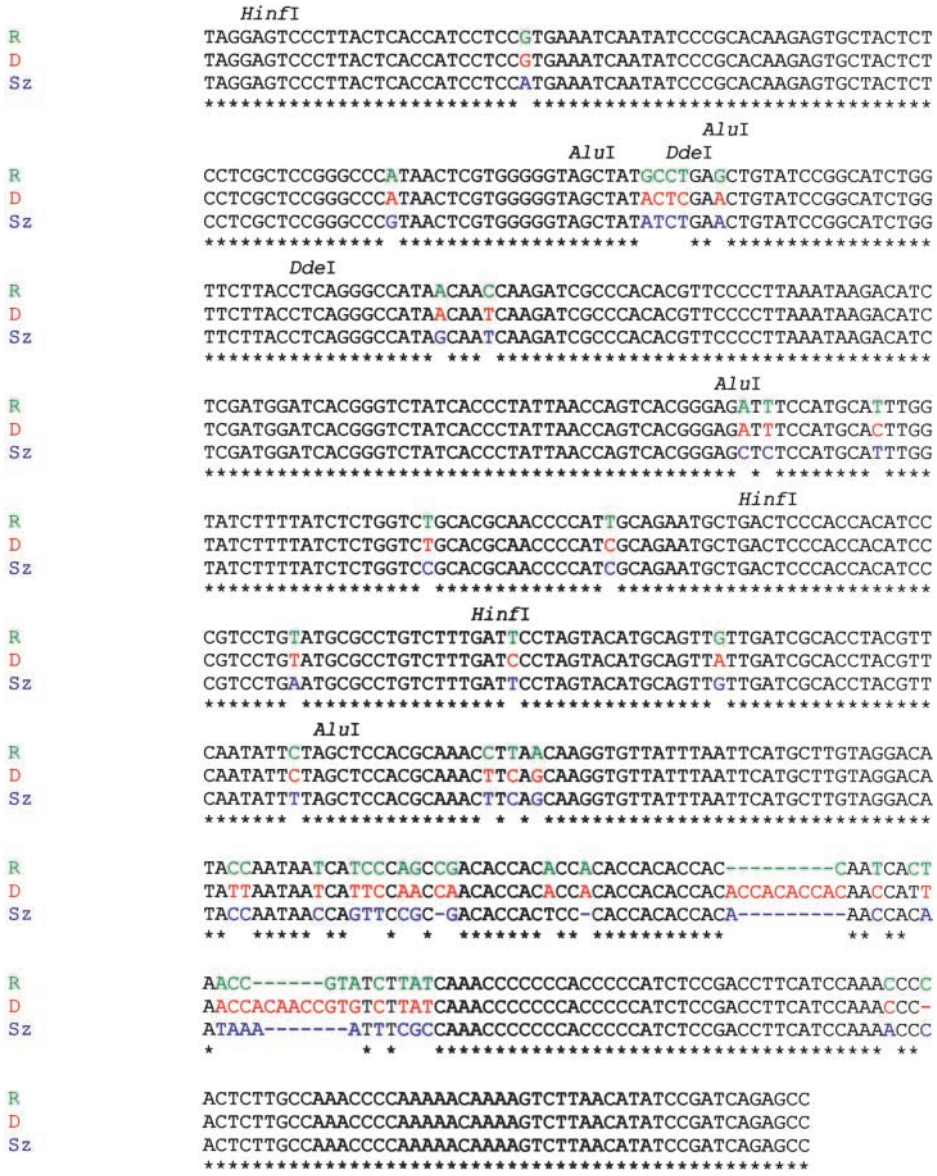


FIGURE 2.—Clustal W (1.7) multiple sequence alignment from three individual alleles from one of the offspring (19255) generated through ECNT. Each of the alleles is representative of one of the sources of mtDNA observed in offspring 19255. R, maternal mtDNA from the recipient oocyte (green lettering represents polymorphic variation); D, maternal mtDNA from the oocyte contributing to the donor blastomere (red lettering); Sz, mtDNA from the sperm used to create the donor blastomere (blue lettering). Asterisk denotes sequence homology within the 590-bp region of the D-loop analyzed. Samples were prepared and sequenced as described in MATERIALS AND METHODS. Restriction enzyme sites *Alu*I, *Dde*I, and *Hin*fI, used to digest PCR products, are also indicated.

