Coexistence of *Bos taurus* and *B. indicus* Mitochondrial DNAs in Nuclear Transfer-Derived Somatic Cattle Clones

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ABSTRACT

We investigated the mitochondrial DNA (mtDNA) composition in one of the largest adult somatic mammalian clones ($n = 20$) reported so far. The healthy cloned cattle were derived from nuclear transfer of an identical nuclear genetic background (mural granulosa donor cells including surrounding cytoplasm) into enucleated oocytes with either *Bos indicus* or *B. taurus* mtDNA. Here we report the first cases of coexisting mtDNAs of two closely related subspecies following nuclear transfer. Heteroplasmy (0.6–2.8%) was found in 4 out of 11 cross-subspecies cloned cattle. Quantitation was performed using "amplification refractory mutation system (ARMS) allele-specific real-time PCR." We determined that the ratio of donor cell to recipient cytoplast mtDNA copy number was 0.9% before nuclear transfer. Therefore, we concluded that the percentage of donor cell mtDNA in the heteroplasmic intersubspecific cloned animals is in accordance with neutral transmission of donor mtDNA. We determined an amino acid sequence divergence of up to 1.3% for the two subspecies-specific mtDNA haplotypes. In addition, intrasubspecific *B. indicus* heteroplasmy of ~1% (but up to 7.3 and 12.7% in muscle and follicular cells of one animal) was detected in 7 out of the 9 *B. indicus* intrasubspecific cloned cattle.

MITOCHONDRIAL DNA (mtDNA) heteroplasmy represents a naturally rarely occurring phenomenon (Chinnery *et al.* 2000). Following intraspecific sexual crosses mammalian sperm mitochondria generate initial heteroplasmy, but are selectively destroyed between the four- and the eight-cell stage of development (Sutovsky *et al.* 1999, and references therein). It is proposed that the ubiquitination of sperm mitochondria that occurs during spermatogenesis tags them for species-specific extinction by the embryo's proteasomes and lysosomes (Sutovsky *et al.* 1999). Remarkably, sperm mitochondria persist in mammalian interspecies crosses as demonstrated for murine (Gyllensten *et al.* 1991; Kaneda *et al.* 1995) and bovine (Sutovsky *et al.* 1999) hybrids. Heteroplasmy can be generated by a number of cell manipulation techniques. Intraspecific heteroplasmic mice have been generated by fusing embryonic cytoplasts with either one-cell embryos (Jenuth *et al.* 1996) or embryonic pronuclei surrounded by some cytoplasm (Meirelles and Smith 1997). Interspecific heteroplasmic mice were also produced by microinjection of somatic mitochondria into pronucleus-stage embryos (Shitara *et al.* 2000). Moreover, embryonic stem cell cybrids were used to introduce mtDNA mutations into the mouse female germline (Sligh *et al.* 2000). Cloning by nuclear transfer of embryonic donor cells resulted in the production of some heteroplasmic cloned animals (Steinborn *et al.* 1998c; Hiendleder *et al.* 1999), whereas in other embryonic cell-derived cloned animals mtDNA arises exclusively from recipient oocytes (Steinborn *et al.* 1998c; Takeda *et al.* 1999). Similar contradictory findings were found after nuclear transfer of fetal or adult somatic donor cells (Evans *et al.* 1999; Steinborn *et al.* 2000). In addition to the neutral transmission of donor cell mtDNA (heteroplasmy at ~1% according to Steinborn *et al.* 2000) reported for cattle but not sheep, a complete absence of donor cell mtDNA transmission has been observed (Evans *et al.* 1999; Steinborn *et al.* 2000).

This work describes the fate of *B. indicus* donor cell mitochondria from a single genetic origin following nuclear transfer into 20 cytoplasts of random genetic origin, which harbored either *Bos indicus* or *B. taurus* mtDNA haplotypes.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AF361442–AF361461, AY029263–AY029268, AY052631, AF416451, AF419237, AF384025, AF419238, AF384022, and AF384026.

