# Uncoupling protein-1 mRNA expression in obese human subjects: the role of sequence variations at the uncoupling protein-1 gene locus

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**Abstract** Uncoupling protein-1 (UCP-1) activity in brown adipose tissue increases thermogenesis, contributes to facultative energy expenditure in humans, and has been implicated in the pathogenesis of rodent obesity. To determine genetic factors controlling UCP-1 expression in humans, we measured intra- and extraperitoneal UCP-1 mRNA abundance levels by a competitive RT-PCR method and compared expression levels with common sequence variations in the β3-adrenergic receptor gene and the distal UCP-1 gene promoter in obese human subjects. While median and average UCP-1 mRNA levels in both the intra- and extraperitoneal tissue were lower in subjects heterozygous for the Trp64Arg mutation in the β3-adrenergic receptor gene, this difference was not statistically significant. However, a strong association of intraperitoneal UCP-1 mRNA abundance with the UCP-1 gene polymorphism at −3826 relative to the transcription start site was observed that explained 19.3% of the interindividual variability. The minor allele imparted a dose-dependent reduction on UCP gene expression. The importance of sequence variations at the UCP-1 gene locus as a common source of UCP-1 mRNA abundance variability was supported by allele-specific expression studies utilizing a newly identified polymorphism in exon 2 of the UCP-1 gene that predicts a substitution of alanine by threonine. In four subjects heterozygous for the −3826 polymorphism, the mRNA species transcribed from the wild-type allele accounted for 63 ± 6% percent of total intraperitoneal mRNA abundance. In one subject homozygous for the minor promoter allele, wild-type mRNA was also more abundant than variant mRNA. Thus, the UCP-1 polymorphism at −3826 is probably only a marker for a frequent mutation causing reduced mRNA expression.—**Esterbauer, H., H. Oberkofler, Y-M. Liu, D. Breban, E. Hell, F. Krempler, and W. Patsch.** Uncoupling protein-1 mRNA expression in obese human subjects: the role of sequence variations at the uncoupling protein-1 gene locus. *J. Lipid Res.* 1998. **39:** 834–844.

**Supplementary key words** uncoupling proteins • mRNA • obesity • β3-adrenergic receptor • RT-PCR

In humans, small decrements in energy expenditure have been shown to increase the risk for obesity (1, 2) and genetic factors determine, at least in part, the resting metabolic rate (3, 4). In rodents, brown adipose tissue (BAT) plays an important role in total-body energy expenditure by burning energy in the form of heat, whereas white adipose tissue stores energy as triglyceride. Central to BAT function is the uncoupling protein-1 (UCP-1) or thermogenin that is an integral component of the mitochondrial inner-membrane of brown adipocytes (5–7). UCP-1 transports fatty acid anions that can reenter the mitochondria after their protonation. The electrochemical gradient that is generated along the inner mitochondrial membrane by respiration dissipates as a result of UCP activation and heat is produced instead of chemical energy (8). More recently, two other members of the UCP gene family, termed UCP-2 and UCP-3, have been identified (9–12). UCP-2 and UCP-3 exhibit structural homology to and share functional properties with UCP-1. In contrast to UCP-1, which is solely expressed in BAT, UCP-2 is expressed in several human tissues including BAT, white adipose tissue, lung, liver, spleen, and macrophages (9, 10). UCP-3 is primarily expressed in muscle and BAT (11, 12).

UCP-1 expression and activity is regulated by the sympathetic nervous system (13, 14) and signalling via the β3-adrenergic receptor has been implicated in UCP-1 activation, as β3-specific agonists enhance energy expenditure and exhibit potent antiobesity effects

Abbreviations: apo, apolipoprotein; BAT, brown adipose tissue; BMI, body mass index; EDTA, ethylenediamine-tetraacetic acid; HDL, high density lipoprotein; RT-PCR, reverse transcription-polymerase chain reaction; SOE, splice overlap extension; UCP-1, uncoupling protein-1; UCP-2, uncoupling protein-2; UCP-3, uncoupling protein-3.

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in rodents (15, 16). In humans, a common Trp64Arg sequence variation in the β3-adrenergic receptor has been associated with a lower metabolic resting rate and earlier onset of non-insulin-dependent diabetes mellitus in Pima Indians (17). In other populations, the same sequence variation was associated with abdominal obesity and resistance to insulin (18) or an increased capacity to gain weight (19). These observations were supported by a recent study showing an additive effect of the β3-adrenergic receptor Trp64Arg variant with a Bcl I polymorphism in the 5′-region of the UCP-1 gene on weight gain in obese subjects (20). In addition, in obese subjects the same UCP-1 polymorphism was associated with lower weight loss during a low calorie diet (21).

To gain insight into possible associations of the β3-adrenergic receptor Trp64Arg variant and the Bcl I UCP-1 gene polymorphism with BAT function, we compared UCP-1 mRNA expression levels among the respective genotypes in morbidly obese subjects. We report here that the Trp64Arg variant of the β3-adrenergic receptor displayed no significant association with UCP-1 mRNA expression levels, while the minor allele of the UCP-1 promoter polymorphism was strongly associated with lower UCP-1 mRNA abundance in intraperitoneal adipose tissue of obese study subjects. Moreover, the association of UCP-1 gene structure and expression level was corroborated by allele-specific expression studies. Thus, part of the variability in UCP-1 gene expression results from common sequence variation(s) at the UCP-1 gene locus.

## METHODS

### Study subjects

This study included 153 unrelated Caucasian subjects with morbid obesity. Tissue samples were obtained from obese subjects who underwent weight reduction surgical treatment through a gastric banding procedure. Study subjects provided informed consent and the study was approved by the institutional review board. After an overnight fast general anesthesia was induced between 8:00 am and 10:00 am by a short-acting barbiturate and maintained by alfentanil-hydrochloride. Fat biopsies were taken from comparable sites of the abdominal subcutaneous fat (referred to as extraperitoneal fat) and the omentum (referred to as intraperitoneal fat) at the beginning of the surgical procedure. Adipose tissue specimens were submerged in ice-cold saline and transported to the laboratory where they were divided into aliquots and frozen at −70°C.

Body mass index (BMI, kilograms per meters squared) was calculated from measurements of weight and height.

