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Letter

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Recently, the relationship of mitochondrial DNA (mtDNA) variants to metabolic risk factors for diabetes and other common diseases has begun to attract increasing attention. However, progress in this area has been limited because (1) the phenotypic effects of variation in the mitochondrial genome are difficult to isolate owing to confounding variation in the nuclear genome, imprinting phenomena, and environmental factors; and (2) few animal models have been available for directly investigating the effects of mtDNA variants on complex metabolic phenotypes in vivo. Substitution of different mitochondrial genomes on the same nuclear genetic background in conplastic strains provides a way to unambiguously isolate effects of the mitochondrial genome on complex traits. Here we show that conplastic strains of rats with identical nuclear genomes but divergent mitochondrial genomes that encode amino acid differences in proteins of oxidative phosphorylation exhibit differences in major metabolic risk factors for type 2 diabetes. These results (1) provide the first direct evidence linking naturally occurring variation in the mitochondrial genome, independent of variation in the nuclear genome and other confounding factors, to inherited variation in known risk factors for type 2 diabetes; and (2) establish that spontaneous variation in the mitochondrial genome per se can promote systemic metabolic disturbances relevant to the pathogenesis of common diseases.

[Supplemental material is available online at www.genome.org.]

Recently, the potential role of the mitochondrial genome in the pathogenesis of type 2 diabetes and other common diseases has begun to attract increasing attention (Wallace 2005). Mitochondrial DNA is exclusively of maternal origin, and some studies have indicated that the inheritance of type 2 diabetes may be biased toward the maternal lineage (Alcolado et al. 2002). Sun et al. (2003) have estimated that mitochondrial DNA mutations might be involved in >20% of cases of type 2 diabetes. In addition, Wilson et al. (2004) have identified a homoplasmic mitochondrial DNA variant associated with the maternal transmission of hypercholesterolemia, hypomagnesemia, and hypertension through four generations of a large, carefully analyzed kindred. Rare forms of maternally transmitted diabetes associated with heteroplasmic mitochondrial DNA variants have also been reported (Mathews and Berdanier 1998; Maassen et al. 2005). These observations provide indirect evidence that sequence variation in the mitochondrial genome might contribute to inherited variation in the risk for type 2 diabetes and related metabolic disorders.

Notwithstanding recent advances in mitochondrial genome research, multiple confounding factors have made it difficult to unequivocally establish a role for mitochondrial DNA variation in the regulation of risk factors for diabetes or other complex metabolic phenotypes. For example, effects of maternal environment or imprinting may contribute to matrilineal transmission patterns of the phenotypes of interest. Variation in copy number and tissue distribution of heteroplasmic mitochondrial DNA mutants can have unpredictable phenotypic consequences. Perhaps the most vexing problem is that the phenotypic expression of even homoplasmic mitochondrial DNA variants may be highly variable because of complex interactions with nuclear gene variants or environmental factors that are difficult to control (Fischel-Ghodsian 2000; Carelli et al. 2003). Thus, in many if not most cases, imperfect correlations between mitochondrial genotype and phenotype have been typically ascribed to incomplete penetrance involving phenomena that are usually not elucidated or explored.

In this study, we investigated whether naturally occurring variation in the mitochondrial genome could be directly linked to risk factors for type 2 diabetes in a rat model in which we have eliminated the confounding effects of variation in the nuclear genome, imprinting phenomena, and environmental factors. We

accomplished this objective by selectively replacing the mitochondrial genome of the spontaneously hypertensive rat (SHR) with the mitochondrial genome of the Brown Norway (BN) rat to create a novel SHR conplastic strain. Because the SHR conplastic strain and the SHR progenitor strain are genetically identical except for differences confined to their mitochondrial genomes, any phenotypic differences between these two strains can be directly attributed to sequence differences in their mitochondrial DNA. Using this approach, we have identified naturally occurring variants in rat mitochondrial genes encoding proteins of oxidative phosphorylation that are directly linked to alterations in mitochondrial enzyme levels and major risk factors for type 2 diabetes.

Results

Risk factors for diabetes

We found that fasting glucose and insulin levels were similar between the SHR conplastic strain and the SHR progenitor strain (Fig. 1A,B). However, after oral glucose loading, both the area under the curve (AUC) for glucose levels and the AUC for insulin levels were significantly greater in the SHR conplastic strain, 664 ± 9 mmol/L/2 h and 18.8 ± 1.3 nmol/L/2 h, respectively, than in the SHR progenitor strain, 603 ± 11 mmol/L/2 h and 13.6 ± 0.6 nmol/L/2 h, respectively (both $P < 0.01$; Fig. 1A,B). Thus, replacing the SHR mitochondrial genome with the BN mitochondrial genome promoted impaired glucose tolerance, a major clinical risk factor for diabetes (Nathan et al. 2007). Fasting and nonfasting triglyceride levels tended to be greater in the conplastic strain than in the progenitor strain, but the differences did not achieve statistical significance (data not shown). The reduced glucose tolerance of the SHR conplastic strain was not associated with excess body weight, as the body weight of the SHR conplastic strain (283 ± 10 g) was slightly lower than that of the SHR progenitor strain (305 ± 10 g; $P < 0.01$).

