

Genetic and Physiologic Analysis of the Role of Uncoupling Protein 3 in Human Energy Homeostasis

Wendy K. Chung, Amy Luke, Richard S. Cooper, Charles Rotini, Antonio Vidal-Puig, Michael Rosenbaum, Melvin Chua, Gemma Solanes, Min Zheng, Long Zhao, Charles LeDuc, Andrew Eisberg, Florence Chu, Ellen Murphy, Mindy Schreier, Louis Aronne, Sonia Caprio, Bowie Kahle, Derek Gordon, Suzanne M. Leal, Rochelle Goldsmith, Antonio L. Andreu, Claudio Bruno, Salvatore DiMauro, Moonseong Heo, William L. Lowe Jr., Bradford B. Lowell, David B. Allison, and Rudolph L. Leibel

By virtue of its potential effects on rates of energy expenditure, uncoupling protein 3 (*UCP3*) is an obesity candidate gene. We identified nine sequence variants in *UCP3*, including Val9Met, Val102Ile, Arg282Cys, and a splice site mutation in the intron between exons 6 and 7. The splice mutation results in an inability to synthesize mRNA for the long isoform (*UCP3L*) of *UCP3*. Linkage (sib pair), association, and transmission disequilibrium testing studies on 942 African-Americans did not suggest a significant effect of *UCP3* on body composition in this group. In vastus lateralis skeletal muscle of individuals homozygous for the splice mutation, no *UCP3L* mRNA was detectable; the short isoform (*UCP3S*) was present in an increased amount. In this muscle, we detected no alterations of *in vitro* mitochondrial coupling activity, mitochondrial respiratory enzyme activity, or systemic oxygen consumption or

respiratory quotient at rest or during exercise. These genetic and physiologic data suggest the following possibilities: *UCP3S* has uncoupling capabilities equivalent to *UCP3L*; other UCPs may compensate for a deficiency of bioactive *UCP3L*; *UCP3L* does not function primarily as a mitochondrial uncoupling protein. *Diabetes* 48:1890–1895, 1999

From the Departments of Pediatrics and Medicine (W.K.C., M.R., M.C., M.Z., L.Z., C.L., A.E., E.M., M.S., R.G., R.L.L.), Division of Molecular Genetics, Naomi Berrie Diabetes Center, Russ Berrie Medical Science Pavilion, the Obesity Research Center (M.H., D.B.A.), St. Luke's/Roosevelt Hospital Center, and the H. Houston Merritt Clinical Research Center for Muscular Dystrophy and Related Diseases (A.L.A., C.B., S.D.), Columbia University College of Physicians and Surgeons; the Rockefeller University (F.C., D.G., S.M.L.); the Department of Internal Medicine (L.A.), Cornell University Medical College, New York, New York; the Department of Preventive Medicine and Epidemiology (A.L., R.S.C., C.R.), Loyola University Medical Center, Maywood; the Department of Medicine (W.L.L.), Northwestern University School of Medicine, Chicago, Illinois; the Division of Endocrinology (A.V.-P., G.S., B.B.L.), Beth Israel-Deaconess Medical Center, Boston, Massachusetts; the Division of Pediatric Endocrinology (S.C.), Yale University School of Medicine, New Haven, Connecticut; the Department of Biological Sciences (B.K.), Marshall University College of Science, Huntington, West Virginia; and the Centre d'Investigacions en Bioquímica I biologic Molecular (A.L.A.), Hospitals Vall d'Hebron, Barcelona, Spain.

Address correspondence and reprint requests to Rudolph L. Leibel, Columbia University, Box 110/Russ Berrie Medical Science Pavilion, 1150 St. Nicholas Ave., New York, NY 10032. E-mail: rl232@columbia.edu.

Received for publication 11 February 1999 and accepted in revised form 10 May 1999.

D.B.A. has accepted speaking honoraria and research grants from Merck, which holds a patent for a combination therapy for the treatment of diabetes and obesity in which *UCP3* is mentioned as a possible therapeutic agent.

Additional information can be found in an on-line appendix at www.diabetes.org/diabetes/appendix.asp. The authors will provide print copies of the appendix upon request.

ANOVA, analysis of variance; FFA, free fatty acid; PCR, polymerase chain reaction; REE, resting energy expenditure; RQ, respiratory quotient; TDT, transmission disequilibrium testing; UCP, uncoupling protein; *UCP3L*, long-form transcript of *UCP3*; *UCP3S*, short-form transcript of *UCP3*.