### Laboratory methods

After an overnight fast, venous blood was collected at 8:00 am into tubes containing EDTA. Plasma glucose was measured by a hexokinase/glucose-6-phosphate dehydrogenase method. Plasma insulin was measured by immunoassay (MEIA, Abbott Laboratories, Abbott Park, IL). Plasma thyroid stimulating hormone (TSH) was measured by an automated immunoassay (Elecsys TSH Immunoassay, Boehringer Mannheim Diagnostics, Mannheim, Germany). Cholesterol and triglyceride were measured by enzymatic procedures using a Hitachi 717 analyzer (Boehringer Mannheim Diagnostics) and the respective enzymatic kits (catalogue Nos. 1489437 and 1058550, Boehringer Mannheim Diagnostics). High density lipoprotein (HDL) cholesterol was determined in supernates after precipitation of plasma with phosphotungstic acid/magnesium chloride. Apolipoprotein (apo) B and apoA-I levels were determined using nephelometric procedures (Array 360, Beckman, Palo Alto, CA). Plasma leptin levels were measured with an RIA kit (Linco Inc., St. Charles, MO) using an antibody raised against highly purified human leptin and recombinant human leptin as tracer and standard. The inter- and intra-assay coefficients of variation were 8% and 7%, respectively.

Total RNA was isolated from 2 g of adipose tissue according to the method of Chomczynski and Sacchi (22). The integrity of the RNA was ascertained by the electrophoretic patterns of rRNA in formaldehyde gels. RNA concentrations were determined by absorbance measurements at 260 nm. Genomic DNA was isolated from adipose tissue specimens and/or peripheral leucocytes using the QIAamp Tissue Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined by absorbance measurements at 260 nm.

UCP-1 mRNA expression levels were determined by competitive reverse transcription-polymerase chain reaction (RT-PCR) as described (23). In brief, splice overlap extension (SOE) (24) was used to construct a 256 bp UCP-1 competitor cDNA fragment that contained a foreign 19 bp sequence instead of a 75 bp deletion at its center. The fragment was cloned into Sma I digested pGEM-3Zf. The primer pairs for the SOE-PCR amplifications were *1*) 5′-ACCTCGCTACACGGGGAC-3′ (UCP-1 upper primer; +450–+467) and 5′-CCTCGAATAAG CTTTGAAATTGATGATGACACTTCT-3′ (SOE primer 1; +562–+542); and *2*) 5′-GCACAGTTGGGCACAC TTTTGT-3′ (UCP-1 lower primer; +761–+740) and 5′-TCAAAGCTTATTCTCGAGGCACTTGGTGTCGGC T CTT-3′ (SOE primer 2; +655–+638). The numbers



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in parentheses designate the 5′ and 3′ ends in the cDNA relative to the translation start site (Genbank accession Nr. U28480 for UCP-1). Primers were synthesized using a Beckman Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, CA). Plasmids containing the respective inserts were linearized with EcoR I and transcribed from the SP6 promoter in the presence of [5-$^{3}$H]UTP (specific activity 10 Ci/mmol; Amersham Life Science, Buckinghamshire, UK) using the Riboprobe-Systems in vitro transcription kit (Promega Corp., Madison, WI). In vitro transcribed RNA was gel purified and the amount of $^{3}$H-labeled RNA synthesized was calculated from its nucleotide composition and the amount of radioactivity incorporated. Radioactivity was determined by liquid scintillation counting (Wallac 1450 Microbeta PLUS, EG&G Berthold, Bad Wildbach, Germany).

To quantify UCP-1 mRNA in adipose tissue samples, 0.6 μg of adipose tissue total RNA was reverse-transcribed along with four increasing doses of competitor RNA using 2 units of *Tth* DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA), 2.5 μm UCP-1 lower primer, 10 mm Tris-HCl, pH 8.3, 90 mm KCl, 10 mm $MnCl_2$ and 2 mm of each dNTP in a 20 μl reaction volume for 30 min at 70°C. PCR amplification was performed using 0.2 μm of each upper and lower UCP-1 primer, 200 μm of each dNTP, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, 2 μCi [α-$^{32}$P]dCTP (specific activity 3000 Ci/mmol; Amersham), and 2.5 units of Amplitaq (Perkin-Elmer Corp.) in a 100 μl reaction volume that was overlaid with mineral oil. Samples were processed through initial denaturation for 5 min at 95°C; 28 cycles of amplification each consisting of 1 min annealing at 61°C (cycles 1–3), at 57°C (cycles 4–6), and at 53°C (cycles 7–28), 1 min extension at 72°C, 1 min denaturation at 95°C, and a final extension at 72°C for 10 min. PCR products were separated on 4% denaturing polyacrylamide gels containing urea. After removal of urea, gels were dried and exposed to X-ray film. Intensities of bands were quantified by scanning autoradiographs with a Model GS-700 Imaging densitometer using the Molecular Analyst software (Bio-Rad, Hercules, CA) and the linear range of films. Signal intensity ratios of wild-type to competitor cDNA were corrected for their molar C/G content and plotted as a function of the known amount of competitor RNA to determine the point of equivalence (i.e., where the molar ratio was 1.0). UCP-1 mRNA abundance in adipose tissue total RNA was normalized for β-actin mRNA abundance determined by RNase protection assay as described previously (23). The method for quantification of UCP-1 mRNA levels has been rigorously evaluated for accuracy and precision. Recoveries of in vitro transcribed UCP-1 RNA in RNA preparations of subjects with low and high UCP-1 mRNA abundance level were 90 and 103%, respectively. The mean intra-assay coefficient of variation was 12% (range 7–19%) and the intra-tissue coefficient of variation in four adipose tissues from different subjects ranged between 13.8 and 20.3% (23). The possibility that UCP-2 and UCP-3 mRNA confounded UCP-1 quantification was excluded by using UCP-2 and UCP-3 cDNA as templates in the PCR reactions described above. Up to 10 fg of gel-purified near full-length UCP-2 or UCP-3 cDNA failed to produce an amplification product in the UCP-1-specific PCR. For preparation of UCP-2 or UCP-3 cDNA, intraperitoneal adipose tissue or muscle total RNA was reverse transcribed by the random hexamer priming method. UCP-2 cDNA was amplified using 5′-ATCAGCATCATGGTTGGGTTC-3′ and 5′-TGA TCAGGTCAGCAGCAGGAG-3′ (+306–+326 and +1249–+1269, Genbank accession Nr. U94592) as primers. Primers for UCP-3 cDNA amplification were 5′-TG GACTGAAGCCTTCAGACGTG-3′ and 5′-ACAACAGT TCTGTAAACATGTG-3′ (+159–+180 and +1189–+1207, Genbank accession Nr. U84763). Amplification conditions for both UCP-2 and UCP-3 cDNA were 35 cycles each consisting of 1 min annealing at 60°C, 1 min extension at 72°C, and 1 min denaturation at 95°C followed by a final extension at 72° for 10 min.