Because reduced skeletal muscle glycogen synthesis is a characteristic feature of pre-diabetic individuals with suboptimal glucose tolerance and of patients with diabetes (Shulman et al. 1990; Petersen et al. 2004), we also measured glycogen content in skeletal muscle of the SHR conplastic and progenitor strains. In the SHR conplastic strain, skeletal muscle glycogen content was significantly decreased compared to that in the SHR progenitor strain (Fig. 1C). We also measured skeletal muscle ATP levels because reduced muscle ATP synthesis is another metabolic characteristic associated with increased risk for diabetes (Petersen et al. 2004). The ATP level in skeletal muscle of the SHR conplastic strain was significantly decreased compared to that in the SHR progenitor strain (Fig. 1D). These results demonstrate that selective replacement of the mitochondrial genome of the SHR with
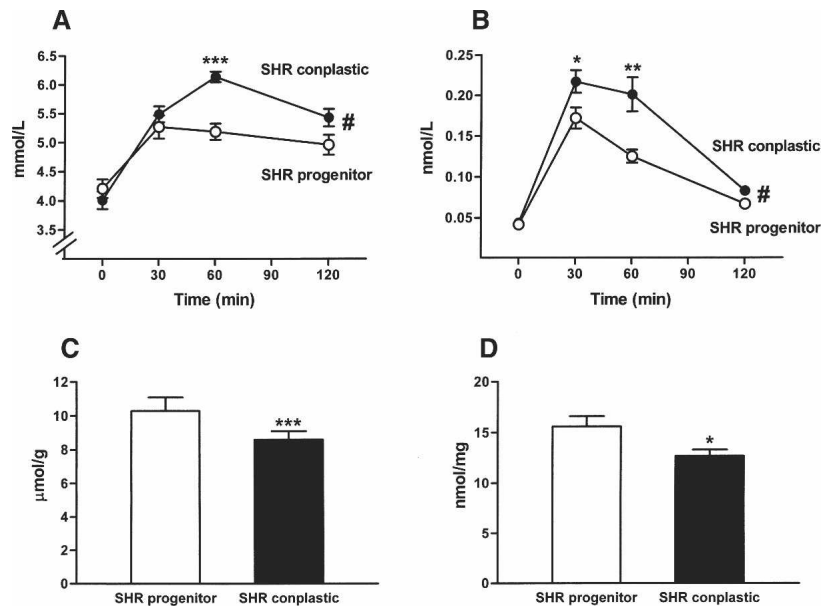

Figure 1. Metabolic differences between the SHR progenitor strain and SHR conplastic strain with identical nuclear genomes but different mitochondrial genomes. (*A*) Blood glucose levels before and after glucose administration in the SHR progenitor strain (open circles, $n = 9$) and the SHR conplastic strain (solid circles, $n = 12$). After oral glucose loading, the SHR conplastic strain showed a significantly greater area under the curve for blood glucose than the SHR progenitor strain, 664 ± 9 mmol/L/2 h versus 603 ± 11 mmol/L/2 h, respectively ($P < 0.01$). Significant differences at individual time points are marked by asterisks. (*B*) Serum insulin levels before and after oral glucose administration. Same symbols as for serum glucose results. After oral glucose loading, the SHR conplastic strain showed significantly greater area under the curve for serum insulin levels than the SHR progenitor strain, 18.8 ± 1.3 nmol/L/2 h versus 13.6 ± 0.6 nmol/L/2 h, respectively ($P < 0.01$). (*C*) Glycogen content in skeletal muscle. Glycogen content in skeletal muscle tissue from the SHR conplastic strain (solid bar, $n = 12$) was significantly lower than in the SHR progenitor strain (open bar, $n = 9$, $P < 0.001$). (*D*) ATP content in skeletal muscle. ATP content in skeletal muscle tissue from the SHR conplastic strain (solid bar, $n = 6$) was lower than in the SHR progenitor strain (open bar, $n = 5$, $P < 0.05$). (***) $P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$ in individual time-point comparisons between the SHR progenitor and conplastic strains; (#) $P < 0.01$ for area under the curve comparisons.

the mitochondrial genome of the BN rat influences several major metabolic risk factors for type 2 diabetes.

In supplemental studies in reciprocally derived female F_1 hybrids that inherited the BN mitochondrial genome, fasting serum insulin levels (0.09 ± 0.02 nmol/L) and triglyceride levels (1.12 ± 0.04 mmol/L) were significantly increased compared to those in F_1 hybrid rats that inherited the SHR mitochondrial genome, 0.05 ± 0.008 nmol/L ($P < 0.05$) and 0.89 ± 0.06 mmol/L ($P < 0.01$), respectively. Despite differences in fasting insulin, the fasting glucose level in rats that inherited the BN mitochondrial genome (4.1 ± 0.2 mmol/L) was not significantly different from that in rats that inherited the SHR mitochondrial genome (4.5 ± 0.2 mmol/L). Insulin-stimulated incorporation of glucose into skeletal muscle glycogen in F_1 rats with the BN mitochondrial genome was significantly lower than that in F_1 rats with the SHR mitochondrial genome, 198 ± 71 nmol glucose/g/2 h versus 517 ± 100 nmol glucose/g/2 h, respectively ($P < 0.05$). These findings in reciprocally derived female F_1 hybrids with identical nuclear genomes but different mitochondrial genomes demonstrate maternal transmission bias in several known metabolic risk factors for type 2 diabetes and are consistent with those in the conplastic strain.