of primate mtDNA and excluded the possibility of contamination by nuclear pseudogenes. Characteristically, HV2 has a series of variable repeats (*acacc*) between nt 464 and nt 475, which do not vary in homoplasmic *M. mulatta* but can vary in heteroplasmic individuals (see Figure 2), a factor not unique to *M. mulatta*.

To identify inheritance patterns following ECNT in nonhuman primates, we isolated and analyzed samples from the two *M. mulatta* NT offspring. We sequenced mtDNA from blood samples for the recipient (16426), her mother (8090), her daughter (19486), an abortus (16426/FT), and the two resultant offspring (19235 and 19255). For the oocyte donor lineage (14893), her mother (13487), and her daughter (17286), we examined white blood cells isolated from serum samples obtained from the ONPRC serum bank. In all cases, three separate sets of samples were isolated and analyzed on three separate occasions using various combinations of primers (see Figure 1), as described in MATERIALS AND METHODS. Figure 3 shows the pedigree of the animals used to generate

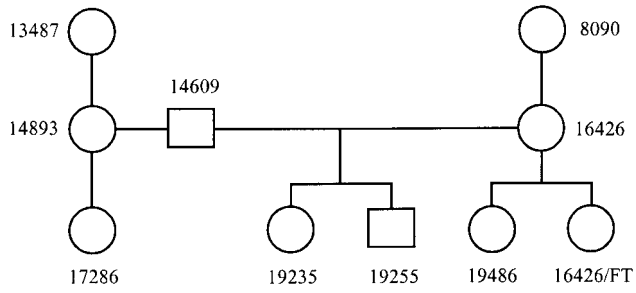


FIGURE 3.—Pedigree chart of the animals analyzed for patterns of mtDNA inheritance to determine mtDNA composition of the two NT-generated offspring (19235 and 19255).

the two NT offspring. Sequence analysis of both the recipient and the donor revealed the presence of heteroplasmy, as previously described (Petri *et al.* 1996).

To ascertain the extent of the heteroplasmy, we subcloned the individual PCR products into the pCR4-TOPO vector. Heteroplasmy was most common in the donor and the recipient between nt 464 and nt 475, which involves the incorporation or loss of one or more *acacc* repeats. The respective sequences and heteroplasmy were confirmed in the donor's and the recipient's respective mothers and daughters and in 16426/FT for the recipient, as well.

Analysis of the offspring (19235 and 19255) through direct sequencing of the PCR products, restriction enzyme digest, and subcloning revealed the variable number of *acacc* repeats and also polymorphic variation unrelated to donor and recipient lineages. This clearly indicated that the two offspring appeared to harbor mtDNA from an additional source. Furthermore, we noted from the recipient pedigree that two bases (nt 341 and nt 388) did not match those of the offspring representative of this lineage. These mismatches are confounding as the multilocus analysis for nDNA of the two offspring, one female and one male, derived from blastomeres isolated from different embryos confirmed the parentage of the two offspring (Meng *et al.* 1997). However, many initial NT protocols have used pooled populations of oocytes as recipients (Takeda *et al.* 1999; Polejaeva *et al.* 2000), which can result in the offspring being cytoplasmically diverse (Takeda *et al.* 1999, 2003). This ultimately demonstrates the necessity to determine parentage through both nDNA and mtDNA analysis.