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MATERIALS AND METHODS

Biological material: Two sets of adult somatic cloned animals were produced by nuclear transfer using primary cultures of quiescent (presumptive G0) mural granulosa cells derived from the genetic origin "E" (denoted E-donor). The cloned animals of set I (E1 and E4–E12; Wells *et al.* 1999) and set II (E13–E22, D. N. Wells, unpublished data) derived from donor cells at passage three (E1 at passage five) and eight of culture, respectively. The embryos that resulted in the cloned animals of set II were either cultured *in vitro* in an improved media formulation termed AgResearch Synthetic Oviduct Fluid medium (AgR SOF; AgResearch, Hamilton, New Zealand; used to produce E13–E16, E21, and E22) or cultured *in vivo* in the oviducts of temporary recipient sheep (E17–E20). AgR SOF is a modified formulation to that described by Gardner *et al.* (1994), containing 8 mg/ml BSA. On day 4–5 of *in vitro* culture, embryos were transferred to fresh AgR SOF containing 10 μM 2,4-dinitrophenol, acting as an uncoupler of oxidative phosphorylation (Thompson *et al.* 2000).

In addition, we analyzed the mtDNA composition in the blood of five other adult somatic cloned cattle [cloned animal L2 and ongoing pregnancies (Wells *et al.* 1998; here referred to as cloned animals L5, L6, L8, and L9)] derived from the genetic origin "L" (denoted L-donor). Quiescent (presumptive G0) granulosa donor cells between passages four and eight of culture were used for their production.

The lack of *a priori* heteroplasmy in recipient cytoplasts was determined in 12 oocyte aliquots (nine randomly taken genetic origins of the "Austrian Simmental" breed), each consisting of 10–15 *in vitro* matured and denuded oocytes.

Straws containing bovine sperm were thawed, and cells were spun down and resuspended in 200 μl phosphate-buffered saline (PBS) buffer. Four 50-μl drops were blotted onto Whatman paper, dried, and individually wrapped for convenient transport.

DNA isolation: The cloned bovine embryos produced previously (Steinborn *et al.* 1998b) and the sample containing exactly 15 cells of the E-donor were lysed in 50 μl K-buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.5% Tween 20). Subsequently, total cellular DNA was isolated as described (Steinborn *et al.* 1998b). DNA isolation from blood, tissues, oocyte aliquots, and sperm spots was performed using the respective DNA isolation kit provided by Macherey & Nagel (Dueren, Germany). For DNA isolation from sperm cells we additionally applied 27 mM dithiothreitol to support their lysis.

Microsatellite analysis: Nine microsatellite loci were analyzed, using the second version of the StockMarks kit for cattle paternity PCR typing (Applied Biosystems, Foster City, CA), which is based on four-dye fluorescent labeling. The fluorescence data collected by the ABI PRISM 310 genetic analyzer were subsequently analyzed using the ABI PRISM Genotyper software for automated genotyping (Applied Biosystems).

Analysis of the mtDNA control region: We amplified the complete bovine mtDNA control region as described (Steinborn *et al.* 1998a) and sequenced the PCR products using the ABI PRISM BigDye terminator cycle sequencing kit and the ABI PRISM 377 sequencer (Applied Biosystems). Note that a potential heteroplasmy of 5–10% in a clone would not impair the determination of the recipient cytoplast-derived mtDNA sequence by sequencing of PCR products.

Demonstration of parental mtDNA diversity using phylogenetic analysis: The phylogeny of mtDNA control region sequences of the E-donor and its cloned offspring was reconstructed with the software package TREE-PUZZLE, version 5.0 (Strimmer and von Haeseler 1996), using the HKY model of sequence evolution (Hasegawa *et al.* 1985) with eight categories of rate heterogeneity. The phylogenetic tree was graphically displayed using TREEVIEW, version 1.6.5. The *B. gaurus* mtDNA (GenBank accession no. AF083371) was used as an outgroup. The two cattle taxa *B. taurus* and *B. indicus* are represented in the tree by the GenBank accession nos. J01394 and L27733.