Mitochondrial DNA sequence analysis

Sequence analysis revealed distinct differences in the mitochondrial genomes of the SHR and BN strains. Comparison of the

Table 1. Mitochondrial DNA variants encoding amino acid substitutions in SHR and BN strains

Gene	Nucleotide	Codon		Amino acid		Number
		BN	SHR	BN	SHR	
mt-Nd2	3956	GTA	GCA	Val	Ala	18
	4352	AAC	AGC	Asn	Ser	150
	4633	ACA	GCA	Thr	Ala	244
	4696	GCA	ACA	Ala	Thr	265
mt-Co1	5326	CTC	TTC	Leu	Phe	2
mt-Atp8	7930	ACC	ATC	Thr	Ile	58
mt-Atp6	8021	GAA	AAA	Glu	Lys	35
mt-Nd4	10227	ATC	ACC	Ile	Thr	23
	11225	GCA	ACA	Ala	Thr	356
	11360	GTC	ATC	Val	Ile	401
	11374	ATC	ATA	Ile	Met	405
mt-Nd6	13647	ATT	GTT	Ile	Val	139
	13974	CTT	TTT	Leu	Phe	30
mt-Cyb	14775	GAC	AAC	Asp	Asn	214
	15135	ATC	GTC	Ile	Val	334

complete mitochondrial DNA sequences revealed polymorphisms of potential functional significance in seven of the 13 messenger RNA (mRNA) genes (Table 1), five of the 22 transfer RNA (tRNA) genes (Supplemental Table 1), and both of the ribosomal RNA (rRNA) genes (Supplemental Table 1). None of the observed differences between SHR and BN mtDNA was associated with heteroplasmy. The mRNA variants are predicted to cause amino acid substitutions in mitochondrial cytochrome *c* oxidase subunit I (COX1), ATP synthase subunit 6 (ATP6), ATP synthase subunit 8 (ATP8), cytochrome *b* (CYTB), NADH dehydrogenase subunit 2 (ND2), NADH dehydrogenase subunit 4 (ND4), and NADH dehydrogenase subunit 6 (ND6) (Table 1). Some of these substitutions are located within 0–3 amino acid residues of mutation sites known to be associated with human disorders including exercise intolerance and Leber hereditary optic neuropathy (LHON). Of the seven variants affecting five tRNA genes, three are located within 4 bp of human mutation sites associated with mitochondrial encephalopathy and focal segmental glomerulosclerosis. A single variant was detected in the 12S rRNA gene *mt-Rn1*, and multiple variants were detected in the 16S rRNA gene *mt-Rn2* that appear to be distant from sites associated with human clinical disorders. In addition, one variant was identified in the origin of replication, and seven variants were identified in the D-loop region (Supplemental Table 2).

Mitochondrial DNA haplotype analysis

We performed haplotype analysis by genotyping 43 rat strains including the SHR and BN progenitor strains for a subset of 16 polymorphisms that included variants causing seven amino acid substitutions in six proteins for oxidative phosphorylation, two silent messenger RNA (mRNA) variants, five transfer RNA (tRNA) variants, and two ribosomal RNA (rRNA) variants. This enabled identification of four haplotype groups including two main categories that encompassed 37 out of the 43 strains tested (Table 2). The SHR strain belongs to a minor haplotype group (group 3) containing only four of the strains tested including SHR, WKY, OKA, and GC. The BN haplotype group (group 4) includes only one other strain, DZB/Gro, and this strain is known to have been derived from a cross of BN rats with AO rats. All of the SHR variants, synonymous and nonsynonymous, were found in many other strains. However, five of the BN variants in genes

encoding amino acid substitutions in proteins of oxidative phosphorylation were not found in any of the other strains except DZB/Gro known to have been derived from BN rats. These genes included *mt-Co1*, *mt-Atp8*, *mt-Nd4*, *mt-Nd6*, and *mt-Cyb* (Table 2).

We also compared the amino acid substitutions in the SHR and BN progenitor strains against the corresponding residues in human, guinea pig, mouse, chimpanzee, macaque, cow, dog, opossum, chicken, zebrafish, and stickleback. Several of the variants observed in the SHR versus BN comparison involved residues that are relatively conserved among multiple species. Of particular interest given the enzyme studies described below is that the BN rat along with the guinea pig and macaque are the only mammals tested that have a leucine at position 2 of COX1 (variant in *mt-Co1* nucleotide no. 5326) (Tables 1, 2). The SHR and 40 other rat strains as well as the mouse, cow, dog, human, chimpanzee, opossum, and chicken have phenylalanine in this position, whereas zebrafish and stickleback have either threonine or alanine.

