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STRUCTURAL ORGANIZATION AND MUTATIONAL ANALYSIS OF THE HUMAN UNCOUPLING PROTEIN-2 (hUCP2) GENE

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ABSTRACT Uncoupling proteins (UCPs) are mitochondrial membrane transporters which are involved in dissipating the proton electrochemical gradient thereby releasing stored energy as heat. This implies a major role of UCPs in energy metabolism and thermogenesis which when deregulated are key risk factors for the development of obesity and other eating disorders. From the three different human UCPs identified so far by gene cloning both UCP2 and UCP3 were mapped in close proximity (75-150 kb) to regions of human chromosome 11 (11q13) that have been linked to obesity and hyperinsulinaemia. At the amino acid level hUCP2 has about 55% identity to hUCP1 while hUCP3 is 71% identical to hUCP2. In this study we have deduced the genomic structure of the human UCP2 gene by PCR and direct sequence analysis. The hUCP2 gene spans over 8.7 kb distributed on 8 exons. The localization of the exon/intron boundaries within the coding region matches precisely that of the hUCP1 gene and is almost conserved in the recently discovered hUCP3 gene as well. The high degree of homology at the nucleotide level and the conservation of the exon /intron boundaries among the three UCP genes suggests that they may have evolved from a common ancestor or are the result from gene duplication events. Mutational analysis of the hUCP2 gene in a cohort of 172 children (aged 7 - 13) of Caucasian origin revealed a polymorphism in exon 4 (C to T transition at position 164 of the cDNA resulting in the substitution of an alanine by a valine at codon 55) and an insertion polymorphism in exon 8. The insertion polymorphism consists of a 45 bp repeat located 150 bp downstream of the stop codon in the 3'-UTR. The allele frequencies were 0.63 and 0.37 for the alanine and valine encoded alleles, respectively, and 0.71 versus 0.29 for the insertion polymorphism. The allele frequencies of both polymorphisms were not significantly elevated in a subgroup of 25 children characterized by low Resting Metabolic Rates (RMR). So far a direct correlation of the observed genotype with (RMR) and Body Mass Index (BMI) was not evident. Expression studies of the wild type and mutant forms of UCP2 should clarify the functional consequences these polymorphisms may have on energy metabolism and body weight regulation. © 1998 Elsevier Science Inc.

Key Words: human uncoupling protein-2, genomic organization, mutational analysis, polymorphism, obesity, energy metabolism, body weight regulation

Introduction

The uncoupling protein (UCP) is a member of the family of mitochondrial anion carriers. UCP creates a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondrial membrane in brown adipose tissue, without coupling to any other energy consuming process. This implies a major role of UCPs in energy metabolism and thermogenesis, which when deregulated are key risk factors in the development of obesity and other eating disorders (1). Recent data have shown that the sympathetic nervous system, via norepinephrine (ß-adrenoceptors) and cAMP, as well as thyroid hormones and PPAR gamma ligands seem to be major regulators of human UCPs expression. UCP1-containing brown adipose tissue, however, is unlikely to be involved in weight regulation in human and adult large-size animals living in a thermoneutral environment because there is little brown adipose tissue present in adults. Fleury *et al.* (1997) discovered a new homologue, UCP2, that is expressed at high levels in skeletal muscle, lung, heart, placenta, kidney, spleen, thymus, leukocytes, macrophage, bone marrow and stomach and at low levels in liver or brain. hUCP2 expression is upregulated in white fat in response to fat feeding (2). In comparison to hUCP1, hUCP2 was found to have a greater effect on the mitochondrial membrane potential when expressed in yeast. It was found to have properties consistent with a role in diabetes and obesity. At the amino acid level human and mouse UCP2 are 95% identical, while human and mouse UCP1 are only 79% identical, suggesting that UCP2 may be critically important to the fitness of an organism. Very recently the molecular cloning of a third analogue, hUCP3, was reported that exists in a long and short form (3, 4). At the amino acid level hUCP2 has about 55% identity to hUCP1 while hUCP3 is 71% identical to hUCP2. hUCP2 has been mapped to human chromosome 11q13, the mouse homologue of UCP2 to chromosome 7, tightly linked to the '*tubby*' mutation, an area of homology of synteny to 11q13. The chromosomal locus of hUCP2 is in proximity (15 cM) of a locus (11q21 - q22) recently uncovered through a genomewide search and found to be linked to percent body fat in Pima Indians (5). Evidence of a potential role of UCP2 in energy metabolism comes from animal models (2) and was further substantiated by linkage studies using three microsatellite markers flanking the hUCP2 gene locus (6). It was found that the three markers encompassing the hUCP2 locus and spanning a 5 cM region on 11q13 are linked to resting energy expenditure in adult human. Interestingly, UCP2 and UCP3 in both mice and human are located within 75-150 kb of each other (7) suggesting that the two genes may have evolved from gene duplication events.

