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Organisation of the coding exons and mutational screening of the uncoupling protein 3 gene in subjects with juvenile-onset obesity

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Summary Uncoupling proteins (UCPs) are mitochondrial transporters that uncouple the cellular respiration releasing stored energy as heat. Recently a third member of the UCP family was identified. Human UCP3 is different from UCP1 and UCP2 by its high and preferential expression in skeletal muscle and consequently the UCP3 gene is an attractive candidate gene for obesity. In this study we have determined the intron/exon organization of the coding region of the UCP3 gene and performed single strand conformation polymorphism (SSCP) analysis and direct sequencing of variants of the gene in 60 Caucasian subjects with juvenile-onset obesity. We detected 4 nucleotide substitutions in the intron regions and 2 silent amino acid variants. During the identification of the intron/exon structure of the gene in a normal healthy male subject with a BMI of 23.5 kg/m^2, a nucleotide substitution replacing a glycine with a serine was identified at codon 84. This variant was neither found among 156 subjects with juvenile-onset obesity nor among 205 control subjects. In a population based sample of 380 young healthy subjects the Gly/Ser84 variant was found in one female subject with a BMI of 25.5 kg/m^2 and a fat mass of 23.7 kg. We conclude it is unlikely that variants in the coding region of the UCP3 gene contribute to the pathogenesis of juvenile-onset obesity among Danish Caucasians. [Diabetologia (1998) 41: 241–244]

Keywords uncoupling protein 3, obesity, genetics, mutation.

Obesity is a multifactorial disorder with a strong genetic component. Studies in twins [1] and in adoptees [2] indicate that most familial aggregation of obesity is attributable to genetic influence rather than to shared family environment. The search for obesity candidate genes has focused on those that have a role in energy metabolism. Uncoupling proteins are mitochondrial transporters that dissipate the electrochemical gradient generated in the electron transfer chain within the mitochondria and thereby uncouple the cellular respiration leading to heat production instead of energy storage. Whereas UCP1 is expressed uniquely in brown adipose tissue and thereby may be of less importance in adult humans, UCP2 has a wide tissue distribution and may play a role in energy balance. Mutational analysis of the coding region of the UCP2 gene has, however, not revealed any genetic variability contributing to the development of obesity in Danish Caucasians [3]. Very recently a third member of the UCP family was reported [4, 5]. At the amino acid level this protein is 57 % identical to UCP1 and 71 % identical to UCP2. It is different from UCP1 and UCP2 by its high and preferential expression in skeletal muscle in humans. Since skeletal muscle is a major contributer to whole body thermogenesis in adult humans, UCP3 may be an important mediator of nonshivering thermogenesis and the UCP3 gene is thus a logical candidate gene for obesity. The objectives of the present study were 1) to de-

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Abbreviations: UCP1–3, Uncoupling protein 1–3; SSCP, single strand conformation polymorphism.

Table 1. Nucleotide sequences of DNA primers used for PCR amplification of the UCP3 gene

intron	sense primer (5' → 3')	antisense primer (5' → 3')	$T_{annealing}/C_{MgCl2}$
1	agg agg ggc cat cca atc	gga ggc acg tct gaa gg	60 °C/2,0 mmol/l
2	aaa ggg act ggg cag agc	ccc att gta ggg gct gc	60 °C/1,5 mmol/l
3	tac cgt ggc gtg ctg gg	gta tgc tgg cct gaa atc	65 °C/1,5 mmol/l
4	gtg acc tgt gcc cag cc	ctc ctt gag gat gtc gta gg	65 °C/1,5 mmol/l
5	tga gga atg cta tcg tca ac	gcc tgg agg tga gtt c	60 °C/1,5 mmol/l
6	ctt ctg tgc cac agt ggt g	act ggt ttc gga cac gtt ag	60 °C/1,0 mmol/l
exon			
2[a]	tgt aaa acg acg gcc agt agc cag gcc aga cat cac	cag gaa aca gct atg acc cag tgg aaa ggt aac gag g	60 °C/1,5 mmol/l
2[b]	tgt aaa acg acg gcc agt aaa ggg act ggg cag agc	cag gaa aca gct atg acc agc ccc tcc ttc cat gtg	60 °C/1,5 mmol/l
3	tgt aaa acg acg gcc agt tgt gca cgc agc ccc ttc	cag gaa aca gct atg acc cta gac ttc cct ggt ctc ttg ac	55 °C/2,0 mmol/l
4	tgt aaa acg acg gcc agt gca gcc ccg cag aga ac	cag gaa aca gct atg acc acg cca tgc tgg gag tcc	55 °C/2,5 mmol/l
5	tgt aaa acg acg gcc agt cca ttt ctc cca ttt ccc	cag gaa aca gct atg acc gcc cac tcc acg gag ttc	55 °C/2,0 mmol/l
6	tgt aaa acg acg gcc agt gag ttg ggg aca aac agt gc	cag gaa aca gct atg acc acg gta gcc aca ttc gaa ag	60 °C/2,0 mmol/l
7	tgt aaa acg acg gcc agt ggg aga gca cac gca tc	cag gaa aca gct atg acc act ggt ttc gga cac gtt ag	60 °C/2,0 mmol/l

