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Functional Organization of the Human Uncoupling Protein-2 Gene, and Juxtaposition to the Uncoupling Protein-3 Gene

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Human and mouse UCP2 genes were cloned and sequenced. Transcriptional start sites were identified using primer extension analysis. The transcription unit of UCP2 gene is made of 2 untranslated exons followed by 6 exons encoding UCP2. *In vitro* translation analysis demonstrated that an open-reading-frame for a putative peptide of 36 residues present in exon 2 did not prevent UCP2 translation and confirmed that the initiation site of translation was in exon 3 as predicted from sequencing data. Short (bp −125 to +93) and long (bp −1383 and +93) CAT-constructs containing DNA upstream of the transcriptional start site of the human gene were made and transfected in adipocytes or HeLa cells allowing characterization of a potent promoter. Analysis of several genomic clones encompassing UCP2 and/or UCP3 genes demonstrated that the 2 genes are adjacent, the human UCP2 gene being located 7 kb downstream of the UCP3 gene.

The coupling mechanism of respiration to ATP synthesis consists in the generation of a proton electrochemical gradient through the inner mitochondrial membrane and the existence of a proton circuit between the respiratory chain and ATP-synthase (1). The analysis of thermogenic brown fat mitochondria which exhibit an uncoupled respiration led to the discovery of a mitochondrial uncoupling protein (UCP) (recently renamed UCP1) which promotes proton transport towards the matrix (1, 2). UCP1 activation decreases the mitochondrial membrane potential, increases respiration rate and uncouples respiration from ATP synthesis, thus generating heat. UCP1 is specific for brown adipose tissue mitochondria and the recent disruption of the UCP1 gene in mice demonstrated that its function is to maintain the normal body temperature during cold exposure (3). Actually, it is known that the level of coupling of respiration to ADP phosphorylation in most cells is below 100%, i.e. the respiration is partially coupled to ATP synthesis. The recent cloning of UCP2 (4, 5) and UCP3 (6–8) identified 2 new members of the uncoupling protein family that are putative candidates for the proton leaks observed in mitochondria. The gross structure of human UCP3 gene was recently described by several groups who located this gene within 75–100 kb of UCP2 gene (9–11). Yamada et al. described the organization of the mouse UCP2 gene (12). A gross organization of the human gene was reported by others (13). Here, we describe the complete organization of human (and mouse) UCP2 gene including determination of transcriptional start sites and translation initiation codon, and characterization of human UCP2 promoter; we also provide evidence that the transcriptional start site of the human UCP2 gene is only 7 kb downstream of the UCP3 gene.

MATERIALS AND METHODS

Materials. Enzymes were purchased from Appligene (Illkirsch, France) and New England Biolabs (Ozyme, Montigny-le-Bretonneux, France); (α-^{32}P)dATP, (γ-^{32}P)ATP and D-threo-(dichloroacetyl-1-^{14}C)-chloramphenicol were obtained from Amersham Corp. (Les Ulis, France). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

Analysis of genomic organization. Human UCP2 gene was cloned from a genomic library (catalog number HL 1067j, Clontech, Palo Alto), using a human UCP2 cDNA. Mouse UCP2 gene was cloned from a genomic library (catalog Number: 946306, Stratagene, La Jolla) using a mouse UCP2 cDNA. Genomic clones were entirely sequenced using the Prism cyclic sequencing kits and an ABI 373 DNA sequencer.

Analysis of transcriptional start site. Determination of human and mouse UCP2 transcriptional start site was made using primer extension analysis of human (white adipose tissue) or mouse (brown

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Abbreviations used: UCP, uncoupling protein; CAT, chloramphenicol acetyltransferase; TK, *Herpes simplex* thymidine kinase.