Because of a possible functional role of hUCP2 in energy metabolism and body weight regulation, the identification and characterization of genetic variants should be of major importance for obesity research. We therefore have initiated a mutational analysis of the hUCP2 gene, which was carried out for all coding exons in a cohort of children of Caucasian origin characterized by low RMR (with deviations up to -25% according to the Harris & Benedict equation (8)). Reduced RMR values have been shown to be a predictive factor for the development of human obesity (Laessle *et al.* personal communication). As a first step we have elucidated the exon/intron structure of the hUCP2 gene (coding and flanking region) by PCR and direct sequence analysis. Mapping of the promotor region and the identification of putative promotor regulatory sequences should give insight into the transcriptional regulation of hUCP2 expression *in vitro* and *in vivo*. Of particular interest will be if hUCP2 expression is regulated by similar mechanisms as hUCP1 (e.g. ß-adrenoceptors, cAMP, and thyroid hormones).

Materials and Methods

Determination of the genomic organization of the human UCP2 gene

Exon / Intron Mapping of the hUCP2 *Gene*

The location of introns within the human UCP2 gene was determined using PCR and sequence analysis. Based on the genomic organization of the hUCP1 gene and the published hUCP2 cDNA sequence, 8 sets of primers were designed to produce 8 overlapping fragments encompassing the entire coding region including the 5'-UTR and the 3'-UTR. The PCR reaction mix of 50 µl contained: 100 ng of DNA, 25 pmol of each primer, 200µM each of dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.001% gelatin and 1 unit *Taq* DNA polymerase (MWG, Germany) (9). Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 95°C/30 sec, 55°C/30 sec, 72°C/1 min for 35 cycles, followed by a final extension step of 7 min at 72°C. For long distance PCR (determination of the size of intervening introns), we used the *Expand™ Long Template PCR System* (Boehringer Mannheim) as recommended by the manufacturer. Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 2 min denaturation at 94°C, 35 temperature cycles were carried out consisting of 94°C for 10 sec, 65°C for 30 sec, 68°C for 3 min (cycles 1 - 10) with elongation times increasing 20 sec/cycle (cycles 11 - 35), followed by a final extension step of 7 min at 68°C. The amplification products were visualized on 1.0 % agarose gels stained with ethidium bromide.

DNA Cloning and Sequence Analysis

Automated sequencing of PCR products was performed to determine the sequences at the exon/intron boundaries as well as the sequence of the entire introns within the coding region and in the 5'-flanking region. PCR products derived from human genomic DNA utilizing the primer pairs described above were isolated by agarose gel electrophoresis, the fragments were polished with T4 DNA polymerase and cloned into the *EcoRV* site of the plasmid Bluescript pBSII SK^+ with the *E.coli* host strain XL1-Blue, using M13 forward (-21) and reverse (-29) primers and/or SK/KS-primers for sequence analysis. Alternatively, the *TOPO TA Cloning Kit* (Invitrogen Inc, USA) was used; sequence analysis was carried out with either M13 forward (-21) or reverse (-29) primers. Sequencing reactions were performed with fluorescently labelled dideoxyterminators using the *PRISM Ready Reaction Dye Terminator Kit* (PE Applied Biosystems, Weiterstadt, Germany) with minor modifications to the supplied manual. Cycle sequencing was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, Weiterstadt, Germany). After an initial 3 min denaturation step at 96°C, 30 temperature cycles were carried out consisting of 10 sec at 96°C, 5 sec at 55°C and 4 min at 60°C. Cycle sequencing reactions were purified by CentriSep columns and run on an ABI 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany). The *DNASTAR* computer program (DNASTAR Inc., Madison, WI, USA) was used for sequence analysis and DNA/Protein homology searches.