Mitochondrial enzyme analysis

To determine whether the observed sequence variation in the mitochondrial genome was also linked to effects on mitochondrial enzyme activity or alterations in mitochondrial protein levels, we studied isolated mitochondria from the SHR conplastic and progenitor strains. Analysis of enzyme activities of selected respiratory chain complexes revealed significantly lower activity of cytochrome *c* oxidase (complex IV) in the SHR conplastic strain compared to the SHR progenitor strain

Table 2. Mitochondrial DNA haplotype grouping of 43 strains of rats

Nucleotide	Genotype[a]			
	Group 1	Group 2	Group 3	Group 4
1716	BN	SHR	SHR	BN
1918	SHR	SHR	SHR	BN
4352[b]	BN	SHR	SHR	BN
5200	BN	SHR	SHR	BN
5237	BN	SHR	SHR	BN
5269	BN	SHR	SHR	BN
5326[b]	SHR	SHR	SHR	BN
7930[b]	SHR	SHR	SHR	BN
8021[b]	BN	SHR	SHR	BN
11,194	BN	SHR	SHR	BN
11,374[b]	SHR	Wistar	SHR	BN
13,647[b]	BN	SHR	SHR	BN
13,693	BN	SHR	SHR	BN
13,974[b]	SHR	SHR	SHR	BN
15,135[b]	SHR	SHR	SHR	BN
15,400	SHR	SHR	SHR	BN

We identified four haplotype groups by genotype analysis of 16 mitochondrial DNA polymorphisms in 43 rat strains including the SHR and BN progenitor strains. The specific variants present at each nucleotide site in the SHR and BN strains are listed in Tables 1 and Supplemental Table 1.
[a]Haplotype Group 1 consisted of 20 strains: ACI/Ztm, BC/CpbU, BDE/Han, BDIV/Ifz, BDIX/Han, BH/Ztm, BUF/Han, COP/OlaHsd, DA/Han, F344/Han, NAR/SaU, NEDH/K, PVG/OlaHsdCp, R/A, RHA/Kun, SDL/Ipvc, SPRD-Cu3/Han, SR/JrIpvc, SS/JrIpvc, WF/Han. Group 2 consisted of 17 strains: AS/Ztm, AVN/Orl, BBWB/Mol, BDII/Han, BS/Ztm, LE/Han, LEW/OlaHsd, LOU/C, MNS/Gro, MW/Hsd, OM/Han, SD/Rij, SDH/Ztu, WF/Gut, WIST/Nhg, WOK.1A/K, WOK/w/K. Group 3 consisted of four strains: SHR/Ola, OKA/Wsl, GC/Kun, WKY/M. Group 4 consisted of two strains: BN/Ola, DZB/Gro. The DZB/Gro strain was derived by a cross of BN rats with AO rats.
[b]Nucleotides causing amino acid substitutions.

(Fig. 2A). In contrast, we found no strain differences in the activities of succinate:ferricytochrome c oxidoreductase (complex II + III), NADH:ferricytochrome c oxidoreductase, rotenone sensitive (complex I + III), or citrate synthase (Fig. 2A). Consistent with the results in the conplastic strain, we also found that mitochondrial cytochrome c oxidase activity was significantly lower in F_1 hybrid rats that inherited the BN mitochondrial genome than in F_1 hybrid rats that inherited the SHR mitochondrial genome, 6484 ± 127 nmol/min/mg protein versus 7137 ± 46 nmol/min/mg protein, respectively ($P < 0.0001$). Thus, the BN strain carries a rare variant in the *mt-Co1* gene that is not found in >40 other strains of laboratory rats (Tables 1, 2) and that is genetically linked to effects on cytochrome c oxidase activity.

Analysis of mitochondrial proteins and DNA copy number

Western blot analysis of mitochondrial protein complexes of oxidative phosphorylation showed decreases in both mtDNA-encoded (COX2) and nuclear-encoded (COX4) subunits of cytochrome c oxidase (complex IV) in the SHR conplastic strain compared to the SHR progenitor strain (Fig. 2B). The level of COX1 also tended to be lower in the SHR conplastic strain than the SHR progenitor strain, but the difference did not achieve statistical significance. In contrast, none of the proteins tested in the other respiratory chain complexes (complexes I, III, or V) appeared to

be reduced in the SHR conplastic strain (Fig. 2B). We did not detect any reductions in mtDNA copy number relative to genomic DNA content or any reductions in tissue citrate synthase activity between the SHR conplastic strain and the SHR progenitor strain (data not shown). These observations indicate that the reductions in cytochrome c oxidase content and enzyme activity linked to the BN mitochondrial genome are relatively selective in nature and not just a consequence of widespread disturbances in mitochondrial biogenesis and/or protein content. Western blot studies of liver mitochondria from reciprocally derived F_1 hybrid rats also revealed effects of mitochondrial genome variation on the protein content of cytochrome c oxidase. In F_1 rats that inherited the BN mitochondrial genome, the mitochondrial content of cytochrome c oxidase subunit 1 was decreased by 25%–30% compared to F_1 rats that inherited the SHR mitochondrial genome ($P < 0.01$). No significant differences were found between the two groups of F_1 hybrid rats in the levels of other major respiratory chain proteins tested from liver, heart, or kidney mitochondria.