$T_{annealing}$ and C_{MgCl2} denote the annealing temperature and the $MgCl_2$ concentration, respectively, used for PCR amplification. [a] Non-translated region, [b] Translated region. Exons and introns are numbered with respect to the non-translated exon upstream of the first coding exon

termine the intron/exon organization of the coding region of the UCP3 gene, 2) to analyse for variations in the coding region of the gene in subjects with juvenile-onset obesity and 3) to evaluate whether identified variants were associated with juvenile-onset obesity in Caucasian subjects or related to estimates of obesity and impaired insulin sensitivity in a cohort of young healthy Danes.

Subjects and methods

Cohort used in the primary mutational scanning. The primary mutational analysis and the subsequent association studies of variants of the UCP3 gene were performed in study groups selected from a population of young Caucasian men of Danish ancestry, who at the age of 18–26 years were examined at the draft board, and who, in addition, had attended school in the municipality of Copenhagen, where height and weight had been measured as part of the school health examinations [6]. The population was further restricted to those who were examined at the Copenhagen City Heart Study Program in 1981–1983 [7] and again in 1992–1994. The cohort of men with juvenile onset obesity included 156 subjects who had a BMI of 31.0 kg/m^2 or more at the draft board examination. From this obese cohort 60 subjects were randomly selected for the initial mutational screening.

Cohort used in the case-control study. As a control cohort 250 draftees were selected at random as every hundredth from the same population [6]. Weight and height at different ages were recorded.

Genomic DNA was obtained from blood samples, drawn at the last examination at the Copenhagen City Heart Study.

Cohort used in the genotype-phenotype interaction study. A cohort of 380 subjects randomly recruited from a population of young healthy individuals aged 18–32 years, who in 1979–1980 and again in 1984–1985 as children had participated in an epidemiological blood pressure survey [8] in a specified part of Copenhagen were genotyped for the Gly/Ser84 mutation of UCP3. All were Danish Caucasians by self-identification. BMI, waist-to-hip ratio and and fat mass, were analysed as previously described [8]. The insulin sensitivity index was estimated from an intravenous glucose tolerance test in combination with injection of intravenous tolbutamide as previously described [8]. Physiological and anthropometric characteristics of this population sample have been reported [8].

Prior to participation informed consent was obtained from all subjects. The study was approved by the ethical committee of Copenhagen and was in accordance with the principles of the Declaration of Helsinki II.

Identification of the intron/exon organisation of the coding part of the UCP3 gene. Genomic DNA was obtained from human leukocyte nuclei using standard methods. In order to amplify the putative introns of UCP3 primer pairs (Table 1) were designed from the partial UCP3 cDNA sequence (Genbank accession number: AF001787) according to the intron/exon organisation of UCP1. Introns were PCR amplified using 300 ng of genomic DNA, 2 units of TaqPlus (Stratagene, La Jolla, Calif., USA), 0.2 μmol/l of each primer and $MgCl_2$ as described (Table 1). PCR conditions were denaturation at 94 °C for 3 min followed by 35 cycles of denaturation for 30 s, annealing at T-anneal (Table 1) for 30 s and extension at 72 °C for 4 min, with a final extension at 72 °C for 9 min. Cycles were performed on a GeneAmp 9600 Thermocycler (Perkin-Elmer/Cetus, Norwalk, Conn., USA). The total PCR product was subsequently run on a 1 % agarose gel and putative intron bands were excised and purified using Microcon 50 spin columns with agarose filters inserted. Direct sequencing was performed using ABI PRISM dRhodamine Dye Terminator Cycle Sequencing Kit and ABI PRISM 377 automatic DNA sequencer (Perkin Elmer, Foster City, Calif., USA).