Quantitation of mtDNA copy number ratios and heteroplasmy: Real-time PCR quantitation was performed using fluoresence measurement by the ABI PRISM 7700 sequence detection system (Applied Biosystems) and the 5′-3′ exonuclease activity of the *Taq* polymerase. The 25-μl amplification reactions contained 67.7 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.01% Tween 20; 0.2 mM dATP, dCTP, dGTP, and 0.4 mM dUTP; 1.25 units *Taq* polymerase; 4.5 mM $MgCl_2$; 200 nM TaqMan probe; 300 nM of each primer; and 10–50 ng total cellular DNA. In general, each sample was amplified in triplicate with standard deviations of threshold cycle (Ct) values not exceeding 0.5 (for Ct values <33). Quantitation was performed at an annealing temperature of 62° or 63° (or lower for primers with a reduced GC content).

The ratio of donor cell to recipient cytoplast mtDNA copy numbers before nuclear transfer was determined by the equation Ratio = $100\%/(1 + E)^{\Delta Ct}$, where $E = 10^{-1/m} - 1$ and ΔCt is the difference between the Ct values of the fusion partners detected in the quantitative real-time PCR assay. The slope (m) being a measure for the efficiency (E) of the real-time PCR is determined by the standard curve $y = mx + b$. The standard curve is generated by plotting the Ct values for serial template dilutions in relation to the logarithm of the dilution factor.

For quantitation of heteroplasmy two sets of experiments were run: (i) The total amount of mtDNA was determined by real-time quantitative PCR (Steinborn *et al.* 1998c) and (ii) the less frequent mtDNA type was quantitated by amplification refractory mutation system (ARMS; Newton *et al.* 1989; Wu *et al.* 1989) allele-specific real-time PCR (Steinborn *et al.* 1998c, 2000). The limitation of the dynamic range in the ARMS allele-specific real-time PCR assay is caused by the late-cycle amplification from the nontarget allele. This phenomenon is an inherent feature of ARMS-PCR. The discrimination of the allele-specific quantitation assay, *i.e.*, the limitation of the dynamic range, was controlled by the use of two homoplasmic nontarget allele controls (bovine total cellular DNA), both carrying the alternative base for the single-nucleotide polymorphism (SNP) under study. We proved that the absolute value of the slope in the equation obtained by plotting the log input *vs.* ΔCt calculated from total mtDNA copy number quantitation and the specific allele-specific quantitation assay was <0.1. This allowed the use of the "comparative Ct method" (ABI PRISM 7700 sequence detection system, Applied Biosystems, 1997) for quantitation of heteroplasmy without running standard curves on the same plate. Briefly, the percentage of donor cell mtDNA in a cloned animal in relation to the total mtDNA content in the donor cell sample, *i.e.*, the level of heteroplasmy, was determined by the equation Heteroplasmy = $100\%/(1 + E)^{-\Delta\Delta Ct}$.

Primers and probes: Nucleotide positions in the names of the oligonucleotides given below represent their localization on the bovine mtDNA genome (numbering according to the GenBank accession no. J01394). For amplification and sequencing of the mtDNA control region we used the primers F15,747-15,766, R383-364, and F16,168-16,185. The fluorogenic probes (MWG-Biotech AG, Germany) for real-time PCR consisted of an oligonucleotide with the 5′-reporter dye 6-carboxyfluorescein (FAM) and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) attached to the 3′ end. For total mtDNA quantitation we used the probe P99-74 and the primers F16,159-16,182 and R131-109. This reverse primer was

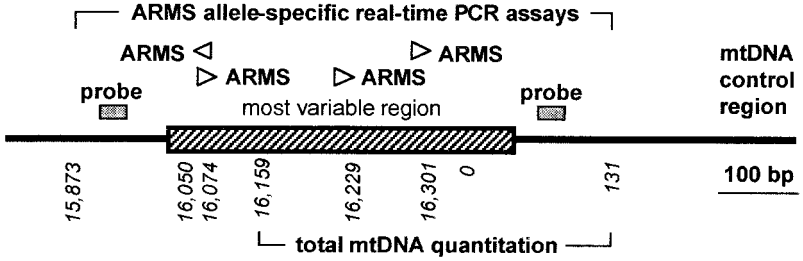

FIGURE 1.—Localization of the fluorogenic probes and ARMS primers (designated ARMS) used for ARMS allele-specific real-time PCR quantitation. Nucleotide positions correspond to the numbering in the GenBank accession no. J01394.