Discussion

The potential role of mitochondria dysfunction in the pathogenesis of a variety of common diseases including type 2 diabetes has attracted considerable attention, although cause and effect rela-
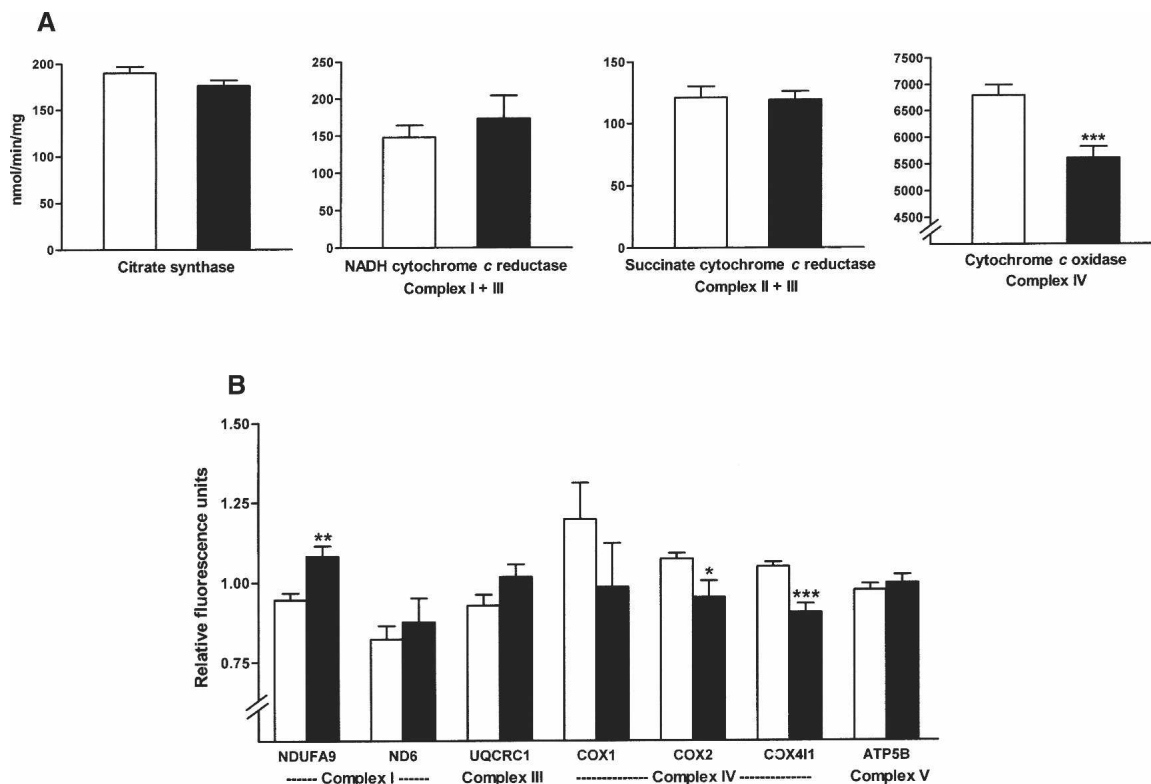

Figure 2. Mitochondrial enzyme activities and protein levels in the SHR progenitor strain and SHR conplastic strain. (*A*) Activity levels of citrate synthase, NADH cytochrome c reductase (NCCR), succinate cytochrome c reductase (SCCR), and cytochrome c oxidase in liver mitochondria isolated from the SHR progenitor strain (open bars) and SHR conplastic strain (solid bars). The activity of cytochrome c oxidase was significantly lower in the SHR conplastic strain ($n = 6$) than the SHR progenitor strain ($n = 6$, $P < 0.001$). (*B*) Mitochondrial protein content in the SHR progenitor strain ($n = 6$) and SHR conplastic strain ($n = 6$). Results are expressed as relative fluorescence units determined after loading equivalent amounts of total mitochondrial protein for each sample. (NDUFA9) 39-kDa subunit of NADH:ubiquinone oxidoreductase; (ND6) mitochondrial NADH dehydrogenase subunit 6; (UQCRC1) core 1 subunit of ubiquinol:ferricytochrome c oxidoreductase; (COX1) mitochondrial cytochrome c oxidase subunit 1; (COX2) mitochondrial cytochrome c oxidase subunit 2; (COX4I1) cytochrome c oxidase subunit 4 isoform 1; (ATP5B) β-subunit of the F_1F_0 ATP synthase; (***) $P < 0.001$; (**) $P < 0.01$; (*) $P = 0.05$ in comparisons between the SHR progenitor and conplastic strains by Student's *t*-test.

tionships have been difficult to sort out (Petersen et al. 2004; Lowell and Shulman 2005; Wallace 2005; Asmann et al. 2006; Calvo et al. 2006). Owing to variation in the nuclear genome and other confounding factors, it has also been difficult to definitively isolate the effects of naturally occurring variation in the mitochondrial genome alone on risk factors for complex diseases including type 2 diabetes. This may explain why associations between mtDNA variants and risk for type 2 diabetes have been identified in some populations but not others (Saxena et al. 2006; Fuku et al. 2007). Even within relatively homogeneous populations, unrecognized variation in confounding factors could either mimic or impair the ability to detect effects of mtDNA on complex phenotypes. Rare forms of diabetes and other clinical disorders have been associated with inheritance of specific mitochondrial DNA variants; however, the clinical expression of such variants has proven highly variable owing to putative effects of heteroplasmy or interactions with nuclear gene variants or environmental factors that have not been well elucidated or explored (Fischel-Ghodsian 2000; Carelli et al. 2003). Thus, uncertainty has persisted regarding the true impact of naturally occurring variation in the mitochondrial genome on complex metabolic phenotypes in vivo.