The Bcl I polymorphism at −3826 relative to the transcription start site of the UCP-1 gene was determined using the primers, PCR and digestion conditions described by Cassard-Doulcier et al. (25). For typing the BstN I polymorphism in the β3-adrenergic receptor gene that results in replacement of tryptophan by arginine (Trp64Arg), the method of Widen et al. was followed (18).

To determine sequence variation in the transcribed sequence of the UCP-1 gene, a genomic fragment spanning exon 1 and 2 was amplified by PCR using 5′-CCAGCTATATAAGTCCCAGCG-3′ (−38–−18) and 5′-GAACAGAGCGGATCCAAGGCTTTT-3′ (+1183–+1206) as forward and backward primer, respectively (Genbank accession Nr. X51952). PCR assays contained 500 ng DNA, 0.2 μm of each upstream and downstream primer, 200 μm of dNTP, 20 mm Tris-HCl, pH 8.8, 10 mm KCl, 10 mm $(NH_4)_2SO_4$, 2 mm $MgCl_2$, 0.1% Triton X-100, and 2.5 units of Pfu-polymerase (Stratagene, La Jolla, CA) in a total volume of 100 μl. The reaction mixture was initially denatured at 95°C for 5 min, followed by 35 cycles of denaturation (95°C, 1 min), annealing (54°C, 1 min), and extension (72°C, 1 min) with a final extension for 10 min at 72°C. PCR products were separated on a 1% agarose gel and eluted from the gel. Sequencing was performed using the Thermo Sequenase dye terminator cycle-sequencing pre-mix kit (Amersham Life Science). In addition to the PCR primers



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above 5′-TTTCGCTCCCTTCTGTT-3′ (+917–+932) and 5′-TAGCTACCTGGAGCCGG-3′(+349–+332) were used as sequencing primers. Aliquots (8 μl) of the sequencing premix were combined with 0.5 pmol template DNA and 0.2 μm primer and were denatured at 96°C for 2 min followed by 25 cycles of denaturation at 96°C for 10 sec, annealing at 54°C for 5 sec, and extension at 60°C for 4 min. The reaction products were ethanol precipitated, resuspended in 25 μl of TSR-reagent (Perkin Elmer Applied Biosystems) and denatured at 95°C for 2 min prior to analysis with an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer Applied Biosystems).

For typing of the Aci I polymorphism in exon 2 of the UCP-1 gene at genomic position +1017, a 290 bp genomic fragment was amplified using the primers spanning nucleotides +917–+932 and +1183–+1206 described. Amplification was performed using 0.2 μm of each upper and lower primer, 200 μm of dNTP, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, and 2.5 units of Amplitaq (Perkin Elmer Applied Biosystems) in a 100 μl reaction volume. Samples were processed through initial denaturation, followed by 35 cycles of 1 min annealing at 60°C, 1 min extension at 72°C, and 1 min denaturation at 95°C, and a final extension at 72°C for 10 min. Reactions were ethanol precipitated, digested with 5 units of Aci I for 1 h at 37°C after addition of buffer supplied by the manufacturer (New England Biolabs Inc., Beverly, MA), analyzed by electrophoresis on 10% polyacrylamide gels, and visualized by staining with ethidium bromide.

For allele-specific expression studies, restriction isotyping of RT-PCR products as well as wild-type- and mutant-specific primer extension was used. Total intraperitoneal adipose tissue RNA (1 μg) was reverse transcribed using the random-hexamer priming method. cDNA aliquots were subjected to PCR. The forward primer, an exon 1–exon 2 junction primer was 5′-CGG CTCCAGGTCCAAGGTGAATG-3′ (+118–+140, Genbank accession Nr. U28480), the reverse primer was 5′-GGTTGCCCAATGAATACTGCCACTC-3′ (+374–+398). PCR reactions contained 1 nm of each upstream and downstream primer, 0.1 mm dNTPs, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, and 0.5 units of Taq-Polymerase (Perkin-Elmer Corp.) in a final volume of 20 μl. cDNA samples were processed through initial denaturation for 1 min at 94°C; 40 cycles of amplification each consisting of 1 sec at 60°C and 1 sec at 94°C. PCR products were diluted 1:1000 in water and subjected to a second PCR amplification using the same forward junction-primer and 5′- CCGCTGTAGAGTTTCATC CGACCTTTC-3′ (+205–+230) as nested antisense primer which carries an artificially introduced G to A substitution (+210) to eliminate a naturally occurring Aci I site which would have complicated interpretation of fragment patterns. PCR amplification was performed using 0.1 μm of each upper and lower primer, 1 μCi [α-$^{32}$P] dCTP (3000 Ci/mmol, Amersham), buffers, dNTPs, and enzyme concentrations as described above. PCR cycling conditions were the same as described, but included a final annealing step of 1 min at 60°C followed by an extension step at 72°C for 5 min. Aliquots of PCR reactions (10 μl) were digested with 5 units of Aci I (New England Biolabs) for 12 h at 37°C and applied to denaturing 10% polyacrylamide gels using 100 mm Tris, 30 mm taurine, 0.5 mm EDTA, pH 9.2, as a buffer system (TTE-buffer) to provide glycerol tolerance. Gels were dried, exposed to X-ray film (X-Omat AR, Eastman Kodak), and autoradiograms were analyzed as described.