used in combination with the probe P99-74 and with one out of three ARMS primers (ARMS16,211-16,229, ARMS16,283-16,301, and ARMS16,056-16,074) in three separate ARMS allele-specific real-time quantitative PCR assays. The probe P15,900-15,929 was used for ARMS allele-specific real-time PCR in combination with F15,873-15,898 and ARMS16,071-16,050. We used the following primer pairs to amplify and sequence the genes coding for NADH dehydrogenase 3 (*MTND3*), NADH dehydrogenase 6 (*MTND6*), cytochrome b (*MTCYB*), cytochrome c oxidase II (*MTCO2*), cytochrome c oxidase III (*MTCO3*), and ATP synthase 6 (*MTATP6*): F9,785-9,804/R10,218-10,194 and F13,860-13,881/R14,468-14,449, F14,383-14,402/R15,757-15,740, F7,266-7,286/R8,132-8,115, F8,944-8,963/R9,885-9,868, and F8,132-8,152/R9,314-9,297, respectively. In addition we applied the primers F14,871-14,890 and R14,955-14,934 for internal sequencing of *MTCYB*.

Determination of the phylogenetic divergence between pairs of species: The estimation of the pairwise relatedness of *Ovis aries* (GenBank accession no. AF010406)/*O. aries musimon* (D84203), *B. taurus* (V00654)/*B. indicus* (AF419237), *Mus musculus* (J01420)/*M. spretus* (AB033700), *Pan troglodytes* (NC_001643)/*Homo sapiens* (AF347015), and *B. gaurus* (AF348596)/*B. taurus* (V00654) was performed by determining their phylogenetic divergence (percentage) on the basis of cytochrome b amino acid sequences.

Accession numbers: Sequences of the mtDNA control region of the recipient cytoplasts and the two donor cell origins used for nuclear transfer were submitted to GenBank under the following accession numbers: AF361442–AF361461 (cloned animals E1–E22; note that E2 and E3 were not investigated) and AY029263–AY029268 (L-donor cell and cloned animals produced to preserve the L-donor). We submitted the following mtDNA-encoded genes of the *B. indicus* donor to the GenBank: *MTND3* (GenBank accession no. AY052631), *MTND6* (AF416451), *MTCYB* (AF419237), *MTCO2* (AF384025), *MTCO3* (AF419238), and *MTATP6* (AF384022). A *MTCO2* variant found in the *B. taurus* mtDNA haplotype of the cloned animal E12 was also submitted (AF384026).

RESULTS

mtDNA copy number ratios of the fusion partners before nuclear transfer: Real-time PCR quantitation (Figure 1) was used to determine the ratio of donor cell to recipient cytoplast mtDNA copy number before nuclear transfer. Therefore we compared the Ct values obtained for the 15 cells of the E-donor and for three cloned embryos (5-cell stage, 12-cell stage, blastocyst stage). For this period of embryogenesis constant mtDNA copy numbers were reported (STEINBORN *et al.* 1998c). In contrast to the use of *in vitro*-produced embryos or metaphase II oocytes for relative quantitation, the use of cloned embryos more authentically considers the intracellular distribution of the oocyte mitochondria before enucleation and the amount of the missing part of the ooplasm that is removed from the recipient oocyte during the enucleation procedure. We determined the following mean Ct values ± standard deviation for the 15 E-donor cells and from the three embryo samples: 27.71 ± 0.01 and 24.59 ± 0.44 (all four samples were amplified in duplicates in the same real-time PCR experiment). Considering the slope of $m = -3.56$ we calculated a ratio of donor cell to recipient cytoplast mtDNA copy number before nuclear transfer of 0.9%.