Polymorphism analysis

Recruitment of index probands and controls

For a total of 68 obese and 104 non-obese children in the age of 7-13 years ascertained in Trier and Kaufbeuren by school physicians, pediatricians and via advertisements in local newspapers, Resting Metabolic Rates (RMR) were determined by indirect calorimetry using the DELTATRAC MBM-100 ventilated hood system (Hoyer, Bremen, Germany). Probands were

grouped according to percent deviation (up to 25% above or below) of their estimated RMR values calculated on the basis of the Harris & Benedict equation (8). Classification to the phenotype obese/non-obese was performed based on the criteria of Must *et al.* (10) with BMI centiles ranging from 85-100 for the obese and from 5-85 for the normal weight children. Written informed consent was obtained from all participants (in all cases their parents). The investigation was approved by the ethics committee of the University of Trier.

Genomic DNA Isolation

EDTA anticoagulated venous blood samples were collected from index probands and controls. Leukocyte DNA was isolated by the salting out procedure according to Miller *et al.* (11) or with a commercially available kit of GENTRA Systems Inc., Minneapolis, USA.

Polymorphism detection

Index probands (25) with significantly reduced RMR were selected for a first polymorphism analysis of the hUCP2 gene. PCR amplification of each individual exon (coding region including 3'-UTR) was performed from genomic DNA. PCR products were subsequently analyzed for possible polymorphisms by direct sequence analysis using automated DNA sequencing as described above. In case of the insertion polymorphism PCR products from exon 8 were first subcloned using the *TOPO TA Cloning Kit* (Invitrogen Inc., USA). For sequence analysis M13 forward (-21) and reverse (-29) primers were used.

Genotyping

a) Base transition in exon 4 (Ala55Val):

Genotyping for the distribution of the Ala55Val polymorphism in the hUCP2 gene was performed by allele-specific PCR (ASP) and/or direct sequence analysis. Allele-specific PCR was carried out in a volume containing 100 ng DNA, 10 pmol of each primer. Samples were processed in a GeneAmp PCR 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 30 sec at 95°C, 30 sec at 61.5°C and 30 sec at 72°C for 35 cycles, followed by a final extension step of 7 min at 72°C. After PCR, aliquots were run on 1.5% agarose gels to determine the respective genotype.

b) Insertion polymorphism (3'-UTR):

PCR reaction mix of 50 µl contained: 100 ng of DNA, 25 pmol of each primer (#1203 / #1204), 200µM each of dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.001% gelatin and 1 unit *Taq* DNA polymerase (MWG, Germany) (9). Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 95°C/30 sec, 55°C /30 sec, 72°C/1 min for 35 cycles, followed by a final extension step of 7 min at 72°C. The PCR amplification products were visualized on 2.0 % agarose gels stained with ethidium bromide. Genotyping was performed according to the size of the resulting PCR product (301 bp versus 256 bp for the insertion polymorphism being present or absent).

Results

Genomic organization of the human UCP2 Gene

Sequence analysis of the resulting PCR fragments confirmed the presence of introns and defined the exact location of the exon/intron boundaries (**Fig. 1**). The hUCP2 gene (coding region plus 5'-/3'- flanking regions) spans over 8.7 kb, distributed on 8 exons and 7 introns.