For allele-specific primer extension studies, a 281 bp fragment (+118–+398) was amplified by PCR using the same set of primers as for the outer PCR reaction described, but with the reverse primer (−374–+398) 5′-biotinylated. PCR reactions contained 2 μl of cDNA, 1 μm of each upstream and downstream primer, 0.2 mm dNTPs, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, and 1 unit of Taq-Polymerase (Perkin Elmer Applied Biosystems) in a final volume of 100 μl. Cycling conditions were as follows: 96°C for 2 min, 45 cycles with denaturation at 96°C, annealing at 60°C, and extension at 72°C, each step for 30 sec. No PCR product was obtained in control reactions using near full-length UCP-2 and UCP-3 cDNA as templates. PCR products concentrated by repeated butanol extraction were mixed with streptavidin-coated magnetic beads (Dynal A.S., Oslo, Norway). Biotinylated DNA-strands were captured and separated from the complementary strand according to the manufacturer's recommendations. Beads containing biotinylated DNA were resuspended in 10 μl water. Primers for wild-type and mutant allele-specific extension were 5′-AAGGTGTCC TGGGAAC-3′ (+167–+182) and 5′-GTCCTGGGAA CAATCA-3′ (+172–+187), respectively. After purification by thin-layer chromatography, 10 pmol of each primer was endlabeled with polynucleotide kinase (New England Biolabs) and [γ-$^{32}$P]ATP (4500 Ci/mmol, Amersham) to a specific activity of $5 \times 10^7$ cpm/nmol. Unincorporated radioactivity was removed by chromatography in G25-spin columns (Pharmacia Biotech, Uppsala, Sweden). After heating mixtures containing approximately 1 pmol of bead-immobilized DNA, 2 pmol of wild-type or mutant primer, 66 mm Tris-HCl, pH 7.5, 33 mm $MgCl_2$, 50 mm NaCl in a final volume of 10 μl to 85°C and cooling slowly to room temperature, 0.1 mm dNTPs (dATP, dTTP, dCTP for wild-type; dGTP, dTTP, dCTP for mutant) and 0.25 mm of the appropriate ddNTPs (ddGTP for wild-type; ddATP for mutant) were added with 0.1 mm DTT and 1



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unit of Sequenase 2.0 (Amersham) to give a final volume of 15 μl. Samples were incubated for 2 min at 30°C and for 1 h at 37°C. Reactions were subjected to electrophoresis in 18% polyacrylamide gels containing 8 m urea using the TTE-buffer system. Gels were exposed to X-ray film without drying. Autoradiograms were analyzed by quantitative scanning. For assay standardization, cDNAs of two patients, carrying either two wild-type or two mutant alleles were amplified using Pfu-polymerase (Strategene) and the outer primer pair (+118–+140, +374–+398). The resulting PCR-fragments were blunt-end cloned into pGEM-3Zf (Promega). Wild-type and mutant plasmids, with or without the variant Aci I site at +190, were PCR amplified and used to ascertain the adequacy of digestion by Aci I in allele-specific expression studies. In addition, various mixtures of plasmids containing the wild-type and mutant allele as inserts served as templates for PCR amplification, restriction with Aci I and primer extension to generate a standard curve to relate molar ratios of templates to their signal intensity ratios.

### Statistical analyses

Allele frequencies were estimated by gene counting. Agreement with Hardy-Weinberg equilibrium was tested using a $\chi^2$ goodness-of-fit test. The standardized pairwise linkage disequilibrium statistic (D′) (26, 27) was calculated to determine the degree of association between the UCP-1 gene markers. Prior to analysis of the effects of the β3-adrenergic receptor and the UCP-1 gene polymorphisms on UCP-1 mRNA expression data, these measures were adjusted, by multiple regression, for the concomitant effects of sex and age. Both the analysis of variance (28) and a more robust non-parametric test, the Kruskal Wallis test (29), were used to investigate the effects of genotype on biochemical measurements and UCP-1 mRNA expression levels. Transformations were made on the original variable if the equal variance and normality assumptions of the one-way ANOVA were rejected. The proportion of interindividual variation in UCP-1 mRNA levels attributable to the UCP-1 polymorphism was calculated using the $R^2$ from the analysis of variance. To compare categorical variables, a contingency $\chi^2$ test was used.

## RESULTS

The observed frequencies of the β3-adrenergic receptor genotype at amino acid residue 64 and the UCP-1 genotype at nucleotide position −3826 relative to the transcription start site are shown at **Table 1**. The estimated allele frequencies were 0.9346 and 0.0654 for the β3-adrenergic receptor and 0.7153 and 0.2847 for the UCP-1 polymorphism, respectively. In addition, the observed genotype frequencies were in agreement with Hardy-Weinberg expectations and no linkage disequilibrium between the two loci was observed in our population. Regarding the β3-adrenergic receptor polymorphism, wild-type and heterozygous subjects exhibited similar distributions of gender, age, systolic and diastolic blood pressure readings, and biochemical variables (Table 1). Compared to wild-type patients, average UCP-1 mRNA levels in the intra- and extraperitoneal adipose deposits were lower in heterozygous subjects, but the difference was not significant (**Table 2**). Simi-

TABLE 1. Characteristics of study subjects by β3–adrenergic receptor and uncoupling protein-1 genotype

| | β3-Adrenergic Receptor Genotype[a] | | Uncoupling Protein-1 Genotype | | |
|---|---|---|---|---|---|
| Variable | Wild-type | Heterozygous | Wild-type | Heterozygous | Mutant |
| Gender (f/m) | 107/26 | 15/5 | 55/15 | 57/9 | 6/2 |
| Age (years) | 36.1 ± 10.5 | 35.8 ± 11.8 | 35.6 ± 10.5 | 37.2 ± 10.9 | 30.4 ± 11.0 |
| BMI (kg/m²) | 43.5 ± 8.6 | 41.8 ± 5.8 | 43.0 ± 8.1 | 42.1 ± 6.8 | 47.2 ± 8.1 |
| RR systolic | 136 ± 17 | 134 ± 19 | 137 ± 17 | 135 ± 18 | 136 ± 15 |
| RR diastolic | 82 ± 11 | 85 ± 14 | 83 ± 10 | 81 ± 12 | 86 ± 14 |
| Glucose (mg/dl) | 92 ± 33 | 87 ± 25 | 91 ± 31 | 94 ± 32 | 76 ± 21 |
| Insulin (μU/ml) | 11.0 ± 8.0 | 10.4 ± 6.5 | 10.0 ± 7.2 | 11.5 ± 8.0 | 14.9 ± 10.0 |
| Cholesterol (mg/dl) | 197 ± 40 | 203 ± 43 | 200 ± 41 | 197 ± 41 | 173 ± 47 |
| Triglyceride (mg/dl) | 354 ± 378 | 367 ± 486 | 341 ± 345 | 385 ± 453 | 336 ± 212 |
| HDL-cholesterol (mg/dl) | 34.1 ± 14.1 | 33.6 ± 10.7 | 35.3 ± 15.5[b] | 33.7 ± 11.6[b] | 23.8 ± 7.8[b] |
| ApoA-I (mg/dl) | 118 ± 29 | 115 ± 18 | 118 ± 29 | 119 ± 27 | 101 ± 26 |
| ApoB (mg/dl) | 99 ± 28 | 102 ± 26 | 101 ± 29 | 98 ± 25 | 96 ± 34 |
| TSH (μU/ml) | 2.4 ± 1.5 | 2.3 ± 1.4 | 2.5 ± 1.6 | 2.2 ± 1.5 | 2.7 ± 1.3 |
| Leptin (ng/ml) | 36.0 ± 17.7 | 36.0 ± 17.3 | 34.3 ± 15.3[c] | 36.4 ± 17.5[c] | 52.5 ± 33.1[c] |