Uncoupling protein (UCP) 3 (*UCP3*, located at chromosome 11q13) is a candidate gene for obesity because of its possible role in uncoupling mitochondrial respiration and its tissue-specific expression in skeletal muscle, an important thermogenic tissue (1–3). Using methods previously described (4), we identified nine DNA sequence variants in *UCP3*, including three that result in alterations in highly conserved amino acids (Val9Met, Val102Ile, and Arg282Cys), and a splice site mutation in the first base pair of the intron between exons 6 and 7 (GgtÆGat) (Table 1 and Fig. 1). (Details on subject phenotypes and sequencing primers can be found in Tables A1–A3 of the on-line appendix at www.diabetes.org/diabetes/appendix.asp.) The splice site mutation was detected only in African-American subjects. Linkage (sib pair), association, and transmission disequilibrium testing (TDT) studies performed on 942 African-Americans from Maywood, IL (Table 2) (5), did not support a significant role for *UCP3* in the determination of body composition of African-Americans. Consistent with this inference, in two women homozygous for the *UCP3* exon 6Æ7 splice variant, we were unable to demonstrate any significant anthropometric or *in vivo/in vitro* metabolic phenotype associated with this mutation.

Unlike *UCP1* and *UCP2*, *UCP3* exists in humans as short- (*UCP3S*) and long- (*UCP3L*) form transcripts of approximately equal abundance (1,6). *UCP3S* transcripts are generated when a cleavage and polyadenylation signal (AATAAA) located in intron 6 prematurely terminates message elongation (6). *UCP3S* is predicted to encode a protein that lacks the last 37 COOH-terminal amino acid residues of *UCP3L*. While the *UCP3L*-predicted protein is similar in length to *UCP1* and *UCP2*, the truncated *UCP3S* protein is unique. Based on homology with *UCP1*, *UCP3L* protein is expected to have six transmembrane domains. The *UCP3S* protein, on the other

TABLE 1
Summary of UCP3 sequence variants

Exon	Nucleotide	Amino acid change	African-American		Asian		Caucasian		Hispanic		Overall	Method of original detection
			Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese		
2	25 G A	Val9Met	0/20	3/16	0/20	0/2	1/158	2/172	0/30	0/24	6/442	Direct sequence
3	288 C T	None	0/10	0/16	0/10	0/2	0/54	0/88	0/12	1/16	1/208	Direct sequence and SSCA
3	297 T C	None	5/12	12/20	10/10	2/2	40/54	68/86	8/12	10/16	155/212	Direct sequence and SSCA
3	304 G A	Val102Ile	4/22	5/18	0/28	0/2	1/188	0/234	0/30	1/24	11/546	Direct sequence and SSCA
5	630 C T	None	10/12	14/16	2/10	1/2	36/62	47/86	10/12	12/16	132/216	Direct sequence and SSCA
5	36 bp 5' of 5' splice site C T	None	0/4	1/6	0/2	—	1/6	3/28	—	0/2	5/48	Direct sequence and SSCA
6	732 G C	None	0/24	0/72	0/26	0/2	0/196	1/260	0/32	0/24	1/636	HPLC system
6	First bp of intron 3' of exon G A	Destroys splice site	0/24	8/72	0/26	0/2	0/196	0/256	0/32	0/24	8/632	Direct sequence and SSCA
7	844 C T	Arg282Cys	0/16	0/12	1/22	0/2	3/170	3/168	1/28	0/22	8/440	Direct sequence

DNA sequence variants (number per total chromosomes analyzed) listed by exon, DNA, and amino acid variant. Nucleotides are numbered relative to Met start as 1 (GenBank accession number U84763). Resulting amino acid changes, if any, are indicated. Variant allele frequencies in African-American, Asian, Caucasian, and Hispanic lean and obese subjects are shown. Lean subjects are defined as having BMI <28.0 kg/m² and obese subjects as having BMI >28.0 kg/m². HPLC, high-performance liquid chromatography; SSCA, single-strand conformation analysis.

hand, should be truncated four to eight residues into the sixth transmembrane domain, and for this reason may be unstable and/or dysfunctional due to the absence of COOH-terminus regions possibly mediating guanine nucleotide and fatty acid regulation (7).

In individuals heterozygous and homozygous, respectively, for the splice site mutation between exons 6 and 7, decreased or absent long isoform of UCP3 (UCP3L) was demonstrated by RNase protection assay of skeletal muscle (Fig. 2). Because of the relatively high allele frequency of the Val9Met and splice variations within the African-Americans in the initial screening study group (Table 1), we conducted association, TDT, and sib-pair linkage analysis in a group of 942 African-Americans from Maywood, IL (Table 2) (5). Furthermore, we physiologically characterized two African-American females homozygous for the splice variant, and an age/sex-matched African-American control homozygous for the wild-type allele (Table 3).

There was no evidence for departure from Hardy-Weinberg equilibrium in the African-American residents of Maywood, IL, for either Val9Met ($P = 0.730$) or the exon 6 splice mutation ($P = 0.904$). The anthropometric and metabolic param-

eters shown in Table 2 were related to these sequence variations by association (8), TDT (9), and Haseman-Elston regression linkage tests (10,11). No significant relationships were demonstrable by any of these tests. (More information on these tests can be found in Tables A4–A8 of an online appendix at www.diabetes.org/diabetes/appendix.asp.) Although lack of significance cannot rule out small effects, these results should be interpreted in conjunction with the physiological data that give further evidence of non-effects.