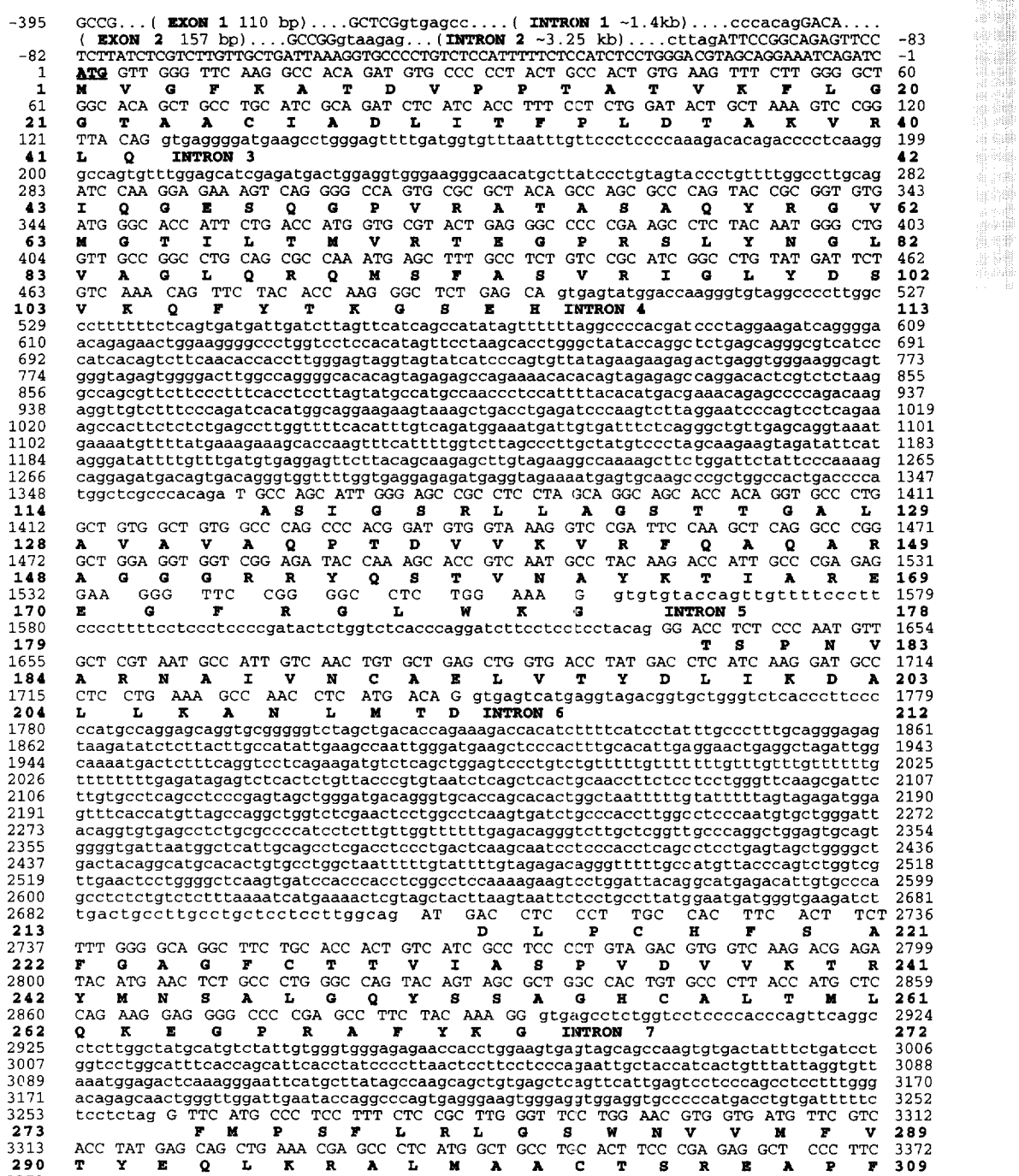

Fig.1 Genomic organization of the human UCP 2 gene. The start codon and stop codon are marked in bold