In the present studies, we have found that conplastic strains of rats with virtually identical nuclear genomes but divergent mitochondrial genomes exhibit differences in mitochondrial cytochrome *c* oxidase activity and major metabolic risk factors for type 2 diabetes. Consistent with the results in the conplastic strains, supplemental studies in reciprocally derived female F_1 hybrids with identical nuclear genomes but different mitochondrial genomes also exhibited differences in cytochrome *c* oxidase activity and metabolic risk factors for type 2 diabetes. Taken together, the present findings provide direct in vivo evidence that isolated sequence variation in the mitochondrial genome, in the absence of confounding variation in the nuclear genome, physical environment, or imprinting phenomena, can be linked to selective effects on mitochondrial enzyme activity and complex metabolic phenotypes related to risk for diabetes. Moreover, any strain differences in maternal environment that might conceivably have influenced the metabolic and mitochondrial phenotypes in the conplastic rats must also be attributable to strain differences in the mitochondrial genome.

Heretofore, few if any animal models have been available for effectively studying the impact of mtDNA variation on glucose metabolism. For example, in the Goto Kakizaki–Wistar rat model of type 2 diabetes, studies in reciprocal F_1 populations with identical nuclear genomes but different mitochondrial genomes failed to show any consistent effects on glucose metabolism that might be attributed to sequence variants in the mitochondrial genome (Gill-Randall et al. 2004). In a study of reciprocal F_1 populations derived by crossing a glucose-intolerant strain of BHE/Cdb rats with Sprague-Dawley rats, Mathews et al. (1999) reported that impaired glucose tolerance was associated with inheritance of a point mutation in the mitochondrial gene encoding ATP synthase subunit 6. However, the Sprague-Dawley rats used to establish the F_1 populations were not clearly inbred, and the phenotyping studies also included F_1 males with different Y-chromosomes. In previous studies in congenic strains, we have found that genes on the Y-chromosome can affect metabolic traits in rats (Kren et al. 2001). Therefore, the differences in glucose metabolism reported between F_1 populations derived from reciprocal crosses of BHE/Cdb rats and Sprague-Dawley rats could

have been related to variation in the nuclear genome rather than variation in the mitochondrial genome.

Because the mitochondrial genome is inherited as a single unit, the present results do not allow us to directly isolate the potential metabolic effects of the individual DNA variants detected in the sequence analysis of the SHR and BN mitochondrial genomes. However, based on the results of mitochondrial haplotype analysis of 43 rat strains together with the proteomic and enzymatic analyses of respiratory chain complexes in mitochondria isolated from the SHR progenitor and conplastic strains, the present findings strongly suggest a role for variant *mt-Co1* in the metabolic perturbations linked to the mitochondrial genome. The SHR conplastic rats that harbor a unique variant encoding an amino acid substitution in the COX1 subunit exhibited lower mitochondrial cytochrome *c* oxidase protein levels and lower mitochondrial cytochrome *c* oxidase enzyme activity than the SHR progenitor rats that harbor the common variant of *mt-Co1*. In contrast, we did not detect reductions in the mitochondrial content or activity of other respiratory chain proteins in the SHR conplastic strain. We also did not detect any reductions in mtDNA copy number or in citrate synthase activity in the SHR conplastic strain. Thus, the conplastic strain was characterized by relatively selective reductions in mitochondrial cytochrome *c* oxidase activity rather than a generalized disturbance in mitochondrial biogenesis and or mitochondrial protein content.

The present findings in the SHR progenitor and SHR conplastic strain have established that naturally occurring variation in the mitochondrial genome, in the absence of variation in the nuclear genome and other confounding factors, can influence risk factors for type 2 diabetes similar to those reported in prediabetic subjects with insulin resistance and patients with type 2 diabetes (Shulman et al. 1990; Petersen et al. 2004; Asmann et al. 2006). Thus, these results directly demonstrate that spontaneous variation in the mitochondrial genome per se can promote systemic metabolic disturbances relevant to the pathogenesis of common diseases. The availability of these kinds of conplastic animal models with different mitochondrial genomes on a fixed nuclear genetic background will now permit the derivation of conplastic cell lines in which the mitochondrial DNA haplotypes linked to metabolic effects in vivo can be directly investigated at the cellular level on the same fixed genetic background studied in vivo. These models will also open the door for future studies in which the phenotypic effects of mitochondrial genome variation can be investigated on precisely manipulated nuclear genetic backgrounds in congenic and co-isogenic strains. In this fashion, it should soon be possible to systematically isolate and characterize in vivo functional interactions between inherited variation in the mitochondrial genome and inherited variation in the nuclear genome.