Resting metabolic rate, respiratory quotient (RQ), metabolic response to graded exercise, and skeletal muscle oxidative enzyme and mitochondrial coupling phenotypes were normal in the two UCP3 (–/–) exon 6 splice variant subjects, who, as expected, had no UCP3L message in skeletal muscle by RNase protection assay. UCP3S mRNA was increased in the –/– subjects, possibly providing compensation for the absent UCP3L (Table 3). Additionally, in five –/– individuals in Maywood, IL, there was no apparent effect on fat mass, BMI, fasting blood glucose, or RQ (one subject only for RQ).

As indicated, UCP3S protein may be unstable and/or dysfunctional. The former might result in underactivation, the latter in lack of suppressability (overactivity) (12). In addi-

Potentially significant sequence variations

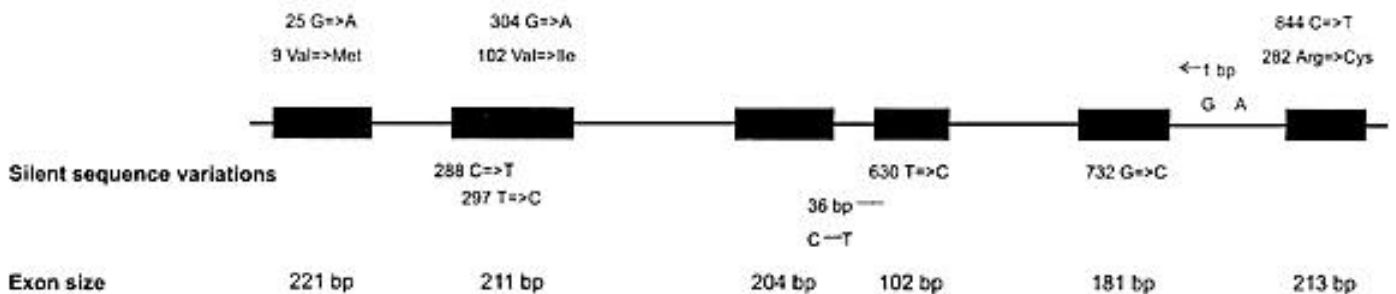


FIG. 1. Summary of sequence variants of UCP3. The genomic structure of UCP3 is shown with the locations of DNA sequence variants resulting in 1) amino acid variations, 2) loss of a splice donor site, and 3) silent exonic and intronic DNA variants that do not result in amino acid changes. Amino acid changes resulting for nucleotide substitutions are listed below the nucleotide change. Arrows indicate the number of base pairs from the splice site at which the nucleotide substitution occurs. Nucleotide numbering is relative to Met start as 1 (GenBank accession number U84763).

TABLE 2
 Characteristics of African-American subjects from Maywood, IL, used in linkage and association studies

Variables	Men	Women	Total
Age	37.7 ± 14.9 (362)	41.2 ± 16.1 (580)	39.8 ± 15.7 (942)
Arm circumference (cm)	31.6 ± 4.6 (356)	31.0 ± 5.7 (557)	31.2 ± 5.3 (913)
BMI (kg/m ²)	26.8 ± 6.4 (362)	30.6 ± 7.9 (578)	29.2 ± 7.5 (940)
FFM (kg)	60.8 ± 9.3 (285)	46.3 ± 6.9 (468)	51.8 ± 10.6 (753)
FM (kg)	22.1 ± 12.4 (285)	34.7 ± 15.3 (468)	29.9 ± 15.5 (753)
FM adjusted for FFM	19.4 ± 10.9 (285)	36.4 ± 13.8 (468)	30.0 ± 15.2 (753)
Percent body fat mass	25.0 ± 8.6 (285)	40.9 ± 8.7 (468)	34.9 ± 11.6 (753)
Plasma glucose (mg/dl)	94.5 ± 36.2 (272)	99.1 ± 45.6 (449)	97.3 ± 42.3 (721)
Glucose adjusted for BMI	97.1 ± 35.6 (272)	97.1 ± 44.5 (447)	97.1 ± 41.3 (719)
Glucose adjusted for FM	98.3 ± 37.5 (240)	96.4 ± 45.6 (393)	97.1 ± 42.7 (633)
Plasma leptin (ng/ml)	7.8 ± 10.0 (341)	28.8 ± 21.4 (554)	20.8 ± 20.6 (895)
RMR (kcal/day)	1,720 ± 324 (47)	1,450 ± 216 (86)	1,545 ± 289 (133)
Adjusted RMR (kcal/day)	1,540 ± 178 (46)	1,544 ± 134 (86)	1,543 ± 150 (132)
RQ	0.85 ± 0.04 (47)	0.84 ± 0.04 (85)	0.84 ± 0.04 (132)

Data are means ± SD (n). FFM, fat-free mass; FM, fat mass; RMR, resting metabolic rate.