The localization of the exon/intron boundaries within the coding region of hUCP2 matches precisely that of the hUCP1 gene and is most likely conserved in the recently discovered UCP3 gene as well (**Table I**). However, the size of each of the introns within the human UCP2 gene differs from its hUCP1 and hUCP3 counterparts. Furthermore, within the 5'-flanking region there is no additional intron in the hUCP1 gene, which distinguishes it from hUCP2 and hUCP3 for which two additional introns (hUCP2) or only one (hUCP3) can be detected. Human UCP3 has been found to exist in two different forms, hUCP3L and hUCP3S. Comparing the genomic structure of hUCP2 to that of the hUCP3 gene, the short form of the human hUCP3 gene differs from its long form by the absence of exon 7 at the C-terminus of the protein. We (Tu *et al.* (12)) and Solanes *et al.* (7) have found that the short form of hUCP3 must be generated by incomplete transcription caused by the presence of a polyadenylation signal (AATAAA) in the last intron terminating messenger elongation. At the amino acid level UCP2 has about 55% identity to hUCP1 while hUCP3 is 71% identical to hUCP2 (**Fig. 2**). The three mitochondrial carrier protein motifs present in hUCP1 are conserved in hUCP2 and in hUCP3. At the carboxyterminus of the hUCP2 protein a Purine-Nucleotide-Binding Domain (PNBD) is found, similar to hUCP1 and hUCP3L (not present in hUCP3S).

Percent Similarity

Percent Divergence

	1	2	3	4		
1		54.9	55.8	54.7	1	hUCP1
2	60.9		70.2	67.8	2	hUCP2
3	60.4	32.9		99.6	3	hUCP3-L
4	60.4	34.5	0.0		4	hUCP3-S
	1	2	3	4		

Fig. 2

Percent Similarity and Percent Divergence of hUCP Proteins. For protein alignment the Clustal Multiple Sequence Alignment was used.

Identification of a point mutation and an insertion polymorphism

Because genetic variations in members of the human UCP gene family may have effects on energy metabolism, body weight regulation and thermogenesis, we have performed a mutational analysis of the hUCP2 gene. DNA sequence analysis of the hUCP2 gene in a cohort of 25 children (aged 7-13) characterized by significantly reduced RMR (based on the Harris & Benedict equation) revealed a base transition in exon 4 (coding region) and an insertion polymorphism in exon 8 (3'-UTR). The base transition in exon 4 consists of a C to T exchange in codon 55 changing an alanine (GCC) to a valine (GTC). The insertion polymorphism consists of a 45 bp repeat located 150 bp downstream of the stop codon in the 3'-UTR (**Fig. 3**).

Evaluation of the genotypes (Allele frequencies, genotype/phenotype correlations)

Allele frequencies

Genotyping the Ala55Val polymorphism in 68 obese and 104 normal weight children revealed allele frequencies of 0.63 for the Alanine and 0.37 for the Valine allele. The allele

frequencies for the insertion polymorphism were 0.71 for the wildtype and 0.29 for mutant allele, respectively. No significant differences in allele frequencies were found between the obese and non-obese groups.

TABLE I

Comparison of the Genomic Organization of hUCP1, hUCP2 and hUCP3

Gene	hUCP1 (Size, bp)		hUCP2 (Size, bp)		hUCP3 (Size, bp)			
					Long Form		Short Form	
No.	Exon	Intron	Exon	Intron	Exon	Intron	Exon	Intron
1	342	612	> 104	~1,400	> 88	~1,800	> 88	~1,800
2	199	> 833	157	~3,250	221	~750	221	~750
3	200	105	225	156	211	~240	211	~240
4	102	?	212	867	204	~1,200	204	~1,200
5	181	?	194	81	102	~470	102	~470
6	> 115		102	971	181	~1,800	331	
7			181	369	> 267			
8			450					
Total	> 1,139		> 1,625	~6,844	> 1,186	~6,260	> 1,157	4,460
	~9,000		> 8,719		> 7,446		> 5,617	