**Transmission of sperm mtDNA following ECNT:** The gametes used to generate the donor blastomeres for NT were derived from the crossing of oocytes from 14893, an *M. mulatta* of Indian origin, with sperm from 14609, a Chinese *M. mulatta* (Meng *et al.* 1997). Such intraspecific crossing in primates might be analogous to interspecific crossing in mice, which can result in leakage of sperm mtDNA (Gyllensten *et al.* 1991; Shitara *et al.* 1998) even though the two *M. mulatta* individuals are from the same genus. We have confirmed through pedigree records that 16426 was of Indian origin (data not shown). Consequently, we subcloned 14609 and sequence analysis revealed the expected variation in homology to that of 14893 and 16426. Furthermore, there was little polymorphic variation between individual mtDNA clones for 14609, with variation limited to nt 71 (g → a), nt 140 (g → a), nt 320 (t → a), and nt 328 (c → t). Significantly, the sequence variants present in 14609, but not in 14893 and 16426, were indicative of the unaccounted heteroplasmic variants in the offspring except for those variants noted at nt 341 and nt 388 for the recipient and her relatives. Figure 1 shows an example of sequences for three allelic variants from 19255. The variation between the polymorphic variants observed in the mtDNA populations matches that of the 0.2% threshold cited as being an acceptable variance between two individuals (Evans *et al.* 1999).

Restriction enzyme digest and AS-PCR (Seibel *et al.* 1994) confirmed the triparental origin of mtDNA in the two primates cloned by NT. Figure 4A shows the relevant fragment sizes for each of the restriction enzymes used. *Dde*I demonstrated that neither offspring possessed the polymorphism present in the recipient at nt 388, but rather the offspring possessed the nucleotide present in the donor and sperm and an extra site arising from the polymorphism present at nt 103 (Figures 2 and 4B). Further investigation revealed that a small number of recipient alleles did possess the nt 103 polymorphism. *Hin*fI supported the presence of recipient mtDNA in each of the offspring (see Figures 2 and 4C), although cutting was similar to that seen for the sperm sample. However, the differential cutting of *Alu*I showed the absence of this particular site at nt 341 in the offspring and its presence in the recipient. The presence of sperm mtDNA was confirmed by the additional *Alu*I site at nt 225 (see Figures 2 and 4D).

To confirm the presence of three mtDNA populations, we used AS-PCR. Each of the alleles was present in the offspring including the sperm mtDNA, as shown in Figure 4E. The specificity of the primers to their intended targets was demonstrated through mismatch assays. For example, Figure 4E shows the nonspecificity of the sperm primer to amplify the respective alleles associated with donor and recipient oocyte sources.

**Regulation of mtDNA transmission in nonhuman primates:** To determine whether heteroplasmic transmission is a normal phenomenon associated with nonhuman primates, we analyzed *acacc* repeat variability in the *M. mulatta* D-loop. Figure 5 shows the heteroplasmic variability in a cohort of intraovarian oocytes (Figure 5A). In this instance, some of the oocytes sampled from female 19601 possessed an extra repeat. However, it is apparent that this female's tissue samples possessed only the extra repeat (Figure 5B), suggesting strict transmission and segregation of somatic mtDNA in a homoplasmic manner.

To substantiate whether leakage of sperm mtDNA is solely associated with NT or a consequence of interspecific crossing in nonhuman primates, we analyzed two offspring generated through artificial insemination (AI; Sánchez-Partida *et al.* 2000). These two AI offspring, 21523 and 21712, were generated through different mothers of Indian origin, 20062 and 13913, respectively, but through the same father, 14609 (Chinese origin), the male used to generate 19235 and 19255. In each instance, we analyzed blood samples from the offspring, both sets of parents, and placental tissue for the presence of sperm-specific mtDNA polymorphisms. Sperm mtDNA was detected in both offspring and their respective placental tissues, but not in the maternal mtDNA sample (see Figure 6).