tion, the absent sixth transmembrane domain may be required for directing UCPs to the inner mitochondrial membrane (13). With any of these effects, one would expect a significant difference in skeletal muscle oxidative activity in subjects producing only UCP3S and no UCP3L if this protein, in fact, functions as an uncoupling protein. The fact that no change in mitochondrial coupling activity was detected in such an individual and the lack of significant differences in the skeletal muscle enzymes of oxidative phosphorylation or in systemic oxygen consumption at rest and during exercise suggest several possibilities: 1) UCP3S has uncoupling capacity equivalent to UCP3L; 2) other UCPs may compensate for deficiency of bioactive UCP3 (as noted, UCP3S mRNA was increased in the muscle of subjects homozygous for the splice mutation); or 3) UCP3L does not function primarily as a mitochondrial uncoupling protein. The lack of statistical linkage of the splice site and Val9Met sequence variants to aspects of body composition suggests that UCP3L or UCP3S may not function in vivo as mitochondrial uncoupling proteins. Against the biological importance of UCP3S is the relative paucity of this mRNA isoform in mice and rats (14).

A recent article by Argyropoulos et al. (15) reporting a study in Gullah-speaking African-Americans and the Mende tribe of Sierra Leone also finds the Val102Ile mutation in exon 3, and the splice site mutation in exon 6. In addition, a single chromosome with an Arg143Stop mutation was identified. No instance of the Val9Met mutation that we found is reported. These authors found the Val102Ile variant homozygous in 4% and heterozygous in 28% of 280 African-Americans and a similar proportion of individuals from Sierra Leone. No consistent phenotypic effects of the Val102Ile allele on obesity/diabetes phenotypes were found (consistent with our results; Table 1). Three instances of homozygosity for the exon 6 splice mutation were detected in the Mende Tribe of Sierra Leone (1%), but no phenotypic information is given regarding these individuals. Heterozygosity for the exon 6 splice mutation was associated with higher RQ ($P = 0.016$), and in the upper quartile of BMI, the frequency of +/- genotypes for exon 6 splice variant was two times that of +/+ genotypes ($P = 0.04$).

The higher RQ is suggested by Argyropoulos et al. (15) to be a proximate obesity phenotype, which, by virtue of decreased fat oxidation, predisposes to obesity. This is an unlikely possibility because, although the "Garlid Model" (16) proposes that UCP1 is an anion transporter that uses free fatty acids (FFAs) to shuttle protons into the mitochondrial matrix (cycling protonophore), these FFAs cannot be oxidized because there is no mitochondrial enzyme capable of adding the necessary CoA group to the FFA. Such FFA-CoA must be formed in the cytoplasm and enter the mitochondria via the carnitine carrier. Finally, as indicated, we found no evidence of association with resting RQ in studies involving 41 men and 71 women, or during graded exercise, to either of the UCP3 sequence variants that we analyzed. It is possible that some of the differences between our results and those of Argyropoulos et al. reflect differences in the populations that we studied. Our detailed physiological studies were performed in only a few subjects and should be repeated in larger groups in individuals. It is also possible that UCPs might indirectly affect the availability of fatty acids for oxidation. Analysis of the phenotypes of mice with knockouts of *Ucp3* should help to answer the questions raised by these two studies of humans with mutations of UCP3.

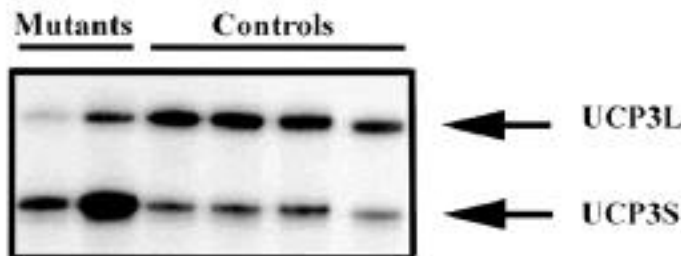


FIG. 2. Ribonuclease protection assays for UCP3 and UCP2 in skeletal muscle. This figure shows UCP3 ribonuclease protection assay of skeletal muscle from two patients who are heterozygous for the UCP3 exon 6 splice variant and four wild-type control subjects. The splice variant is predicted to result in production of only UCP3S from such an allele, since the splice donor site is destroyed by the mutation. Heterozygous splice site mutation subjects both have a ratio of UCP3S:UCP3L of 5.15:1, rather than the 0.093:1 ratio observed in subjects without splice donor site alteration.

RESEARCH DESIGN AND METHODS

Information on detection of sequence variants in *UCP3* can be found in Table 1 (4). Details on mutation detection in *UCP3* by single-strand conformation polymorphism and direct sequencing can be found in Table A1 of the on-line appendix at www.diabetes.org/diabetes/appendix.asp (4).