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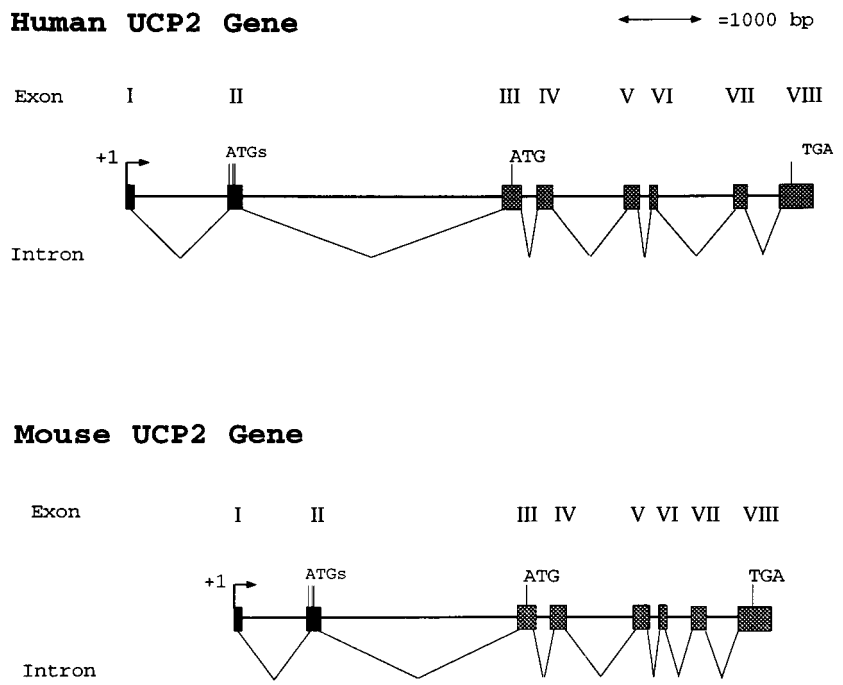

FIG. 1. Human and mouse UCP2 gene structure. Upper part: human UCP2 gene; lower part: mouse UCP2 gene. Each gene is made of 8 exons numbered from I to VIII. The transcriptional start site was determined using primer extension analysis (see Figure 3). Exons I and II (black boxes) do not contain sequence corresponding to translated region of UCP2; exon II contains 3 ATG whereas an ATG codon initiating a sequence corresponding to UCP2 is in exon III (see Figure 4).

adipocytes) RNA. For human UCP2 gene, a 5′-ATCATGGCCCGATCCCCTTGGTTTTCCATAG oligonucleotide corresponding to the estimated exon 2 of the gene was 5′-end-labeled using polynucleotide kinase and (γ-^{32}P)-ATP. For mouse UCP2 gene, a 5′-ACCATGGCCCGATCCCCTCGATTTTC-CATAG oligonucleotide corresponding to estimated exon 2 of the gene was 5′-end-labeled. Hybridization of primer to 1 μg poly(A^+) RNA of mouse brown adipocyte or 5 μg poly(A^+) RNA of human subcutaneous white adipose tissue, and extension reaction were done according to published procedure (14).

Identification of UCP2 translation initiation codon. Two different mouse UCP2 cDNAs were inserted in pSELECT plasmid (Promega, Charbonniéres, France) in front of the SP6 promoter, one containing the 8 exons, and another one starting at exon 3 and therefore lacking the 3 ATGs and the open reading frame present in exon 2. The cDNA lacking exons 1 and 2 was obtained by digestion by Hind III. After SP6-promoter-driven transcription, RNA was added to a cell-free translation system (*in vitro* translation kit manufactured by Promega, Charbonniéres, France) containing labelled methionine (Redivue L-(^{35}S) Methionine, Amersham, Les Ulis, France). Translation products were analyzed by electrophoresis and autoradiography.

Chloramphenicol-acetyl-transferase (CAT)-constructs and promoter studies. Fragments of DNA corresponding to positions bp −1383 to +93 or positions bp −125 to +93 of human UCP2 gene (transcriptional start site taken as +1 position) were cloned in pBL-CAT6 vector (15) using *Sac* I (bp −1383) or *Sac* II (bp −125) restriction sites, to give −1383 CAT-plasmid and −125 CAT-plasmid. A genomic fragment corresponding to positions bp −1383 and bp −48 (*Sma* I site) of human UCP2 gene was cloned in front of the promoter of the *Herpes simplex* virus thymidine kinase (TK) promoter to generate −1383/−48 TK-CAT or −48/−1383 TK-CAT plasmid. The different CAT constructs were entirely sequenced. Transfection experiments of 1B8 or HeLA cells and CAT assay were made as previously described (16).