[a]β3-Adrenergic receptor wild-type and heterozygous genotype refer fo Trp64Trp and Trp64Arg, respectively. Uncoupling protein-1 wild-type, heterozygous, and mutant genotype refer to A/A, A/G, and G/G, respectively, at −3826 relative to the transcription start.
[b]$P < 0.05$, [c]$P < 0.096$; analysis of variance adjusted for age and gender.



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TABLE 2. Effect of β3-adrenergic receptor and UCP-1 genotype on UCP-1 mRNA expression level in adipose tissues of obese subjects

| Tissue | UCP-1 mRNA Expression Level | | | | |
|---|---|---|---|---|---|
| | β3-Adrenergic Receptor Genotype | | UCP-1 Genotype | | |
| | Wild-type | Heterozygous | Wild-type | Heterozygous | Mutant |
| | *amol/fmol β-actin mRNA* | | | | |
| Intraperitoneal adipose | 3.548 ± 2.320 | 3.212 ± 3.233 | 4.432 ± 2.554[b,d,e] | 2.878 ± 2.271[b,d,f] | 1.456 ± 0.802[b,e,f] |
| n[a] | 66 | 13 | 36 | 36 | 6 |
| Extraperitoneal adipose | 0.477 ± 0.379 | 0.411 ± 0.471 | 0.555 ± 0.454[c] | 0.390 ± 0.398[c] | 0.388 ± 0.216[c] |
| n | 46 | 11 | 25 | 27 | 4 |

[a]Number of subjects.
[b]$P < 0.0004$; [c]$P < 0.063$, analysis of variance with gender and age as covariates; [d]$P < 0.0107$; [e]$P < 0.0038$; [f]$P < 0.2019$, Scheffe-test.

larly, median UCP-1 mRNA levels were lower in carriers of one variant allele than in subjects with two wild-type alleles (intraperitoneal tissue: 3.050 vs. 2.166 amol/fmol β-actin mRNA; extraperitoneal tissue: 0.370 vs. 0.125 amol/fmol β-actin mRNA). Again, the difference in median levels of UCP-1 mRNA did not reach statistical significance ($P < 0.261$ for both intra- and extraperitoneal adipose tissues, Kruskal Wallis test). In addition, separate analyses in females or males did not reveal significant differences in UCP-1 mRNA levels.

Age, gender, clinical and biochemical variables stratified by UCP-1 genotype are shown in Table 1. Average values of plasma leptin and insulin tended to increase in going from the wild-type to the homozygous mutant genotype, while plasma cholesterol, HDL cholesterol, and apoB tended to decrease. Among these variables, only the average and median levels of HDL cholesterol differed significantly among the genotypes ($P < 0.05$). The difference in average leptin plasma levels was of borderline significance ($P < 0.096$), but no association was observed between leptin plasma concentrations and intra- or extraperitoneal UCP-1 mRNA expression levels in the entire study group. The average level of intraperitoneal UCP-1 mRNA expression differed significantly by UCP-1 genotype in that the variant allele was associated with a dose-dependent reduction of mRNA expression levels (Table 2). Median levels of UCP-1 mRNA expression showed a similar pattern (3.625, 2.442, and 1.050 amol/fmol β-actin mRNA for wild-type, heterozygous, and mutant subjects, respectively; $P < 0.001$, Kruskal Wallis test). Average extraperitoneal UCP-1 mRNA expression levels exhibited marginally significant differences among UCP-1 genotype classes. Consideration of the β3-adrenergic receptor genotype class and/or BMI as covariates did not alter the significant association of intraperitoneal UCP-1 mRNA expression with UCP-1 genotype ($P < 0.001$). Adjustment for UCP-1 genotype class did not improve the association of UCP-1 mRNA levels with the β3-adrenergic receptor genotype.

We next calculated the contribution of differences in UCP-1 genotypes to the interindividual variation in UCP-1 mRNA expression levels. In our obese subjects, 19.3% of the interindividual variation in intraperitoneal UCP-1 mRNA abundance was attributable to the UCP-1 polymorphism, while the contribution of the UCP-1 polymorphism to the interindividual variation of UCP-1 expression in extraperitoneal adipose deposits was 10.2%.

To substantiate the influence of the UCP-1 gene on interindividual variability of UCP-1 mRNA expression in our patients, we searched for sequence variations in the transcribed region of the gene that would allow allele-specific expression studies. We amplified genomic DNA from several subjects heterozygous for the Bcl I site at −3826 and analyzed sequences extending from the transcription start site into exon 2. In 4 of 12 patients studied, a G to A substitution at position +1017 downstream of the transcription start site was identified by sequencing (**Fig. 1**). The G to A transition was confirmed by sequencing of the complementary strand and predicted a substitution of alanine by threonine at amino acid residue 64 of the mature protein. In addition, the G to A substitution created a restriction site for the enzyme Aci I, thereby permitting verification of sequencing results (Fig. 1) and providing a fast method for genotyping.

The allelic frequency of the G to A substitution in exon 2 of the UCP-1 gene was 0.0804 in our sample of obese subjects. The relationship of this polymorphism with the polymorphism at −3826 of the UCP-1 gene transcription start site is displayed in **Table 3**. Standardized linkage disequilibrium statistics showed a disequilibrium coefficient of 0.92152 reflecting a highly significant, but not perfect association between the two markers.