Absence of *a priori* heteroplasmy in the recipient cytoplasts: Human oocytes have been shown to contain mtDNA molecules with homopolymeric tract heteroplasmy and a small proportion of oocyte mtDNA molecules harbor the 4977-bp deletion (NAGLEY and WEI 1998 and references therein). Although we have used neither homopolymeric tract polymorphisms nor insertion/deletion substitution to differentiate our target alleles we wanted to exclude the possibility that the heteroplasmy was already present in the recipient cytoplasts. Therefore, we analyzed 12 samples, each of which contained 10–15 *in vitro* matured and denuded oocytes from nine randomly chosen slaughtered cows (from three cows two aliquots were obtained). The target allele *AC16,300/1GT* was used for ARMS allele-specific real-time PCR (assay sensitivity 0.1%). As expected, we could show the absence of heteroplasmy in these oocytes (Ct values for the oocyte aliquots between 22.5 and 24.6; standard deviations obtained for duplicate amplification ≤0.5).

Analysis of mtDNA composition in the cloned animals: First, using microsatellite analysis we confirmed that nuclear DNA in all cloned animals matched the nuclear DNA isolated from the donor cell line (data not shown). To identify E-donor-specific SNPs necessary for the quantitation of heteroplasmy in the cloned animals we performed sequencing analysis of the mtDNA control region. Regardless of a clear *B. taurus* morphology it revealed the unexpected occurrence of *B. indicus* haplotypes in the E-donor and in some recipient oocytes. To illustrate the diversity of mtDNA haplotypes used for nuclear transfer we performed phylogeny reconstruction (Figure 2). It indicated two highly diverged clades (*B. taurus* clade and *B. indicus* clade) composed of 11 and 9 cloned animals, respectively. The clades were supported by quartet puzzling values of 100%.

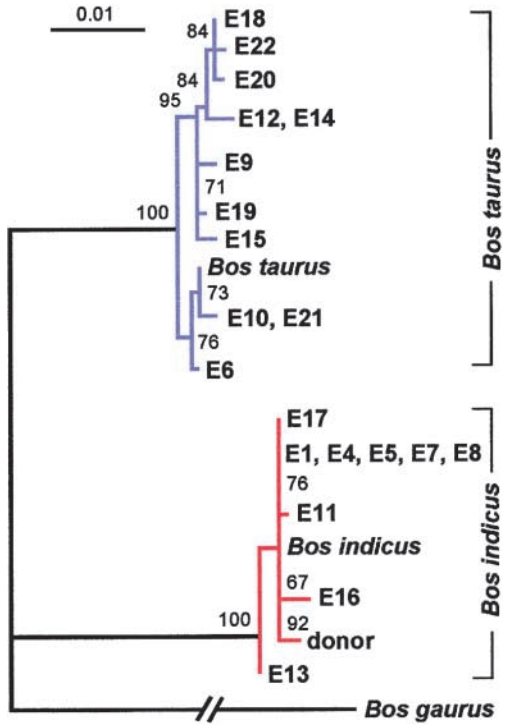

FIGURE 2.—Maximum-likelihood tree of complete mtDNA control region sequences from the cloned cattle E1–E22 and their donor cell. A *B. gaurus* sequence was used as an outgroup. For each of the two cattle taxa *B. taurus* and *B. indicus*, a representative sequence obtained from GenBank was included in the tree. Measures of support are quartet puzzling values.

The *B. taurus* mtDNA haplotypes exhibited pronounced variation with only two pairs (one individual from set I and set II within each pair) of identical sequence. The *B. indicus* sequences were less variable since one haplotype was shared by 5 cloned animals. These 5 cattle produced in set I of the nuclear transfer experiments potentially originated from a single pair of ovaries or from cows belonging to one maternal lineage.