Analysis of DNA between UCP3 and UCP2. To analyze the region in between human UCP3 and UCP2 genes, we used the genomic clones described above, a genomic clone isolated from a library screened with a PCR fragment corresponding to exons 6 and 7 of human UCP3 gene, and P1 clones purchased from Genome Systems (Saint-Louis, MO). The sequencing of the DNA region between mouse UCP3 and UCP2 gene was made using DNA isolated as previously described (17).

RESULTS AND DISCUSSION

Intron-exon structure of human and mouse UCP2. To isolate the human UCP2 gene, a 14 kb clone was isolated from a library using a mouse UCP2 cDNA and exon 2 of the human gene as probes. After digestion by *Bam* H1 and *Sac* I, the restriction fragments were cloned into pSport plasmid and sequenced. It revealed that this clone was lacking the most 5′ exon(s) and was ending at 8 kb downstream of the 3′ end of the last 3′ exon. A second screening of the genomic library identified another 18 kb clone, refered to as U2GL2, containing all the exons and 4 kb of DNA upstream of the putative exon 1. Two genomic clones were isolated from a mouse genomic library using mouse UCP2 cDNA as a probe. Sequencing of the insert of a first phage revealed a 10-kb DNA fragment going from 250 bp upstream of the first 5′ exon of mouse UCP2 cDNA, to 4 kb downstream of the end of the last 3′ exon of UCP2 cDNA. Sequencing of the second clone showed a 15.7 kb DNA fragment covering 7kb upstream of the first 5′ exon to