The Aci I site created by the G to A transition in exon 2 permitted separation and quantification of mRNA transcribed from different alleles. To establish the reliability of the quantification procedure, we cloned exon 2-containing cDNA fragments into pGEM-3Zf. Plasmids

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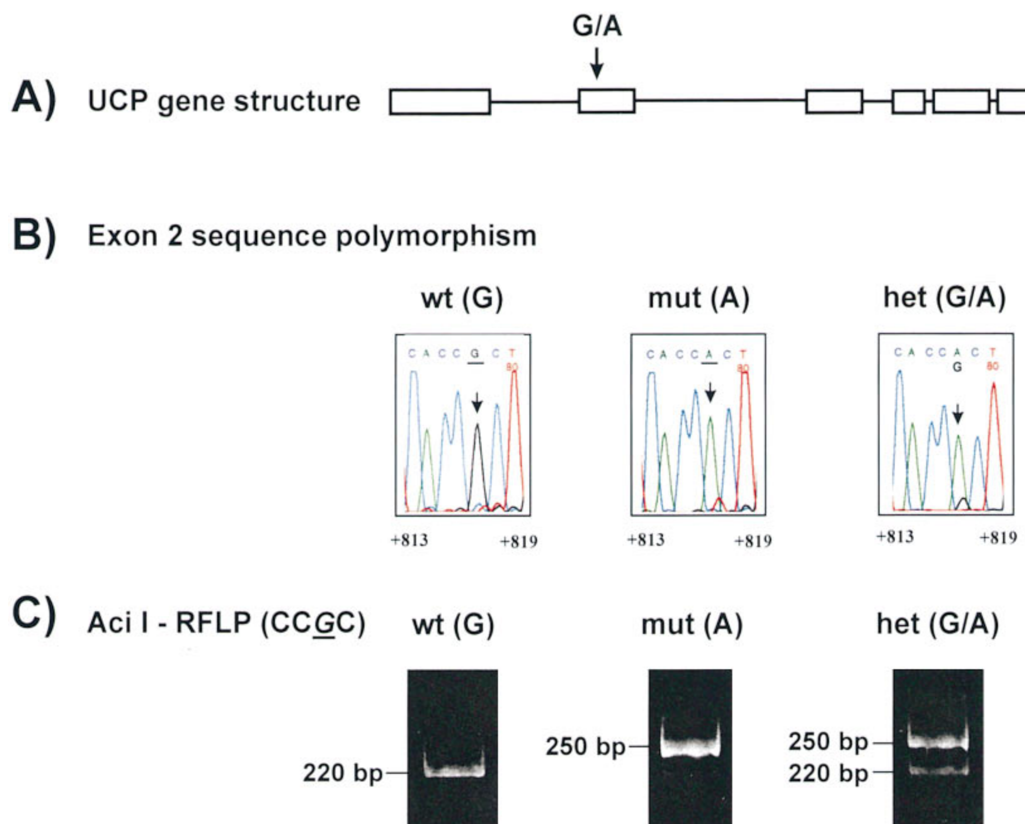


**Fig. 1.** Identification of sequence polymorphism in the transcribed region of the human UCP-1 gene. A: UCP-1 gene structure. Boxes refer to exons separated by lines representing introns. B: Profiles obtained by automated dye terminator cycle-sequencing of the coding strand of exon 2 in wild-type, mutant, and heterozygous subjects showing the substitution of G by A. C: Electrophoretic patterns of PCR products encompassing the G to A substitution in exon 2 after restriction with Aci I in the subjects shown in B.

harboring the wild-type G and the variant A were identified by sequencing, mixed at different weight ratios, and amplified by PCR in the presence of $^{32}$P-dCTP. PCR products were digested with Aci I, and subjected to denaturing polyacrylamide gel electrophoresis. After correction of signal intensities of fragments representing the wild-type and variant allele for their G/C content, a linear relationship was obtained between molar input ratios and the signal intensity ratios of the discriminatory bands (R = 0.975). This experiment also showed complete digestion of the wild-type band, while digestion of the variant product generated no additional bands. In all study subjects, the abundance of the wild-type mRNA exceeded that of the variant mRNA (**Fig. 2**, **Table 4**).

TABLE 3. Distribution and genotype frequencies identified by the Aci I and Bcl I polymorphisms in the uncoupling protein-1 gene of obese subjects

| | B1B1 | B1B2 | B2B2 |
|---|---|---|---|
| A1A1 | 69 | 46 | 6 |
| A1A2 | 1 | 19 | 1 |
| A2A2 | 0 | 0 | 1 |

A1, A2, B1, and B2 refer to the major and minor alleles at the Aci I and Bcl I gene locus; allele frequencies for the Aci I and Bcl I polymorphisms and their haplotypes are A1 (0.9196), A2 (0.0804), B1 (0.7168), B2 (0.2832), A1B1 (0.7121), A1B2 (0.2074), A2B1 (0.0047), and A2B2 (0.0757). D′ = 0.92152, $\chi^2$ = 26.89, $P$ = 0.0000.

To corroborate differences in allele-specific expression by independent methods, we used a quantitative allele-specific primer extension (**Fig. 3**). By using mixtures consisting of different molar ratios of plasmids harboring the wild-type or variant cDNA, we established that the primer extension with both wild-type and mutant-specific primers could reliably quantitate different amounts of mutant and wild-type templates. Analysis of RNA samples isolated from intraperitoneal adipose tissues of five subjects revealed an excellent agreement among the three methods (Table 4). In four subjects heterozygous for the polymorphic site at −3826 of the UCP-1 gene, the expression level of the wild-type mRNA accounted for 63.4 ± 6.0% of total



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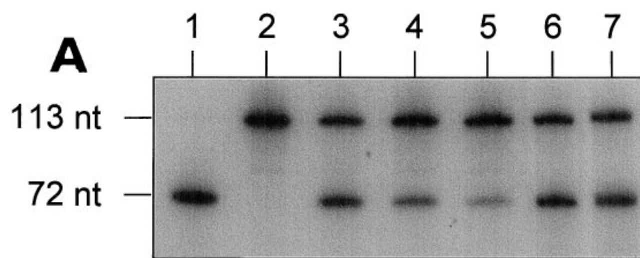


**Fig. 2.** UCP-1 allele-specific expression studies using restriction isotyping of mRNA. Autoradiogram of Aci I digested PCR products showing cloned wild-type cDNA in lane 1, cloned variant cDNA in lane 2, mixtures of cloned wild-type and variant cDNA in molar ratios of 2, 1, and 0.4 in lanes 3, 4, and 5, respectively, and Aci I-digested RT-PCR products of intraperitoneal adipose tissue total RNA from two patients in lanes 6 and 7, exhibiting a molar excess of the wild-type mRNA product.