Genotyping of African-Americans. Polymerase chain reaction (PCR) was performed using fluorescent-labeled sense primers, and the product of digestion was analyzed by electrophoresis in an ABI 377 DNA sequencer using Genescan 202 and Genotyper programs. (Further details can be found in Tables A4 and A5 of the on-line appendix at www.diabetes.org/diabetes/appendix.asp.)

Ribonuclease protection assay for UCP3 and UCP2 in skeletal muscle. Total RNA was obtained from muscle biopsy samples by guanidium thiocyanate-phenol chloroform extraction. Partial human cDNA probes for *UCP2* and *UCP3* were generated as previously described by reverse transcriptase-PCR using total RNA from muscle (3,6). The PCR products were subcloned into PGMT easy TA cloning vector (Promega, Madison, WI). A linearized template for the anti-sense and sense probes were prepared using *SpeI* and *NcoI*. Anti-sense probe in the RNase protection assay (17), made as previously described (3,6), protects *UCP2* mRNA of 210 bp, a *UCP3L* mRNA of 293 bp, and a *UCP3S* mRNA of 193 bp. A 75-bp cDNA corresponding to 18S ribosomal RNA (gift of M. Jakubowski, Beth Israel-Deaconess Hospital, Boston, MA) was used as an internal control (17). RNA transcripts for *UCP2*, *UCP3*, and 18S were quantified by solution hybridization RNase protection and phosphorimager analysis (3,6). Protected bands were

visualized by autoradiographs and quantified by phosphorimager analysis (Image-Quant software; Molecular Dynamics, Sunnyvale, CA). *UCP2* is expressed in arbitrary phosphorimager units, *UCP3* in attomoles (Table 3).

Indirect calorimetry, bicycle ergometry, and body composition (Columbia University). Resting energy expenditure (REE) was measured by indirect calorimetry performed in the Clinical Research Center at Columbia Presbyterian Medical Center with a Delta Trac II MBM-200 Metabolic Monitor (Sensor Medics, Yorba Linda, CA) fitted with a ventilated hood. Body composition was measured by dual plateau beam absorptiometry. Oxygen consumption (metabolic efficiency) during exercise was assessed by an incremental exercise efficiency test on an electronically braked bicycle ergometer (Ergo-metrics 800S; Ergoline, Frankfurt, Germany), on which the work rate was increased by 25 W every 4 min until 16 min of exercise (range 0–75 W) were completed. Subjects were encouraged to pedal at a cadence of 50–60 rpm during the test. Expired gas analysis was performed continuously during the test with a commercially available Sensormedic 2900 metabolic cart (Yorba Linda, CA) in the mixing chamber mode. Results are expressed as on minute averages during the final minutes of each workload (Table 3). Body composition was determined as previously described (Table 3) (18).

Quantitation of skeletal muscle enzymes and mitochondrial coupling (Columbia University)

Quantitation of skeletal muscle enzymes. Biochemical analysis of respiratory chain enzyme activities and citrate synthase in skeletal muscle homogenate

TABLE 3
Phenotypes of African-Americans with exon 6 splice site mutation

	Subject 1	Subject 2	Subject 3
Age (years)	40	40	41
<i>UCP3</i> splice site genotype	–/–	–/–	+/+
Sex	F	F	F
Ethnic group	African-American	African-American	African-American
Diabetes	Type 2	None	None
Height (cm)	162.5	152	161
Weight (kg)	113.6	52.2	64
BMI (kg/m ²)	43.0	22.4	24.6
FFM (kg)	67.1	36.6	46.1
FM (kg)	46.5	15.6	17.9
REE (kcal/day)	1,876	940	1,330
REE (kcal · m ⁻² · day ⁻¹)	872	638	794
REE (kcal · kg ⁻¹ FFM · day ⁻¹) (normal)	27.9 (29.5 ± 5.0)	25.7 (27.5 ± 5.0)	28.2 (27.5 ± 5.0)
RQ	0.72	1.09*	0.82
Exercise (Vo ₂ l ⁻¹ · min ⁻¹ · kg ⁻¹ FFM and RQ) (Vo ₂ /RQ)			
0 W	9.60/0.77	9.24/0.80	11.6/0.98*
25 W	12.35/0.82	13.6/0.82	13.9/0.84
50 W	16.3/0.89	20.8/0.87	19.0/0.85
75 W	Unable to do/—	29.5/0.97	26.2/0.98
Cytochrome c oxidase (IV) (control ± SD)	6.235 (6.44 ± 0.44)	2.861 (2.915)	3.878 (2.915)
Succinate cytochrome C reductase (II+III) (control)	0.531 (0.701 ± 0.228)	0.435 (0.491)	0.505 (0.491)
NADH-cytochrome reductase (I+III) (control)	0.880 (1.020 ± 0.377)	1.703 (0.715)	0.52 (0.715)
NADH dehydrogenase (I) (control)	24.26 (35.48 ± 7.07)	20.14 (25.50)	23.33 (25.50)
Succinate dehydrogenase (II) (control)	1.132 (1.00 ± 0.526)	2.253 (1.513)	1.291 (1.513)
Normal coupling of mitochondria†	Yes	Not done	Not done
<i>UCP3S</i> mRNA (amol/μg RNA)	65.7	56.9	12.1
<i>UCP3L</i> mRNA (amol/μg RNA)	0	0	34.8
<i>UCP2</i> mRNA (phospho units/μg RNA)	19.0	24.8	22.1