Methods

Strain derivation

We selectively replaced the mitochondrial genome of a highly inbred strain of spontaneously hypertensive rats (SHR; SHR/OlaIpcv strain) with the mitochondrial genome of a highly inbred strain of Brown Norway (BN) rats (BN/Crl strain) to create a novel conplastic strain harboring the BN mitochondrial genome on the SHR nuclear genetic background. The official name designation of this conplastic strain is SHR/OlaIpcv-mt[BN/Crl] and is referred to hereafter as the SHR conplastic strain. We chose the

SHR-BN model because mtDNA sequence analysis revealed clear differences in the SHR and BN mitochondrial genomes including homoplasmic variants that encode amino acid substitutions in seven structural proteins involved in oxidative phosphorylation (Tables 1, 2; Supplemental Tables 1, 2). We substituted the BN mitochondrial genome on the background of the SHR because the SHR genetic background has proven favorable for identifying effects of other DNA variants on glucose and lipid metabolism (Pravenec et al. 2001).

We derived the conplastic strain using the "supersonic" breeding strategy of Behringer to accelerate the breeding process (Behringer 1998). In each backcross generation derived from the (male SHR × female BN) F_1 hybrids, we superovulated 4-wk-old females and then mated them overnight with SHR males to generate fertilized ova. We induced superovulation by administration of pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) given 48 h apart. We collected fertilized ova by flushing the oviducts on day 1 of pregnancy, and then we transferred seven to nine two-cell embryos into the oviducts of mature pseudopregnant SHR recipients that had been mated overnight with sterile vasectomized males. This transfer of embryos into the oviducts of pseudopregnant recipients occurred on day 1 of pseudopregnancy and produced pregnant SHR foster mothers that delivered litters ~3 wk later from which female offspring were obtained for use in creating the next backcross. We superovulated the female offspring when they reached 4 wk of age and immediately mated them to SHR males to repeat the cycle, thereby compressing the length of each backcross generation to a total of ~7 wk.

In the present experiments, we studied male conplastic rats that were the product of seven to nine generations of backcrossing. After seven to nine backcross generations, a developing congenic/conplastic line will be identical to its inbred partner across 98.4%–99.6% of the genome (Silver 1995). In the present studies, genotype analysis of polymorphic markers distributed at ~10-cM intervals across the nuclear genome confirmed lack of divergence in nuclear DNA between the SHR conplastic strain and the SHR progenitor strain. Thus, there is a very high probability that the conplastic and progenitor strains used in this study are homozygous identical at any given locus in the nuclear genome.

Without sequencing the entire nuclear genomes of both strains, no amount of genotyping can exclude all possible residual heterozygosity with absolute certainty. Therefore, we have also included supplemental studies in female F_1 hybrid rats derived from reciprocal crosses of the SHR and BN progenitor strains (see Supplemental Methods describing the studies in female F_1 hybrids). Female F_1 offspring derived from reciprocal crosses of highly inbred strains have identical nuclear genomes but different mitochondrial genomes, depending on which maternal strain is used to establish each cross. The F_1 offspring derived by crossing male SHR × female BN rats inherit the BN mitochondrial genome, whereas the F_1 offspring derived by crossing male BN × female SHR inherit the SHR mitochondrial genome. Because both the SHR and BN progenitor strains have been inbred for well over 40 generations, all F_1 rats must have identical nuclear genomes. While the studies in female reciprocal F_1 hybrids can exclude variation in the nuclear genome, they do not exclude potential effects of variation in maternal environment and genomic imprinting phenomena. However, the studies in conplastic strains effectively eliminate the confounding effects of imprinting and maternal environment. Thus, together with the supplemental studies in female reciprocal F_1 hybrids, the present studies in conplastic rats provide a highly rigorous means of isolating the effects of mitochondrial genome variation on complex phenotypes.

Risk factor phenotyping

After weaning, we fed the conplastic and progenitor rats standard chow and at 10 wk of age, switched them to a semisynthetic diet (Hope Farms, Netherlands) containing 60% fructose, as high-fructose diets have been shown to increase susceptibility of SHR to genetic variants that influence glucose metabolism (Pravenec et al. 1999). We performed glucose tolerance testing by administering an oral glucose load of 300 mg/100 g body weight after an overnight fast in 12-wk-old male rats. We drew blood from the tail without anesthesia for measurements of glucose, insulin, and triglycerides as previously described (Pravenec et al. 2003). We obtained samples of gastrocnemius muscle by snap-freezing in liquid nitrogen and measured glycogen content enzymatically as glucose using the glucose oxidase method in chloroform–methanol sediment after acid hydrolysis as previously described (Vrana et al. 1978). We also processed snap-frozen samples of gastrocnemius muscle in liquid nitrogen for measurement of ATP content by high-pressure liquid chromatography as previously described (Flachs et al. 2002). The protocols used to study the reciprocal F_1 hybrids are described in the Supplemental Methods. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), which corresponds fully to the European Community Council recommendations for the use of laboratory animals 86/609/ECC. The experiments were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences, Prague.