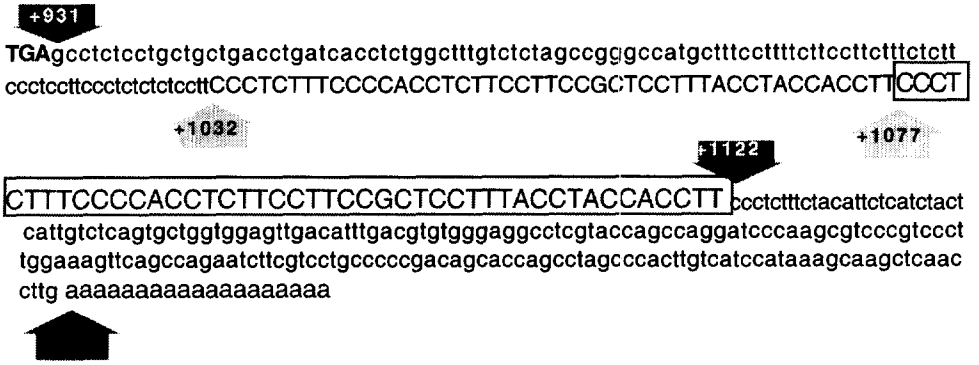

Fig. 3

The insertion polymorphism consists of a 45 bp repeat (boxed residues) located at bp 1077 to 1122 (+1 for the translational start codon ATG), 150 bp downstream of the stop codon TGA in the 3'-UTR of the hUCP2 gene.

Genotype/Phenotype correlations

Although a first set of samples indicated a higher frequency of the Valine allele in children with significantly reduced RMR this finding could not be confirmed when the allele frequencies were compared grouped according to RMR:

Group 1 (up to -25% deviation of predicted RMR): p = 0.351 (57)*
Group 2 (no deviation of predicted RMR): p = 0.388 (58)*
Group 3 (up to +25% deviation of predicted RMR): p = 0.360 (57)*
*= number of cases studied, with p-values ≤ 0.01 considered as being significant

The rate of the Ala55Val polymorphism was independent of percent deviation RMR (measured versus predicted). There was no direct effect of the polymorphism and the amount of deviation RMR, only sex had a significant effect on RMR (male >female), p = 0.001; a possible direct interaction of sex and the hUCP2 polymorphism on RMR turned out to be not significant (with p = 0.084).

Similar results were obtained for the insertion polymorphism in the 3'-UTR. So far a direct correlation of the observed genotype with reduced RMR and BMI was not evident.

Discussion

The recent molecular cloning of two new members of the human UCP gene family, UCP2 and UCP3, somehow has revolutionized obesity research namely because of their potential physiological role as mediators of energy metabolism, body weight regulation and thermogenesis. Flier and Lowell (1997) themselves characterized the identification of the UCP2 homologue as a major breakthrough towards understanding the molecular basis for energy expenditure, and considered these findings likely to have important implications for the cause and treatment of human obesity (1). This is largely due to the fact that hUCP1 containing brown adipose tissue is unlikely to be involved in weight regulation in adult large-size animals and in humans living in a thermoneutral environment (2), primarily because of its very limited abundance. In contrast, UCP3 as a new member of the UCP gene family is preferentially expressed in skeletal muscle and brown adipose tissue; it therefore represents a candidate protein for the modulation of the respiratory control in skeletal muscle (4). The chromosomal mapping of both hUCP2 and hUCP3 to human chromosome 11q13 in close proximity to each other, and positive linkage data in Pima Indians indicating that this region may contain genetic markers responsible for energy expenditure and body weight regulation, have made hUCP2 and hUCP3 strong candidate genes in the molecular pathogenesis of human obesity.