A

| | Recipient (16426) | Donor - oocyte (14893) | Donor - sperm (14609) |
|---|---|---|---|
| *Dde*I | 282<br>256<br>218 | 538<br>218 | 538<br>218 |
| *Hin*fI | 343<br>281<br>94<br>38 | 381<br>281<br>94 | 343<br>281<br>94<br>38 |
| *Alu*I | 295<br>268<br>182<br>11 | 295<br>279<br>182 | 295<br>182<br>147<br>132 |

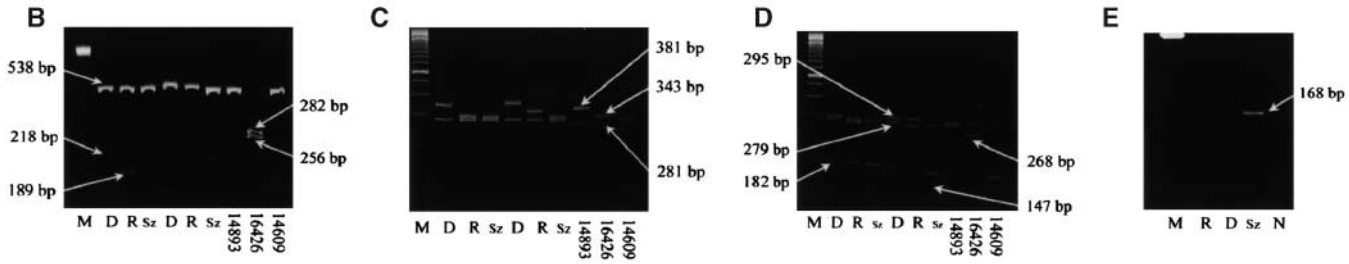


FIGURE 4.—Restriction enzyme digest and AS-PCR. Restriction enzyme digestion was performed using *Dde*I, *Hin*fI, and *Alu*I to demonstrate differential cutting of the three mtDNA sources for the NT-generated offspring. Products were generated through PCR and digested as described in MATERIALS AND METHODS. (A) The fragment sizes generated by each of the respective restriction enzymes. (B) *Dde*I demonstrates the differential cutting associated with the recipient (16426) and the contrasting patterns observed in the offspring. (C) The differential cutting of donor oocyte mtDNA (14893) with *Hin*fI. (D) *Alu*I demonstrates the presence of the sperm mtDNA. (E) AS-PCR to detect mtDNA specific to the sperm lineage. Animal numbers refer to those indicated in the pedigree chart in Figure 2. M, 1-kb ladder (GIBCO BRL, Grand Island, NY); R, recipient mtDNA observed in the offspring; D, donor oocyte blastomere mtDNA; Sz, donor sperm blastomere mtDNA; N, negative control, *i.e.*, no DNA present.

## DISCUSSION

Our analysis demonstrates that the only two nonhuman primate offspring generated through NT inherit three populations of mtDNA: (1) maternal mtDNA from the recipient oocyte; (2) maternal mtDNA from the oocyte that, following fertilization, contributed to the donor blastomere; and (3) paternal mtDNA from the sperm fertilizing the embryo from which the donor blastomere was isolated. This represents a unique case of triparental heteroplasmy. Other micromanipulation studies have shown that the introduction of "foreign" mtDNA into an oocyte at fertilization or a zygote can facilitate its transmission to the offspring, along with that of the recipient, resulting in varying degrees of biparental heteroplasmy. This has been demonstrated in both the human through CT (BRENNER *et al.* 2000) and the mouse by pronuclear transfer (JENUTH *et al.* 1996; MEIRELLES and SMITH 1997). Furthermore, other studies following NT have indicated similar patterns of mtDNA transmission from both ECNT (STEINBORN *et al.* 1998a,b, 2000) and SCNT (HIENDLEDER *et al.* 1999; TAKEDA *et al.* 2003). In each instance, the contribution of donor mtDNA appears to be low, except in a few cases (TAKEDA *et al.* 2003; reviewed in ST. JOHN *et al.* 2004).

Following coitus, mtDNA transmission is restricted to a few molecules thought to pass through an oogenic "bottleneck" (JANSEN and DE BOER 1998) or mediated by a restriction event (JENUTH *et al.* 1996), hypothesized to take place during very early oogenesis. The persistence of both donor and recipient mtDNA following NT indicates that the restrictive nature of mtDNA transmission is violated following oocyte and embryo reconstruction. Our results indicate that a similar mechanism is present in *M. mulatta* to ensure that all somatic tissues are homoplasmic for both the nucleotide composition and the extra *accac* repeat (see Figure 5). The variability of this repeat sequence detected in the oocyte may be observed in only those oocytes recovered through superovulation protocols prior to ART or following ovariecotomy, as they are likely to be less viable and lost to atresia (see ST. JOHN 2002).