Units for enzymes are μmol · l⁻¹ · min⁻¹ · g⁻¹. Normal values refer to range over many control subjects. Control values for subjects 2 and 3 refer to a control sample of healthy frozen muscle run in the same assay. Two African-American women, homozygous for the exon 6 splice variant (–/–) and an age-, sex-, and race-matched control underwent phenotypic measures of body composition, energy expenditure, and skeletal muscle oxidative phosphorylation (see METHODS). Subject 1 had early-onset obesity beginning at the age of 5 years and had gastric stapling performed at age 36 years. Her medical conditions include adolescent-onset type 2 diabetes, sleep apnea, dilated cardiomyopathy, and congestive heart failure and hypertension. Subjects 2 and 3 had never been overweight and had no significant past medical history. Additionally, subject 2 is a monozygotic twin. Her co-twin was phenotypically normal by both BMI and body fat measurements, but was unavailable for further study. The three subjects were studied in the postabsorptive state. Vastus lateralis muscle was obtained by Bergstrom needle biopsy (subjects 2 and 3) (3) or by open surgical biopsy (subject 1). Muscle fragments from subjects 2 and 3 were immediately frozen in liquid nitrogen and held at –80°C until being processed for RNA. Muscle tissue from subject 1 was studied fresh. *Subject was hyperventilating at 0 W. †Mitochondrial coupling was assayed as described in METHODS.

was performed as described (19). In particular, cytochrome c oxidase was determined spectrophotometrically by decrease in absorbance at 550 nm of reduced cytochrome c (20). Reduced cytochrome c was prepared fresh before each experiment by adding a few grains of sodium hydrosulfide (dithionite) to a 1% solution in 10 mmol/l K-phosphate buffer, pH 7.0.

Mitochondrial coupling. Mitochondria were isolated from 200 mg of freshly obtained vastus lateralis muscle (21). Mitochondrial oxygen uptake was determined polarographically (22) with a Clark oxygen electrode at 30°C. Freshly isolated mitochondria were added to respiratory buffer consisting of 10 mmol/l succinate, 0.3 mol/l mannitol, 0.2 mol/l EDTA, 5 mmol/l MgCl₂, 10 mmol/l KCl, 1 mg/ml bovine serum albumin, and 10 mmol/l potassium phosphate (pH 7.4). ADP was added to a final concentration of 0.2 mmol/l, followed by addition of the uncoupling agent, dinitrophenol (carbonyl cyanide p-(tri-fluoromethoxy) phenyl hydrazone [FCCP]), to a final concentration of 10 μmol/l (Table 3, subject 1).

Indirect calorimetry and body composition (Maywood, IL). Blood was drawn for determination of leptin (23) and blood glucose. The number of hours postprandial was not consistent for all participants, i.e., not all bloods were fasting samples. Blood was drawn into EDTA-containing vacutainers, and plasma was separated and stored at -80°C. Plasma glucose was assessed in duplicate using the glucose oxidase method (YSI 2300 Glucometer; YSI Yellow Springs, OH). Anthropometry, body composition, and calorimetry were performed as previously described (24,25). (Further details can be found in Tables A4 and A5 of the on-line appendix at www.diabetes.org/diabetes/appendix.asp.)

Association, TDT, and Haseman-Elston regression linkage testing. For each type of analysis, we conducted univariate tests for each variable and for each polymorphism (exons 2 and 6), and followed these with multivariate analogues of the univariate tests, where possible. In the classical association test, only association was tested by regressing the respective phenotype on the number of alleles of the rarer type (0, 1, or 2) for the polymorphisms under consideration, and the number of alleles was squared to allow for dominance effects while controlling for sex and age, (age)², and (age)³. When necessary, data were transformed to achieve approximate normality and homoscedasticity via a Box-Cox-type transformation (26). Because ordinary least squares regression analysis requires that the residuals be independent, some strategy was needed to deal with the presumably correlated residuals due to having related individuals in the data set. Therefore, an association test proposed by George and Elston (8), and applied and implemented in the ASSOC program of the SAGE software (27), was used. There was a weakly significant association of blood glucose concentrations with the exon 2 Val9Met polymorphism, such that individuals homozygous for the more common (Val9) allele had higher fasting glucose concentrations. This result persisted after controlling for BMI. There was also a marginally significant association ($P = 0.052$) for exon 2 Val9Met with BMI. (Further details can be found in Tables A6–A8 of the on-line appendix at www.diabetes.org/diabetes/appendix.asp.)