Using quantitative ARMS allele-specific PCR we analyzed the mtDNA composition of adult cloned cattle produced by nuclear transfer of the E-donor into enucleated oocytes of random genetic origin. The allele-specific assays were designed for donor-specific SNPs (Figure 1) to quantitate the contribution of the donor mtDNA. We found the first four cases of intersubspecific *B. indicus*/*B. taurus* heteroplasmy ($n = 11$, Table 1). We also detected intrasubspecific *B. indicus* heteroplasmy in 7 animals ($n = 9$, Table 2). In contrast, we detected a complete absence of heteroplasmy following either inter- or intrasubspecific nuclear transfer in 9 (7 and 2 cloned animals, respectively) of the 20 cloned animals derived from the E-donor (Tables 1 and 2). No traces of donor mtDNA were detectable in the female germline of the intrasubspecific cloned animals E4 and E5, although their investigated tissues showed respective levels of heteroplasmy of up to 6.0 and 12.7%. In our hands

TABLE 1

Intersubspecific heteroplasmy: *B. indicus* donor mtDNA in *B. taurus* recipient oocytes

Cloned animals	Tissues analyzed (% *B. indicus* donor mtDNA)
E6, E9, E10, E12	Blood (0)[a], muscle (0)[a], skin (0)[a], follicular cells (0)[a]
E14, E19, E21	Blood (0)[a]
E15	Blood (0.6)
E18	Blood (1.1)
E20	Blood (1.8)
E22	Blood (2.8)

[a] Assay sensitivity, 0.1%.

sensitivity levels of 0.1% were routinely achieved for ARMS allele-specific quantitative real-time PCR (assays in STEINBORN *et al.* 2000; assay for the target allele *AC16,300/1GT* of this work). The reduced sensitivity/allelic discrimination of the *B. indicus*-allele-specific quantitation assay designed for the target allele *C16,050T* (0.5%) is most probably due to the low GC content (reduced annealing temperature of 43°) of the genomic environment surrounding this SNP. Additional sequencing of 1683 bases (*MTCO2*, *MTATP6*, and adjacent 318 bp of *MTCO3*) did not reveal another SNP between the E-donor and the cloned animals E1, E4, E5, E8, and E11.

Recently, the use of adult somatic nuclear transfer in animal conservation was demonstrated by cloning the last surviving cow (denoted L-donor in this work) of the Enderby Island cattle breed to preserve the female genetics of this endangered breed (WELLS *et al.* 1998). Enderby Island Shorthorns had adapted to harsh subantarctic conditions. Here we analyzed the mtDNAs of five cloned cattle derived from the L-donor. Nuclear genetic identity between the L-donor cell and the five cloned animals derived from this donor was demonstrated by microsatellite analyses (data not shown). Sequencing of the mitochondrial control region of the five cloned animals yielded two *B. taurus* haplotypes (cloned animals L2 and L9) and three *B. indicus* haplotypes, whereas the L-donor harbored a *B. indicus* haplotype. Only L9 was heteroplasmic, harboring 0.9% donor mtDNA, whereas the L2 sample was homoplasmic, exhibiting only the *B. taurus* recipient cytoplast-derived mtDNA. The quantitation of the L-donor mtDNA in the three cloned individuals with cytoplast-derived *B. indicus* mtDNA was not possible due to the absence of polymorphisms. Since the control region represents the most variable part of the mtDNA, we did not continue to search for SNPs in the coding region.

In addition to the last surviving Enderby Island cow, analysis of samples stored from other now deceased Enderby Island animals revealed 12 *B. indicus* mtDNAs and only one *B. taurus* haplotype (data not shown).

TABLE 2

Intrasubspecific *B. indicus* heteroplasmy

Cloned animals	Tissues analyzed (% *B. indicus* donor mtDNA)
E1	Blood (1.4)
E4	Blood (2.4), muscle (6.0), skin (2.1), follicular cells (2.5), oocytes (0)[a]
E5	Blood (2.1), muscle (7.3), skin (1.9), follicular cells (12.7), oocytes (0)[a]
E7	Skin (0)[a], follicular cells (0)[a]
E8	Blood (0.9), muscle (0.7), skin (1.6), follicular cells (1.0)
E11	Blood (0.6), muscle (0.7), skin (1.8), follicular cells (1.0)
E13	Blood (0)[a]
E16, E17	Blood (0.6)

[a] Assay sensitivity, 0.5%.