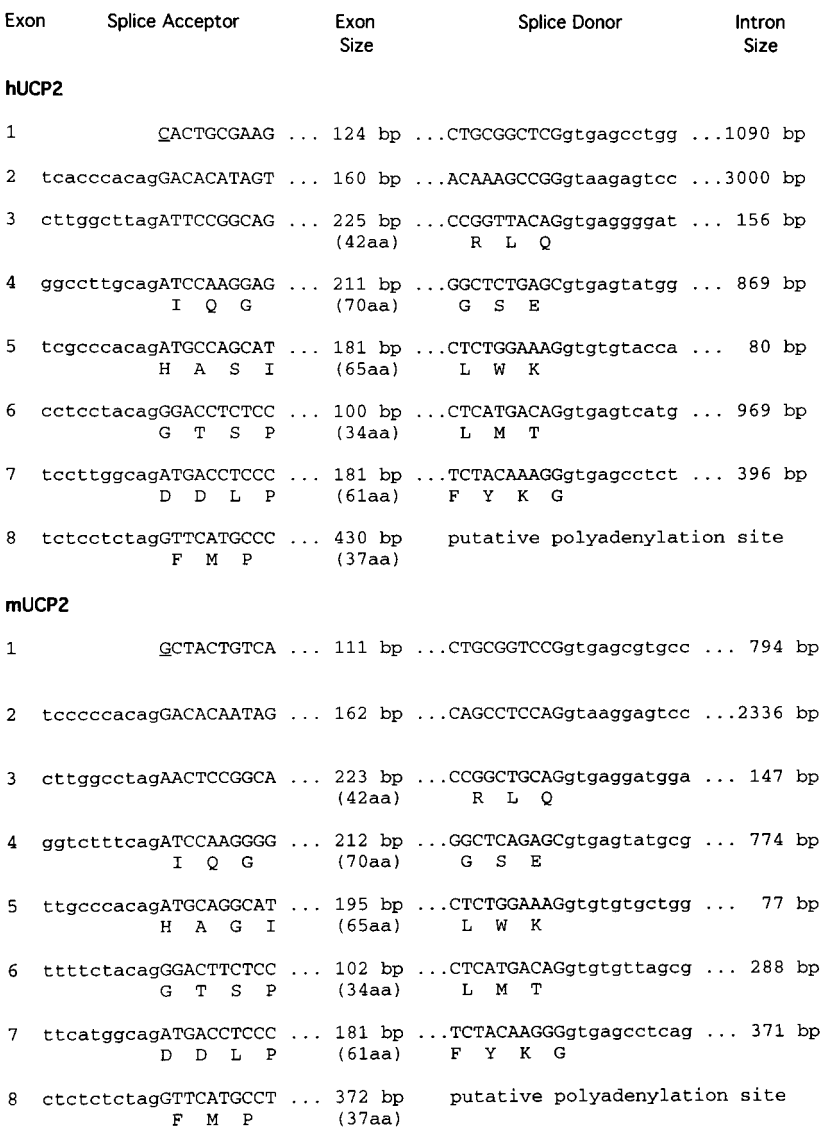

FIG. 2. Organization of human and mouse UCP2 gene. Upper pannel: hUCP2, human UCP2 gene; lower pannel: mUCP2, mouse UCP2 gene. Location of splice donors and splice acceptors. Uppercase letters refer to exonic sequence and lowercase to intronic sequence. Length of exonic and intronic fragments, and number of amino acid residues per exon are given. Untranslated region in human and mouse exon 3 is 100 and 97 bp long respectively. Untranslated region in human and mouse exon 8 is 320 and 266 bp long respectively. Amino acids (one letter-code) adjacent to the splice sites are shown below the nucleotide sequence. Transcriptional start sites (see Figure 3) are underlined. The GenBank accession numbers for human and mouse genomic sequences are AF 096288 and AF 096289 respectively.

2 kb downstream of the last 3′ exon. The intron-exon structure of human and mouse UCP2 genes is shown in Figures 1 and 2. Human and mouse UCP2 genes are made of 8 exons. Alignment of genomic sequences with cDNA sequences (4) or ESTs present in GenBank demonstrated that the translated regions of UCP2 were located in exons 3 to 8. Neither human nor mouse exons 1 and 2 contain open reading frames corresponding to UCP2. In fact, human and mouse exon 2 contains a 111 bp open reading frame preceded by three ATGs in frame, and encoding a putative unknown peptide of 36 aminoacids. Human and mouse Exon 8 contain TGA codon followed by a polyadenylation site. The total lengths of human and mouse UCP2 gene (from exon 1 to exon 8) are 8.0 and 6.3 kb respectively. Both genes are characterized by the intron 2 that is longer than the other introns. The number of amino acids per exon is given in Figure 2. Using an oligonucleotide corresponding to expected exon 2 of mouse or human UCP2 gene, primer extension analysis demonstrated the presence of transcriptional start sites in mouse or human exon 1 (Figure 3). A double band was detected in mouse gene indicating the presence of transcriptional start sites in exon 1 at 370 or 369 bp upstream of ATG codon present in the third exon. Similar data were obtained for the human gene, the most 5′ transcrip-