Mitochondrial enzyme activities, protein content, and DNA copy number

We used spectrophotometric methods (Jesina et al. 2004) to measure enzyme activities of citrate synthase (CS), ferrocytochrome c:oxidoreductase (cytochrome c oxidase [COX], complex IV), succinate:ferricytochrome c oxidoreductase (succinate cytochrome c reductase [SCCR], complexes II + III), and NADH:ferricytochrome c oxidoreductase, rotenone sensitive (NADH cytochrome c reductase [NCCR], complexes I + III) in liver mitochondria isolated using differential centrifugation techniques (Bustamante et al. 1977). Enzyme activity results are expressed per milligram of mitochondrial protein.

We used SDS-PAGE and Western blotting with subunit-specific primary mouse monoclonal antibodies (MitoSciences) to measure mitochondrial levels of a panel of respiratory chain proteins using methods similar to those previously described (Jesina et al. 2004). We measured mitochondrial levels of the β-subunit of the F_1F_0 ATP synthase (ATP5B); mitochondrial cytochrome-c oxidase subunit 1 (COX1); mitochondrial cytochrome c oxidase subunit 2 (COX2); cytochrome c oxidase subunit 4 isoform 1 (COX4I1); core 1 subunit of ubiquinol:ferricytochrome c oxidoreductase (UQCRC1); 39-kDa subunit of NADH:ubiquinone oxidoreductase (NDUFA9); and mitochondrial NADH dehydrogenase subunit 6 (ND6). We quantified the protein levels by detection of infrared fluorescence using anti-mouse Alexa Fluor 680-labeled secondary antibodies and an Odyssey Infrared Imaging System (LI-COR Biotechnology). Results are expressed as relative fluorescence units determined after loading equivalent amounts of total mitochondrial protein for each sample.

We used real-time PCR to measure the DNA copy number of mitochondrial genes *mt-Co1*, *mt-Nd4*, *mt-Atp8*, and *mt-Cyb* relative to the amount of the nuclear gene cytochrome c oxidase, subunit IV, isoform 1 (*Cox4i1*) as an internal control. For real-time PCR, total DNA was isolated using standard methods and analyzed by real-time PCR testing using QuantiTect SYBR Green reagents (QIAGEN) on an Opticon continuous fluorescence detector (MJ Research) with an annealing temperature of 58°C.

Mitochondrial DNA levels were normalized relative to the internal genomic DNA control with relative amounts of mtDNA to genomic DNA determined in triplicate using the preferred method of Muller et al. (2002). The primers used to amplify mitochondrial genes were *mt-Co1* upstream, 5′-GGAGCAGTATTCGC CATCAT; *mt-Co1* downstream, 5′-CGGCCGTAAGTGAGATG AAT; *mt-Cyb* upstream, 5′-CATCAGTCACCCACATCTGC; *mt-Cyb* downstream, 5′-TGGATGGAATGGGATTTTGT; *mt-Nd4* upstream, 5′-CTCACAACACACCCCCTACC; *mt-Nd4* downstream, 5′-TCCCATAACCCCCTAGCTTT; *mt-Atp8* upstream, 5′-TGCCA CAACTAGACACATCCA; *mt-Atp8* downstream, 5′-TGTGGGGG TAATGAAAGAGG. The primers used to amplify the nuclear gene *Cox4i1* were upstream, 5′-ACTACCCCTTGCCTGATGTG; and downstream, 5′-ACAGATCTGCGCTCACACAC.

DNA sequence and haplotype analysis of mitochondrial genomes

We amplified the entire rat mitochondrial genome from DNA extracted from liver tissue of a 12-wk-old male SHR (SHR/NCrl strain) using the polymerase chain reaction (PCR) and 34 overlapping primer pairs. Sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer using the BigDye Terminator v 3.1 Cycle sequencing kit (Applied Biosystems). We then searched for variants by comparing the SHR sequence results to the published mitochondrial genome sequence (accession no. AC000022) of the BN rat (BN/SsNHsdMCW strain). We confirmed all variants causing amino acid changes by resequencing the specific regions of interest in at least three SHR/NCrl rats (two liver samples and one kidney sample) and one BN rat (liver tissue from BN/Crl strain). We performed haplotype analysis by genotyping 43 rat strains including the SHR and BN progenitor strains for a subset of 16 polymorphisms that included variants causing seven amino acid substitutions in six proteins for oxidative phosphorylation, two silent messenger RNA (mRNA) variants, five transfer RNA (tRNA) variants, and two ribosomal RNA (rRNA) variants (Table 2). Genotyping was performed by PCR-restriction fragment length polymorphism analysis (RFLP) or direct sequencing. We used the EMBOSS tool Align (needle; gap penalty O = 10, Ex = 0.5, and matrix = DNAfull) for pairwise global alignment of mtDNA sequences and the Transeq tool with the option "Vertebrate Mitochondrial" code for translation of mtDNA sequence to amino acid sequence (Rice et al. 2000) To compare protein sequences, we visualized alignments with the program SeaView (Galtier et al. 1996).

Statistical analysis

We used the Student's *t*-test to analyze for differences between group means with statistical significance defined as $P < 0.05$. All results are expressed as means ± standard errors of the means (SEM).

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