TABLE 3

Type of amino acid differences in mtDNA-encoded subunits between *B. taurus* and *B. indicus*

Complex	Amino acid position	Amino acid substitution
I	*MTND3*	
	82	A → T
I	*MTND6*	No
III	*MTCYB*	
	356	V → I
	372	I → V
IV	*MTFCO2*	
	57	D → N[a]
IV	*MTCO3*	
	171	V → I
	238	G → A
V	*MTATP6*	
	7	T → A
	69	T → A
	154	M → V

The amino acid substitutions (single-letter code) occurred in the complexes I–IV of the electron transport system or in the ATP synthase complex (complex V). Numbers indicate the amino acid position inside each subunit.

[a] Substitution found only in the cloned animal E12 (*B. taurus* haplotype).

Phylogenetic divergence between *B. taurus* and *B. indicus* based on mtDNA-encoded peptides: To estimate the extent of the possible mtDNA sequence divergence between two (sub)species, which may allow the generation of inter(sub)specific heteroplasmy, sequencing of parts of the *B. indicus* mtDNA was performed. On the basis of the haplotype classification presented in Figure 2 we selected the E-donor and randomly two representative *B. indicus* mtDNAs (cloned animals E1 and E11) for sequencing and subsequent comparison with the *B. taurus* reference. We analyzed six randomly selected mtDNA-encoded proteins from the electron transport system complexes I, III, and IV (there is no mtDNA-encoded subunit of complex II) and the ATP synthase complex (complex V). Among them are three highly conserved proteins (encoded by *MTCO2*, *MTCO3*, and *MTCYB*; classification reviewed in Blier *et al.* 2001) and proteins that are regarded as more variable [encoded by *MTND6* and *MTATP6* (Blier *et al.* 2001) and *MTND3*]. We found intersubspecific differences in four of the six protein sequences investigated (Table 3), yielding divergence rates between 0% (*MTND6*) and 1.3% (*MTATP6*). While nonconservative changes were not detected, out of a total of eight differences, three were conservative (between amino acids of the same family) and five were semiconservatives (hydrophobic or charged to neutral amino acids). By chance, we additionally detected a *B. taurus MTCO2* variant (Table 3, semiconservative, 0.4% divergence).

DISCUSSION

We report intrasubspecific *B. indicus* and intersubspecific heteroplasmy in adult somatic cattle cloned by nuclear transfer. The ratios quantitated for the coexisting *B. indicus* parental mtDNAs in seven of nine intrasubspecific heteroplasmic cloned animals (Table 2) were similar to those reported by us earlier for heteroplasmic cloned *B. taurus* cattle (Steinborn *et al.* 2000). We showed that *B. indicus* mtDNA was not present *a priori* in the recipient oocytes used for nuclear transfer. Moreover, each heteroplasmic cloned individual was quantitated at two sites (donor cell-specific ARMS primers ARMS16,211-16,229 and ARMS16,283-16,301 for detection of intersubspecific heteroplasmy and primers ARMS16,056-16,074 and ARMS16,071-16,050 for quantitation of intrasubspecific *B. indicus* heteroplasmy). At each of these four sites the donor mtDNA exhibited a rare polymorphism compared to the cloned animals (Steinborn *et al.* 1998a and data not shown). Thus, it is very unlikely that these rare mutations were, by chance, present *a priori* in the recipient cytoplasts used for nuclear transfer. The fact that the percentage of donor mtDNA in our heteroplasmic cloned cattle was at a level expected for neutral transmission of parental mtDNAs makes the theory of *a priori* heteroplasmy of the recipient cytoplasts even more unlikely. Together with the fact that homo- and heteroplasmy occurred within one clonal origin (*i.e.*, the same nuclear DNA), the utilization of a nontarget allele control (bovine total cellular DNA carrying the alternative base for the SNP under study) excludes a hypothetical attribution of nuclear mitochondrial pseudogenes (for review see Bensasson *et al.* 2001) to the heteroplasmic signal.