In cell culture, both interspecific and intraspecific

**A**

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E4  TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
E5  TCCCAGCCGACACCACACCACACCACACCACACATAACTAACCG
E6  TCCCAGCCGACACCACACCACACCACACCACACATNACTAACCG
E7  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
E1  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
E3  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
E8  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
E9  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
E2  TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG
    *************************     **************
```

**B**

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E2          TCCCAGCCGACACCACACCACACCA-----CACATCACTAACCG

Heart       TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
Colon       TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
L. Brain    TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
Liver       TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
Kidney      TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
Muscle      TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
Skin        TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG

E4          TCCCAGCCGACACCACACCACACCACACCACACATCACTAACCG
            *************************     **************
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FIGURE 5.—Sequence alignment for oocytes E1–9 (A) and tissues (B) sampled from an *M. mulatta* female (19601). Each oocyte is homoplasmic for one allele or another. Asterisk denotes sequence homology within the region of the D-loop analyzed. Samples were prepared and sequenced as described in MATERIALS AND METHODS.

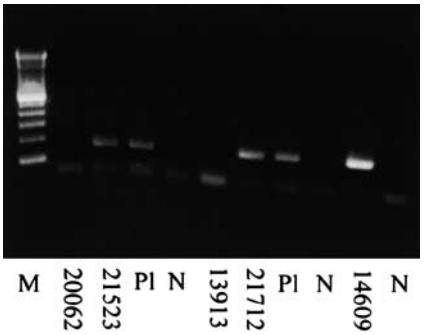


FIGURE 6.—Sperm mtDNA was detected in the interspecific offspring 21523 and 21712 (lanes 2 and 6) and placental samples (lanes 3 and 7) through AS-PCR following AI. The presence of the sperm mtDNA was confirmed by amplifying the sperm sample from the father (lane 9), 14609 for both cases, and the specificity of the primers was determined by the failed amplification of the offspring's respective maternal mtDNA sources, 20062 and 13913 (lanes 1 and 5). Lanes 4, 5, and 10 are negative (*i.e.*, no DNA) controls. M, 1-kb ladder (GIBCO BRL).

transfections can produce viable cybrids, the fusion of an enucleated somatic cell. However, the generation of cybrids from differing species suggests that foreign mtDNA can repopulate a cell only when it does not have to compete with the recipient cell's own mtDNA, as with human and nonhuman ape primate cybrids (MORAES *et al.* 1999). This would suggest that mtDNA transcription and replication are under the control of the donor cell's nuclear background, as evidenced by the comparison between rat and mouse xenomitochondrial transformation. In this instance, the greater diversity between the fusion partners results in a greater degree of compromised ATP production through impaired OXPHOS function (DEY *et al.* 2000; MCKENZIE and TROUNCE 2000).

Interestingly, the donor blastomeres used to propagate 19235 and 19255 were from a mixture of pre- and post-eight-cell-staged embryos and could constitute between 10 and 20% of the total mtDNA population following fusion of the donor blastomere with the recipient cytoplasm (STEINBORN *et al.* 1998a). The further dilution of mtDNA, evident in more advanced embryos, could result in considerably less mtDNA being transmitted to the offspring, as in cattle (STEINBORN *et al.* 1998b). Consequently, for those offspring possessing two or more mtDNA populations, it is vital to determine whether donor mtDNA will outcompete its recipient counterpart. Crossing of strains or subspecies can result in mtDNA sequence variance with subsequent differential amino acid composition affecting protein compatibility to the electron transfer chain (ETC) and reduced ATP synthesis.