Identification of mutations in the UCP3 gene. SSCP analysis was performed on the entire coding region, including intron-exon boundaries and the non-translated region of the first coding exon of the UCP3 gene. PCR amplification of the 6 exons (7 segments) was carried out in a volume of 25 μl, containing 100 ng of genomic DNA prepared from peripheral blood leukocytes, 0.2 μmol/l of each primer, 0.313 units of Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, Conn., USA), $MgCl_2$ as stated (Table 1) and including 0.125 μl of a 37-MBq/ml solution (α-^{32}P)dCTP (Amersham, Buckinghamshire, UK). Primers (Table 1) were designed from the partial intron sequencing described above. The reverse primer used for am-

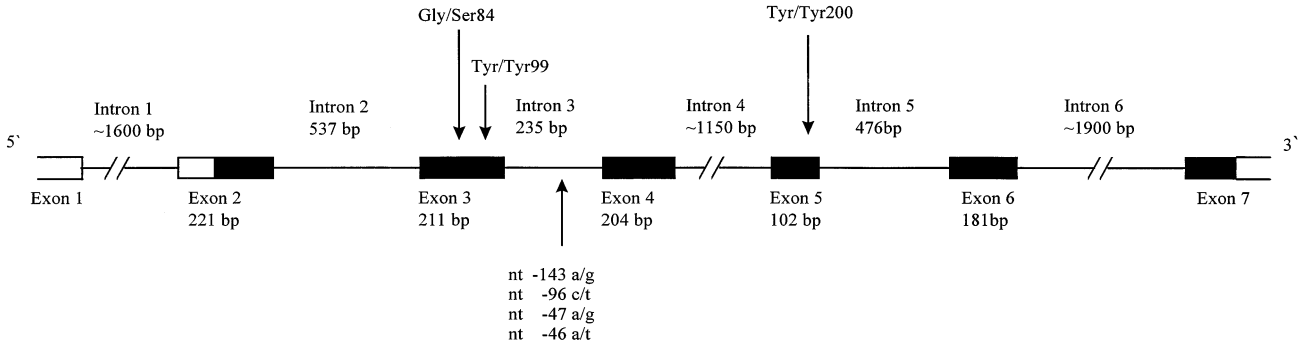

Fig. 1. Intron-exon structure of the coding region of the human UCP3 gene. Lines and boxes indicate introns and exons, respectively. Exons and introns are numbered with respect to the exon upstream of the first coding exon. The coding regions are indicated by filled boxes whereas the non-translated sequences are depicted by open boxes. The sizes of the segments are indicated. The nucleotide positions of intron variants are indicated with respect to the splice acceptor site. The codon number of the mutations identified in exons are shown

plification of the last coding exon was designed from the 3'UTR sequence. PCR conditions were denaturation at 94 °C for 3 min followed by 35 cycles of denaturation for 30 s, annealing at T-anneal (Table 1) for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 9 min. PCR products were mixed with loading buffer, denatured and allowed to partially re-anneal in order to generate heteroduplices and were finally analysed by non-denaturing gel electrophoresis at two different conditions as previously described [9]. In our laboratory these conditions provide a sensitivity of the SSCP of more than 90 % which is consistent with estimates from other groups [10]. Segments showing variation in migration were re-amplified by PCR, purified using Microcon 100 microconcentrators and sequenced directly using ABI PRISM Dye Primer Cycle Sequencing Kit with Amplitaq DNA Polymerase FS and ABI prism 377 (Perkin Elmer, Foster City, Calif., USA). All nucleotide variants were identified on both strands.

Screening for the Gly/Ser84 amino acid mutation in the UCP3 gene. The DNA segment containing codon 84 was amplified as described above, including primers used for amplification of the second coding exon (Table 1). Restriction fragment length polymorphisms were detected after digestion overnight with 2 units of MspI. Fragments were resolved on a 3 % agarose gel and visualised by staining with ethidium bromide.