The heteroplasmic intersubspecific adult somatic cloned cattle demonstrate the nuclear-mitochondrial compatibility between *B. taurus* and *B. indicus*. Even among

the Enderby Island cattle, separated under harsh subantarctic conditions for 150 years (Bunn 1998), we found haplotypes from both subspecies. The unexpected occurrence of *B. indicus* mtDNA haplotypes in the New Zealand population with European cattle (*B. taurus*) morphology was confirmed by another group (S. H. Phua, K. G. Dodds, R. Spelman and A. M. Crawford, personal communication) reporting a similar *B. indicus/B. taurus* haplotype ratio for New Zealand Jersey cattle. The *B. taurus/B. indicus* nuclear-mitochondrial compatibility is also in accordance with reports on the descendants of hybridization between *B. indicus* and *B. taurus* (Troy *et al.* 2001) and can be concluded from the successful intersubspecific embryonic cell cloning between *B. indicus* and *B. taurus*, which for unknown reason(s) led to a shift to homoplasmy during embryogenesis (Meirelles *et al.* 2001).

In general, the reason(s) leading to heteroplasmy or homoplasmy in the described inter- or intrasubspecific nuclear transfers remains unknown. The mtDNA transmission pattern in the cloned animals did not correlate with donor cell passage similarly as reported previously (Steinborn *et al.* 2000) or with mtDNA haplotype. The comparison of the set I (conventional *in vitro* culturing of cloned embryos) and the set II (improved media formulation or *in vivo* culturing) animals that were cloned from the E-donor showed an increased occurrence of intersubspecific heteroplasmy (heteroplasmic animals/total number of animals, 0/4 *vs.* 4/7, respectively). It remains unclear whether the occurrence of heteroplasmy in intersubspecific cattle reflects a renormalization in the expression of genes involved in mitochondrial biogenesis due to the improvement of culture conditions. Genetic changes and perturbations in development that result from manipulating mammalian embryos are well documented (references in Wilmut 2002).

Our finding of intersubspecific heteroplasmy in healthy cloned mammals has important implications in view of the ongoing discussion concerning the use of animal cloning for preserving genetic variation, recreating species and populations already extinct or on the verge of extinction (Lanza *et al.* 2000; Lee 2001). It is a first demonstration that the mtDNA of the endangered species can also be rescued by nuclear transfer (in addition to the nuclear genome of the donor cell) to derive offspring with the original mitochondria. The necessity to ascertain that the mtDNA of the endangered species is also rescued was discussed recently (Meirelles *et al.* 2001). Segregation toward donor mtDNA might be possible in subsequent generations due to random genetic drift as shown for intraspecific mice (Jenuth *et al.* 1996). However, tissue-specific and age-related selection for different mtDNA genotypes in those mice (Jenuth *et al.* 1997) or even lack of mtDNA maintenance (Moraes *et al.* 1999) in interspecific hybrids of closely related species was also found. Closely related interspecific mice crosses show reduced fitness, indicating a misbalanced nuclear-mitochondrial interaction (Nagao *et al.* 1998).

Recently, two wild endangered species, a gaur (*B. gaurus*) and a European mouflon (*O. aries musimon*: http://www.ncbi.nlm.nih.gov/htbin-post/taxonomy/), were successfully cloned by either interspecific or intersubspecific nuclear transfer using recipient oocytes collected from *B. taurus* and European *O. aries*, respectively (Lanza *et al.* 2000; Loi *et al.* 2001). The gaur clone died soon after birth. Comparing cytochrome b amino acid sequences a divergence of 3.2% (partial sequences), 0.3%, and 0.5% was revealed for *O. aries musimon/O. aries* and *B. indicus/B. taurus*, respectively. For the *B. gaurus/B. taurus* interspecies nuclear transfer clone an impairment of nuclear-mitochondrial interaction cannot be excluded, whereas the divergence between *B. indicus/B. taurus* and *O. aries musimon/O. aries* seems to be tolerated.

Due to the limited supply of oocytes and surrogate animals, the cloning of highly endangered or extinct species will require inter(sub)specific nuclear transfer. However, the nuclear-cytoplasmic composition should be considered if repopulation under specific (extreme) environmental conditions is attempted (see above). In light of these limitations, future research may reveal whether inter(sub)specific cloning can participate in the efforts to conserve endangered species.

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