UCP-1 mRNA abundance. One of the five patients studied carried the variant nucleotide in the distal UCP-1 promoter on both alleles (Table 4). In this patient, a comparable excess of wild-type mRNA was found with all three methods and the ratio of wild-type to variant mRNA expression level average 2.44. Hence, sequence variations at the UCP-1 gene locus other than that at −3826 relative to the transcription start site must have influence UCP-1 mRNA expression, at least in this patient.

## DISCUSSION

This study shows that common sequence variation(s) at the UCP-1 gene locus account for a major fraction of the interindividual variation in UCP-1 mRNA expression levels of our obese patients. Our conclusion is supported by *i*) a highly significant association between the mRNA expression level and the polymorphism in the distal promoter of the UCP-1 gene, and *ii*) significant differences in abundance levels of UCP-1 mRNA species transcribed from different alleles.

The newly discovered exon 2 polymorphism that allowed allele-specific expression studies occurs at amino acid residue 64 in the hydrophilic region between the transmembrane α-helices I and II of UCP-1 (11). Major functional consequences are therefore not expected from this substitution. Interestingly, alignment of the UCP-1 sequence with published sequences of UCP-2 and UCP-3 showed a conserved threonine at the respective position of UCP-2 and UCP-3. Our studies on the allelic frequencies in obese subjects (Table 3) and a population of lean subjects (data not shown) indicates that threonine 64 is encoded by the rare allele of the UCP-1 gene.

Despite a large body of information in rodents, little is known about the contribution of uncoupled mitochondrial respiration in brown fat to energy expenditure in human subjects. Our recent study showed that intraperitoneal UCP-1 mRNA expression levels in morbidly obese individuals were significantly lower than in lean controls (23). While these results are consistent with a causal role of UCP-1 in the pathogenesis of obesity, such an association may reflect a secondary phenomenon and could result from an expansion of white adipocyte number and/or mass at the expense of brown adipocytes. Consequently, UCP-1 mRNA expression levels in total adipose tissue would be reduced. The current study shows, however, that the UCP-1 expression level within brown adipocytes is a major determinant of total tissue UCP-1 mRNA abundance, as sequence variations in the UCP-1 gene explain 19.3% of the variance in its expression. Thus, the UCP-1 gene locus has a major impact on mRNA expression levels, at least in obese subject-populations. Further insight in the role of UCP-1 in human obesity may be gained from studies on the relationship of the polymorphism with UCP-1 mRNA abundance in lean subjects.

The allelic frequency of the UCP-1 polymorphism at −3826 in our population was very similar to the frequencies reported in other obese populations (20, 21, 25, 30). Previous studies found no difference in allelic

TABLE 4. Allele-specific expression of UCP-1 mRNA in obese subjects

| Subjects | BclI UCP-1 Genotype[a] | UCP-1 mRNA Abundance | Aci I Wild-type mRNA Abundance | | | |
|---|---|---|---|---|---|---|
| | | | Method 1 | Method 2 | Method 3 | Mean ± SD |
| | | *amol/fmol β-actin mRNA* | *% of total UCP-1 mRNA* | | | |
| 1 | B1B2 | 1.833 | 70 | 73 | 68 | 70.3 ± 2.5 |
| 2 | B1B2 | 2.750 | 62 | 59 | 68 | 63.0 ± 4.6 |
| 3 | B1B2 | 1.500 | 57 | 54 | 56 | 55.7 ± 1.5 |
| 4 | B1B2 | 1.667 | 64 | 63 | 67 | 64.7 ± 2.1 |
| 5 | B2B2 | 1.110 | 71 | 72 | 70 | 71.0 ± 1.0 |

Methods 1, 2, and 3 refer to methods of restriction digestion of RT-PCR products, primer extension for wild-type mRNA, and primer extension for mutant mRNA, respectively. Results of each method are the means of duplicate determinations.

[a]B1 and B2 refer to the wild-type and variant allele, respectively.



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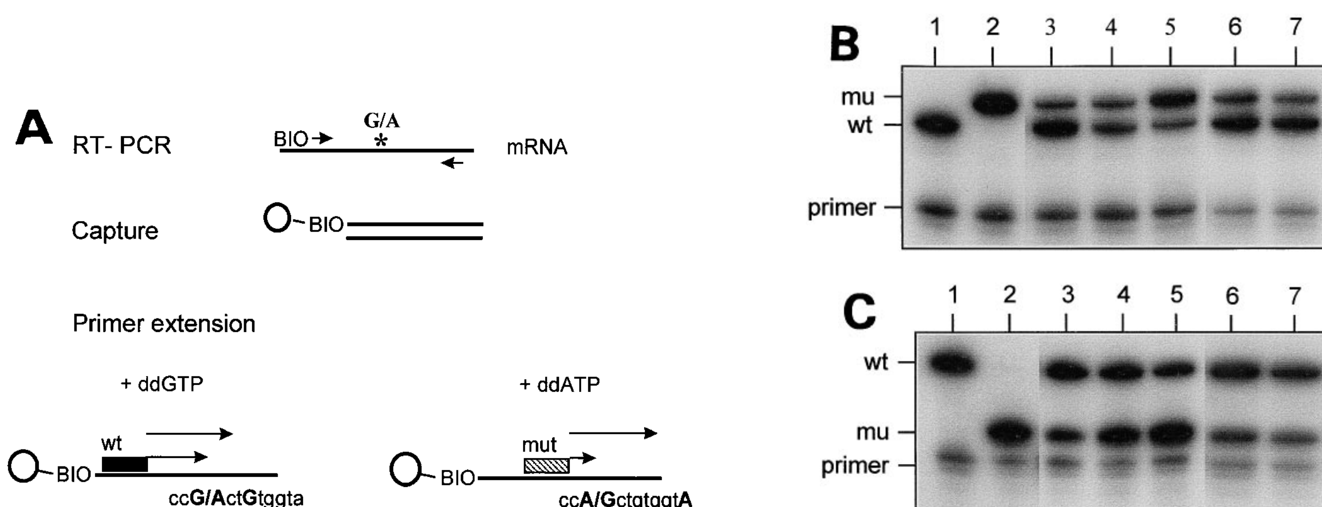