Two types of sibling-based TDTs were used (28). In these tests, only individuals within sibships in which there are two or more siblings with different genotypes are used. The first of these sib-TDTs is a mixed-model analysis of variance (ANOVA) in which genotype is the fixed effect and sibship is the random effect. Covariates were sex and polynomials of age.

For the sibling-based TDT, the polymorphism in exon 6 had marginally significant linkages/associations with many of the variables relating to body composition, such that individuals inheriting the rarer (splice mutation) of the two exon 6 alleles tended to be less obese (BMI $P = 0.05$, percent fat $P = 0.015$, fat mass $P = 0.013$). There were no significant results with the Val9Met polymorphism in the TDT analysis. Nonsignificant results were also replicated by another type of permutation-based sib-TDT (29). The results of Fisher combination in Table A7 (in on-line appendix) were obtained from the mixed model ANOVA sib-TDT and parent-based TDT.

For linkage analyses, all phenotypes were residualized for sex and polynomials of age, and the residuals were then transformed to approximate normality via a Box-Cox transformation (26) using the Unicorn software (11). Subjects were placed into sib-pair units, and the maximum likelihood estimate of the probability of sharing 0, 1, and 2 alleles identical by descent (IBD) at each polymorphism was calculated using Mapmaker Sibs software (30).

Using these IBD probabilities, a Haseman-Elston (10) test was conducted. All sibling pairs were treated as independent. However, alternative versions of the Haseman-Elston test that differentially weight dependent sibling pairs, or only use one pair from each sibship, yielded virtually identical results. Allele frequencies were calculated using the PedManager software, which estimates allele frequencies based on the genotypes of the founders in the pedigree (31). Specifically, extended pedigrees were decomposed into nuclear families using the "nuclear families" option in the PedManager software. Only pedigrees consisting of more than one sibling were included.

Several other tests of linkage were conducted but not reported here in detail. In brief, a nonparametric analogue of the Haseman-Elston test using ranks of squared intrapair differences (32,33) was conducted. Additionally, these tests

were conducted using the number of alleles (0, 1, or 2) IBD as the predictor variable (rather than the estimated proportion of alleles IBD), and weighting by the maximum likelihood estimates of the probability that the pair shared 0, 1, or 2 alleles IBD (33). We also used a modified version of a multivariate Haseman-Elston test described elsewhere (29,34), and a new variance components version described by Elston et al. (35).

All results are based on Haseman-Elston regression of estimated IBD proportion on intra-sibling-pair squared differences of phenotypes adjusted for age and sex. Based on the Haseman-Elston test, none of the linkage results were statistically significant.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grants DK52431 (R.L.L.), DK30583 (R.L.L.), T32DK07559 and HL53353 (R.S.C.), DK53477 (B.B.L.), HG00008 and R29DK47256 (D.B.A.), R01DK5176 (D.B.A., M.H.), P30DK26687 (D.B.A., M.H., R.L.L.), Telethon-Italy (C.B.) FIS-Beca Ampliacion de Estudios-Spain (A.L.A.), and the Nutrition Research Foundation.

We are grateful to Renata Lee and Lynn Orviet at the Rockefeller University DNA Core for their technical assistance, to Jeanine Albu and Sharon Rha for assistance with subject recruitment and phenotyping, to Maria Pospischil for oligonucleotide synthesis, and to Mary Prudden for manuscript preparation.

REFERENCES

1. Boss O, Samec S, Paolini-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 408:39–42, 1997
2. Gong DW, He Y, Karas M, Reitman M: Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta 3-adrenergic agonists, and leptin. *J Biol Chem* 272:24129–24132, 1997
3. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB: UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Commun* 235:79–82, 1997
4. Chung WK, Power-Kehoe L, Chua M, Chu F, Aronne L, Huma Z, Sothorn M, Udall JN, Kahle EB, Leibel RL: Exonic and intronic allelic variation in the leptin receptor (OBR) of obese humans. *Diabetes* 46:1509–1511, 1997
5. Rotimi CN, Cooper RS: Familial resemblance for anthropometric measurements and relative fat distribution among African Americans. *Int J Obesity* 19:875–880, 1995
6. Solanes G, Vidal-Ouig A, Grujic D, Flier JS, Lowell BB: The human uncoupling protein-3 gene: genomic structure, chromosomal localization, and genetic basis for short and long form transcripts. *J Biol Chem* 272:25433–25436, 1997
7. Miroux B, Frossard V, Raimbault S, Ricquier D, Bouillaud F: The topology of the brown adipose tissue mitochondrial uncoupling protein determined with antibodies against its antigenic sites revealed by a library of fusion proteins. *EMBO J* 12:3739–3745, 1993
8. George VT, Elston RC: Testing the association between polymorphic markers and quantitative traits in pedigrees. *Genet Epidemiol* 4:193–201, 1987
9. Allison DB: Transmission-disequilibrium tests for quantitative traits. *Am J Hum Genet* 60:676–690, 1997
10. Haseman JK, Elston RC: The investigation of linkage between a quantitative trait and a marker locus. *Behav Genet* 2:3–19, 1972
11. Allison DB, Gorman BS, Kucera EM: UNICORN: a computer program for transforming data to normality. *Educ Psychol Measurement* 55:625–629, 1995
12. Ricquier D, Bouillaud F: The mitochondrial uncoupling protein: structural and genetic studies. *Prog Nucleic Acid Res Mol Biol* 56:83–108, 1997
13. Sirrenberg C, Endres M, Folsch H, Stuart RA, Neupert W, Burnner M: Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5. *Nature* 391:912–915, 1998
14. Gong DW, He Y, Reitman ML: Genomic organization and regulation by dietary fat of the uncoupling protein 3 and 2 gene. *Biochem Biophys Res Commun* 256:27–32, 1999
15. Argyropoulos G, Brown AM, Willi SM, Zhu J, He Y, Reitman M, Gevaso BM, Spruill I, Garvey WT: Effects of mutations in the human uncoupling protein 3 gene on the respiratory quotient and fat oxidation in severe obesity and type 2 diabetes. *J Clin Invest* 102:1345–1351, 1998
16. Jezek P, Orosz DE, Modriansky M, Garlid KD: Transport of anions and protons by the mitochondrial uncoupling protein and its regulation by nucleotides and