The transmission of sperm mtDNA tends to be species specific. Drosophila can transmit both oocyte and sperm mtDNA to the offspring independent of intra- or interspecific crossing (KONDO *et al.* 1992). Mussels possess gender-specific mtDNA genomes with the sperm mtDNA molecule being transmitted through to males only, along with oocyte mtDNA (FISHER 1990; HOEH *et al.* 1991). In mammals, sperm mtDNA persists in those offspring generated through interspecific crossing, for example, *Mus musculus* and *M. spretus* (GYLLENSTEN *et al.* 1991), although this sperm mtDNA is not transmitted to subsequent generations (SHITARA *et al.* 1998). The sperm mtDNA detected in the two *M. mulatta* ECNT offspring and in those offspring generated through AI is indicative of those interspecific murine crossings.

In mammals, sperm mitochondria are eliminated by the eight-cell stage in intraspecific crosses (KANEDA *et al.* 1995; SUTOVSKY *et al.* 1996; CUMMINS *et al.* 1997, 1998b, 1999). This appears to be mediated through the process of ubiquitination, a postfertilization event (SUTOVSKY *et al.* 1999), which ensures that spermatogo-

nia are labeled during spermatogenesis for subsequent destruction in the early embryo. However, certain studies have shown that mtDNA leakage can arise. In murine studies, round spermatids injected into activated oocytes resulted in the persistence of these mitochondria in 1% of eight-cell- or later-staged embryos (Cummins *et al.* 1998b). Furthermore, sperm mtDNA persisted to the blastocyst stage in one set (3/6) of abnormal human embryos generated through IVF (St. John *et al.* 2000). It is likely that the three pronuclear embryos failed to regulate their cytoplasms and consequently the ubiquitination process was not initiated or completed (St. John *et al.* 2004). However, in the human it is also apparent that the robustness of the ubiquitination process is limited. This is reflected by the report of a male patient harboring a mitochondrial myopathy derived from his father's sperm mtDNA (Schwartz and Vissing 2002).

Poor quality sperm and semen samples have been reported to harbor higher levels of mtDNA mutation and rearrangements. These include point mutations (see Holyoake *et al.* 1999, 2001; Spiropoulos *et al.* 2002) and multiple and large-scale mtDNA deletions (see Kao *et al.* 1995, 1998; Lestienne *et al.* 1997; Cummins *et al.* 1998a; Reynier *et al.* 1998; St. John *et al.* 2001; O'Connell *et al.* 2002). In the case of the male patient with the mtDNA myopathy, the onset of mtDNA disease suggests a considerable positive selective advantage for the rearranged sperm mtDNA genome with an ability to outreplicate oocyte mtDNA by at least 1:1000 (Ankel-Simons and Cummins 1996).

The transmission of sperm mtDNA following NT would be restricted to blastomere-NT-generated offspring only and would not affect the outcomes related to SCNT. This diverse pattern of mtDNA transmission associated with ECNT adds to the debate directed at defining those cells likely to be the most appropriate donor cells for NT (Wakayama and Yanagimachi 2001). However, the physiological consequences of generating heteroplasmic offspring through NT and cytoplasmic transfer (Barritt *et al.* 2001; St. John 2002) require considerable investigation before these techniques are applied to the study of disease and in the clinical setting. This is especially so in light of the recent report of unsuccessful human pregnancies arising from pronuclei transfer (Zhang *et al.* 2003). Furthermore, for those offspring generated with higher levels of donor mtDNA transmission (for example, Takeda *et al.* 2003), it is necessary to determine whether both alleles are transcribed, as well as being replicated, and consequently contribute to the protein composition of the various ETC subunits. The consequence of transcribing only one allele is the possible onset of mtDNA-type depletion syndromes (Larsson *et al.* 1994; Poulton *et al.* 1994), which would severely affect offspring survival. Perhaps the most likely approach for avoiding this outcome would be the generation of offspring derived through autologous NT, where the donor cell originates from the same female source, as described for the derivation of SCNT human embryonic stem cells (Hwang *et al.* 2004). However, care must be taken in establishing whether cultured cells maintain their mtDNA genetic integrity and are subject to mutation or large-scale deletion as characteristic of an unpackaged mtDNA genome (reviewed in St. John *et al.* 2004).

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