**Fig. 3.** UCP-1 allele-specific expression studies using wild-type- and mutant-specific primer extension. A: Experimental strategy showing use of a biotinylated sense primer for RT-PCR (top), capture of the amplified UCP-1 sense strand via streptavidin beads (middle), and primer extension using wild-type-specific primer and ddGTP (bottom left) or mutant-specific primer and ddATP (bottom right); arrows represent the expected extension products of the wild-type and mutant templates which are terminated by G (wild-type-specific extension) or A (mutant-specific extension). B: Autoradiogram of primer extension products using the wild-type-specific primer. Lanes 1–7 show primer extension products of cloned wild-type cDNA, cloned mutant cDNA, mixtures of wild-type and mutant cDNA in molar ratios 2, 1, and 0.4, respectively, adipose tissue total RNA from the two patients shown in Fig. 2. C: autoradiogram of primer extension proucts using the mutant-specific primer. Lanes 1–7 show extension products of templates described in B.

frequencies between obese and normal populations (20, 21, 30). Nevertheless, the minor allele was associated with obesity indices such as higher weight gain during adult life or reduced weight loss on a low calorie diet. Thus, the association of the variant allele with altered energy homeostasis may depend on its interaction with other obesity-causing factors. Alternatively, the sequence variation at −3826 may be associated with a number of distinct haplotypes exhibiting different frequencies in obese and lean subjects. Our study shows that the −3826 polymorphism is probably only a marker for expressional differences, but not the causative mutation, as allele-specific differences in mRNA abundance levels were observed in one subject homozygous for the variant allele. The putative causative sequence variation(s) may affect the binding of *trans*-acting factors regulating UCP-1 gene expression. The proportion of the variance that results from variation at the UCP-1 gene locus itself may differ from our estimate and identification of the causative UCP-1 gene mutation(s) will be essential to quantify the actual contribution of gene structure to its expression level and to evaluate its possible role in obesity.

UCP-1 mRNA abundance was lower and the association of the UCP-1 promoter polymorphism was weaker in extraperitoneal adipose deposits than in intraperitoneal tissues. These differences may reflect tissue-specific differences in the number, mitochondrial content or UCP-1 gene expression of brown adipocytes. Previous studies in humans have demonstrated that UCP-1 mRNA expression levels correlate with those of the β3-adrenergic receptor (31). Signal transduction via this receptor is thought to play an important role in catecholamine-induced lipolysis and in the regulation of energy homeostasis in rodents. In addition, an interaction of the UCP-1 polymorphism with the Trp64Arg mutation in the β3-adrenergic receptor on weight gain has been reported in obese humans (20). Hence, β3-adrenergic receptor activity, which is reportedly much lower in the subcutaneous than in the intraabdominal adipose tissue (31), could have contributed to the difference in the UCP-1 expression level between intra- and extraperitoneal adipose tissue and to the strength of its association with the Bcl I polymorphism.

Our study failed to show a significant effect of the Trp64Arg mutation on UCP-1 mRNA expression levels even though mRNA concentrations tended to be lower in subjects carrying one mutant allele. Because the Trp64Arg variant showed associations with various indices of obesity and/or insulin sensitivity in many (17–19, 32) but not all (33) of the populations studied, this variant is probably a susceptibility gene for obesity and may contribute to decreased energy expenditure. Indeed, Japanese women with the Trp64Arg variant had lower resting metabolic rates and exhibited a greater resistance to weight-loss treatment than women with



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the wild-type allele (34). The absence of a statistically significant association between β3-adrenoreceptor genotype and UCP-1 mRNA abundance, even in our female study subjects, does not necessarily exclude an effect of the Trp64Arg variant on UCP-1 gene expression, but may reflect differences in study populations and/or study design. Possible effects of the Trp64Arg variant on metabolic variables and UCP-1 mRNA abundance may have been masked by confounding variables including the genotype effects implicated by our study. Moreover, the number of study subjects may have been too small to detect possible interactions between the β3-adrenergic receptor genotype and the −3826 UCP gene polymorphisms on UCP-1 mRNA expression levels. As the Bcl I polymorphism in the UCP-1 gene seems to be only a marker for UCP-1 mRNA expression levels, statistical adjustment for this polymorphism to compensate for the genotypic effects may have been inadequate. Hence, the conclusion that the Trp64Arg variant does not influence UCP-1 mRNA expression would be premature and a larger study sample or a better knowledge of other factors that regulate UCP-1 mRNA expression levels will be necessary to fully characterize the role of the Trp64Arg variant on UCP-1 gene expression. Moreover, the Trp64Arg variant could influence translational or posttranslational events in UCP-1 gene expression not measured in our study.

The borderline significant association of the UCP-1 genotype with plasma leptin levels (Table 1) is reminiscent of the increased levels of leptin in transgenic mice (35, 36) in whom the diphtheria toxin-A chain was placed under the control of the UCP-1 gene promoter to ablate BAT (37). These transgenic animals exhibited reduced brown fat and marked obesity. Administration of exogenous leptin to these mice had no effect on food intake or body weight implying resistance to the anti-obesity action of leptin (38). Targeted inactivation of the UCP-1 gene in transgenic mice resulted in defective thermoregulation, but not in obesity or hyperphagia (39). Interestingly, UCP-2, a widely expressed protein that shares functional properties and structural similarities with UCP-1 (9, 10), was selectively upregulated in BAT of UCP-1-deficient mice. Thus, loss of UCP-1 activity may have been compensated for by UCP-2. Additional evidence for an important role of UCP-2 in energy metabolism comes from studies in rat pancreatic islets and white adipocyte preparations linking leptin's thermogenic action with increased UCP-2 gene expression (40). These studies support a model in which UCP-2 can function as a backup mechanism for reduced UCP-1 expression in brown adipose tissue with leptin being a critical signalling component. In humans, very little is known about the importance of UCP-2 and UCP-3, another recently discovered member of the UCP gene family (11, 12), and possible relationships among expression levels of the UCP family members remain to be determined in obese and/or lean subjects.

In summary, this study identifies the UCP-1 gene locus itself as a common cause of reduced UCP-1 gene activity in obese subjects. Clearly, the functional mutations must be identified in order to compare their frequencies in lean and obese subjects and to determine their pathophysiological consequences. Such knowledge might be essential for obesity treatment aimed to correct or enhance energy expenditure via induction of UCP-1.

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