Results

Analysis of the human UCP3 gene demonstrated that the gene is comprised of at least 7 exons of which 6 exons are coding (Fig. 1). Exons are numbered with respect to the exon upstream of the first coding exon. All coding exons, the 3' region of exon 1, which is non-translated and the 5' region of exon 7, including some of the non-translated region and the entire intron 2, 3 and 5 were sequenced. In addition intron 1, 4 and 6 were sequenced approximately 100 bp from the splice sites. The sequences surrounding splice junctions were in accordance with consensus sequences for RNA splicing. The sequence of the exons and adjacent introns have been deposited in the Genbank database (accession numbers: AFO26955-58).

SSCP-heteroduplex scanning and sequencing of migration variants of the UCP3 gene in 60 subjects with juvenile-onset obesity showed 6 nucleotide substitutions, 2 of which were located in exons and 4 in introns. In the third intron 4 variants were detected: 46 bp upstream of exon 4 an a → t substitution was identified, 47 bp and 143 bp upstream, respectively, an a → g substitution was identified and 96 bp upstream a c → t substitution was found. In exon 3 at codon 99 and in exon 5 at codon 200 two silent variant were identified, Tyr(tat) → Tyr(tac) and Tyr(tac) → Tyr(tat), respectively. At codon 84 a g → a substitution resulting in a Gly (ggc) → Ser(agc) replacement was detected during the determination of the intron/exon structure of the gene in a normal, healthy male subject, with a BMI of 23.5 kg/m^2 and a waist-to-hip ratio of 0.93. This amino acid variant was neither found among 156 subjects with juvenile-onset obesity nor among 205 control subjects. In the cohort of 380 young healthy subjects the Gly/Ser84 variant was identified in one female subject with a BMI of 25.5 kg/m^2, a fat mass of 23.7 kg, a waist-to-hip ratio of 0.82 and an insulin sensitivity index of 15.2×10^{-5} (minx pmol/l)$^{-1}$. Values in the total cohort were (mean (SD)): 23.6 (3.7) kg/m^2, 17.1 (7.8) kg, 0.82 (0.07) and 15.2×10^{-5} (9.3×10^{-5}) (minx pmol/l)$^{-1}$, respectively.

Discussion

The recently cloned human UCP3 gene is an obvious candidate for a mediator of regulated thermogenesis in skeletal muscle. With the purpose of performing mutational analysis on genomic DNA we have in the present study delineated the intron/exon structure of the coding region of the gene. However, since the UCP3 mRNA transcript is predicted to be equal to or greater than 2.2 kb [5] and since the transcriptional start of the gene has not yet been reported, the UCP3 gene may comprise more than the identified 7 exons.

The mutational analysis of the UCP3 gene demonstrated several nucleotide variants. None of the identified intron nucleotide substitutions or the two silent exon variants are located in the vicinity of splice junctions. It is therefore unlikely that the variants affect the splicing of the exons. We detected one exon variant changing an amino acid. This Gly/Ser84 replacement is a conservative amino acid change and when extrapolated from the structure of UCP1, which at the amino acid level is 57 % homologous to UCP3, the Gly/Ser84 substitution is not predicted to be located within any functional domains of the gene. Moreover, as judging from the BMI, fat mass and waist-to-hip ratio of the two carriers of the variant our results indicate that this amino acid substitution in its heterozygous form has no major impact on the pathogenesis of obesity. Yet, it should be noted that the glycine at codon 84 is conserved in human UCP1, 2 and 3, respectively and the residue is located within one of the putative transmembrane regions of the gene. Structure-function studies at the cellular level are therefore needed to exclude a potential minor biological influence of the gene variant.

Despite a high sensitivity of the SSCP screening method used in this study, we failed to detect any frequent amino acid variants with potential impact on the function of the UCP3 protein. However, this does not exclude a role for the UCP3 gene in contributing to obesity in subsets of patients with specific forms of obesity. Furthermore, localisation and mutational screening of the regulatory regions and studies of the expression of the UCP3 gene in obese subjects are necessary in order to exclude the UCP3 gene as a risk factor for altered energy metabolism. In summary, genetic variation in the coding region of the human UCP3 gene is not involved in the pathogenesis of juvenile-onset obesity in Danish Caucasian subjects.

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