- fatty acids: a new look at old hypotheses. *J Biol Chem* 269:26184–26190, 1994
17. Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE: Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *J Clin Invest* 97:2553–2561, 1996
 18. Rosenbaum M, Ravussin E, Matthews DE, Gilker C, Ferraro R, Heysmfield SB, Hirsch J, Leibel RL: A comparative study of different means of assessing long-term energy expenditure in humans. *Am J Physiol* 270:R496–R504, 1996
 19. DiMauro S, Servidei S, Zeviani M, DiRocco M, DeVivo DC, DiDonato S, Uziel G, Berry K, Hoganson G, Johnson SD: Cytochrome c oxidase deficiency in Leigh Syndrome. *Ann Neurol* 22:498–506, 1987
 20. Wharton DC, Tzagoloff A: Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol* 10:245–250, 1967
 21. Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, Munnich A: Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 228:35–51, 1994
 22. Poe M, Estabrook RW: Kinetic studies of temperature changes and oxygen uptake concomitant with substrate oxidation by mitochondria: the enthalpy of succinate oxidation during ATP formation by mitochondria. *Arch Biochem Biophys* 126:320–330, 1968
 23. Luke A, Long A, Cooper R, Rotimi CN, Forrester T, Wilks R, Bennett F, Ogunbiyi O, Compton JA, Bowsher RR: Leptin and body composition among Nigerians, Jamaicans, and U.S. Blacks. *Am J Clin Nutr* 67:391–396, 1998
 24. Luke A, Duzaro-Arvizu R, Rotimi C, Prewitt TE, Forrester T, Wilks R, Ogunbiyi O, Schoeller DA, McGee D, Cooper RS: Relation between body mass index and body fat in population samples from Nigeria, Jamaica and the United States. *Am J Epidemiol* 145:620–628, 1997
 25. Luke A, Rotimi CN, Prewitt TE, Schoeller DA, Kushner RF, Cooper RS: Resting metabolic rate: no black/white difference found among men (Abstract). *FASEB J* 12:A204, 1998
 26. Box GE, Cox DR: An analysis of transformations. *J R Stat Soc* 26 (Series B):211–252, 1964
 27. Elston RC: *Statistical Analysis for Genetic Epidemiology*. Version 3.1. Cleveland, OH, Case Western Reserve University, 1997
 28. Allison DB, Heo M, Kaplan N, Martin ER: Development of sibling-based tests of linkage in the presence of association for quantitative traits that do not require parental information. *Am J Hum Genet* 64:1754–1764, 1999
 29. Allison DB, Thiel B, St. Jean P, Elston RC, Infante M, Schork NJ: Multiple phenotype modeling in gene-mapping studies of quantitative traits: power advantages. *Am J Hum Genet* 63:1190–1201, 1998
 30. Kruglyak L, Lander ES: High-resolution genetic mapping of complex traits. *Am J Hum Genet* 56:1212–1223, 1995
 31. Boehnke M: Allele frequency estimation from data on relatives. *Am J Hum Genet* 48:22–25, 1991
 32. Puri ML, Sen PK: A class of rank order tests for a general linear hypothesis. *Ann Math Stat* 40:1325–1343, 1969
 33. Kruglyak L, Lander ES: A nonparametric approach for mapping quantitative trait loci. *Genetics* 139:1421–1428, 1995
 34. Amos CI, Elston RC, Wilson AF, Bailey-Wilson JE: A more powerful robust sib-test of linkage for quantitative traits. *Genet Epidemiol* 6:435–449, 1989
 35. Elston RC, Buxbaum S, Jacobs KB, Olson JM: Haseman and Elston revisited (Abstract). *Genet Epidemiol* 15:546, 1998