In order to better understand a possible implication of hUCP2 and hUCP3 in the development of human obesity we have set out to elucidate the genomic structure of the hUCP2 gene and to search for genetic variants which may be of functional relevance. Analyzing the overall genomic structure of hUCP2 and its counterparts hUCP1 and hUCP3 revealed a high degree of homology, particularly within the coding region. Major differences of the exon/intron structure are only found at the boundaries of these genes. Cassard (13) note that the human UCP1 gene spans 13 kb and contains a transcribed region that covers 9 kb of the human genome (similar to hUCP2), which is split into 6 exons only. hUCP3 contains at least 7 exons spread over 8.5 kb. In contrast to hUCP2, hUCP3 generates two transcripts, UCP3L and UCP3S which are predicted to encode long and short forms of the UCP3 protein differing by the presence or absence of 37 amino acid

residues at the C-terminus. These 37 residues are encoded by exon 7 which is missing in UCP3S. Very recent data from Surwit *et al.* (14) and our group (Lentes *et al.*, in preparation) suggest that the hUCP3 gene maps 5' to the hUCP2 gene and that the extreme 3'-end of exon 7 of hUCP3 and the transcriptional start site of hUCP2 are less than 10 kb apart from each other. This strongly implies that the organization of the UCP3/UCP2 gene locus is a result of a gene duplication event. The hUCP1 gene on the other hand was assigned to human chromosome 4 (4q31) (13). The high degree of homology at the nucleotide level and the conservation of exon/intron boundaries among the three UCP gene suggests that they may have evolved from a common ancestor or are the result from gene duplication events. However, despite their sequence similarity, all UCPs are distinguished by their different pattern of expression: hUCP1 with a 1.9 kb mRNA expressed exclusively in human perirenal brown adipose tissue, plays an important role in generating heat and burning calories, as well as in the regulation of body temperature, body composition, and glucose metabolism. Compared to hUCP1, a 1.6 kb hUCP2 mRNA is widely expressed in adult human tissues (2) including tissue rich in macrophage; expression levels are upregulated in white fat in response to fat feeding. hUCP3, the third analogue discovered by Vidal-Puig *et al.* (3) and Boss *et al.* (4), is distinguished from hUCP1 and hUCP2 by its abundant and preferential expression in skeletal muscle in humans, and brown adipose tissue and skeletal muscle in rodents. Since skeletal muscle and brown adipose tissue are believed to be important sites for regulated energy expenditure in humans and rodents, respectively, hUCP3 may be an important mediator of adaptive thermogenesis (3).

To understand better the regulation of hUCP2 gene expression *in vivo* we are currently in the process to map the major transcription initiation sites as well as the promoter region in order to identify important regulatory sequences (e.g. CRE, thyroid hormones (TRE), transcription factor binding sites etc.) utilizing 5'-RACE and genome walking. For instance, in case of the hUCP1 gene neither a CCAAT sequence nor a Sp1 binding motif were detected (13). Comparing the promoter region of these two genes should be helpful for the identification of putative promoter elements important for UCP2 gene regulation *in vivo*.

With regard of hUCP2 being a potential mediator of energy metabolism and body weight regulation we have screened for possible polymorphisms within the hUCP2 gene. Two frequent genetic variants could be detected; genotyping of these variants (an Ala55Val polymorphism in exon 4 as well as a 45 bp insertion polymorphism in exon 8) in a cohort of 68 obese and 104 non-obese children did not show any significant differences in allele frequencies between the two groups. No direct interaction of these variants with RMR or BMI could be observed. In terms of function, the Ala55Val polymorphism in the hUCP2 gene most likely has no effect on membrane potential (D. Ricquier, personal communication); the insertion polymorphism in the 3'-UTR probably has also no obvious functional consequences however it may have an effect on UCP2 mRNA stability. This does not completely exclude a potential role of hUCP2 in energy metabolism and body weight regulation. The set-up of suitable expression systems (e.g. yeast) should be of extreme value to determine functional correlates of UCP2 as well as for the characterization of newly identified genetic variants.

Finally, the close proximity of hUCP3 to hUCP2 on chromosome 11q implies that instead of hUCP2 a malfunctionning of hUCP3 or a deregulation of its gene expression may well be a causative factor for the development of human obesity. Therefore the identification of genetic variants in the hUCP3 gene and its flanking regions will be of great importance to determine whether and to what extent UCP3 may play a role in energy metabolism and thermogenesis. The search for such mutations and/or polymorphisms is currently underway.

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