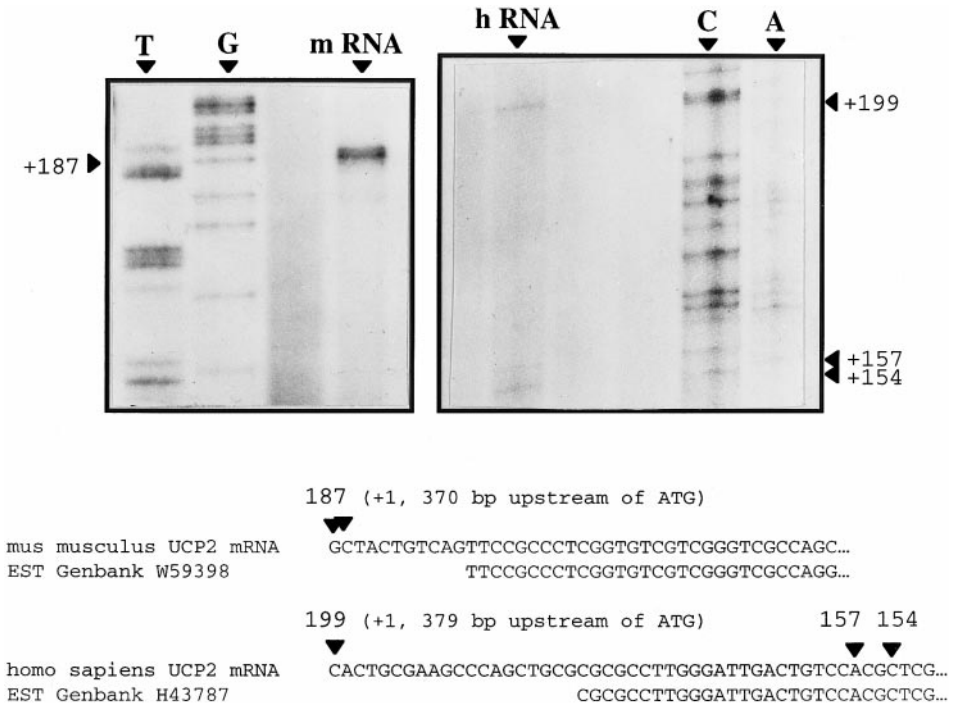

FIG. 3. Identification of mouse and human UCP2 gene transcriptional start sites. Upper part, primer extension analysis of mouse (left-hand side panel) or human (right-hand side panel) UCP2 gene. Primer extension analysis was carried out using RNA from mouse brown adipocyte (m RNA) or RNA from human adipose tissue (h RNA), and oligonucleotides corresponding to central region of expected exon 2 (see Methods section). Positions bp + 187 and + 199 refer to the higher molecular weight bands obtained with mouse and human RNA, respectively; these positions were calculated relatively to the 5′ end of oligonucleotide used for primer extension reaction and were taken as +1 position. The sequence of the 5′ end of the mRNA corresponding to mouse or human extension product is shown in the lower part of the figure. An alignment to the 5′ end of the most 5′ EST sequences related to UCP2 and present in GenBank is shown.

tional start site being 380 bp upstream of the ATG in exon 3. In the case of the human gene, two other transcriptional start sites were detected 43 and 46 bp downstream of the most 5′ site (positions 157 and 154 in Figure 3).

Translation initiation codon. The sequence of the short open reading frame present in exon 2 is highly conserved in mouse and man, the level of homology at the nucleotide and protein level being 92% and 84% respectively (Figure 4). Moreover, the nucleotide sequence surrounding the 3 ATGs present in exon 2 of mouse or human UCP2 gene is in rather good agreement with the usual structural requirements of initiation sites (partially shown in Figure 4, ref. 18). Therefore, the possibility existed that translation of the short conserved peptide would prevent the translation of UCP2 protein. To investigate such hypothesis, we inserted either the full-length UCP2 cDNA, or a shorter DNA lacking exons 1 and 2, in a transcription vector (see Methods). The transcripts were added to an *in vitro*-translation-system. Figure 4 shows that a unique 33 kd product of the size of UCP2 was obtained even when the 3 ATGs and the 36 aminoacid open reading frame of exon 2 were present. Therefore the presence of an open reading frame upstream of UCP2 cDNA does not influence the translation of UCP2 at the expected codon.

The present data show that human and mouse UCP2 genes are very similar in their organization. During the completion of these studies, Yamada et al. described the organization of the mouse UCP2 gene and reported similar data (12). The UCP2 gene has an organization which is somehow different from rat, mouse or human UCP1 gene which does not contain untranslated exons (19–21). Solanes et al. reported that the human UCP3 gene contains at least 7 exons including 1 untranslated exon (9). Interestingly, the coding regions for UCP1, UCP2 or UCP3 are all contained in 6 exons. Moreover, alignment of this region of the 3 genes show conserved residues. This strongly supports a common origin of the 3 genes of the uncoupling protein family. It would be interesting to know the genomic structure of the recently described plant UCP related to mammalian UCPs (22).

Human UCP2 promoter. The DNA upstream of transcriptional start site of human UCP2 gene does not contain neither TATA box nor CAAT box and is GC-rich, contrary to the UCP1 gene (19–21). A DNA fragment of 4 kb upstream of the transcriptional start site of human UCP2 was sequenced. CAT constructs were

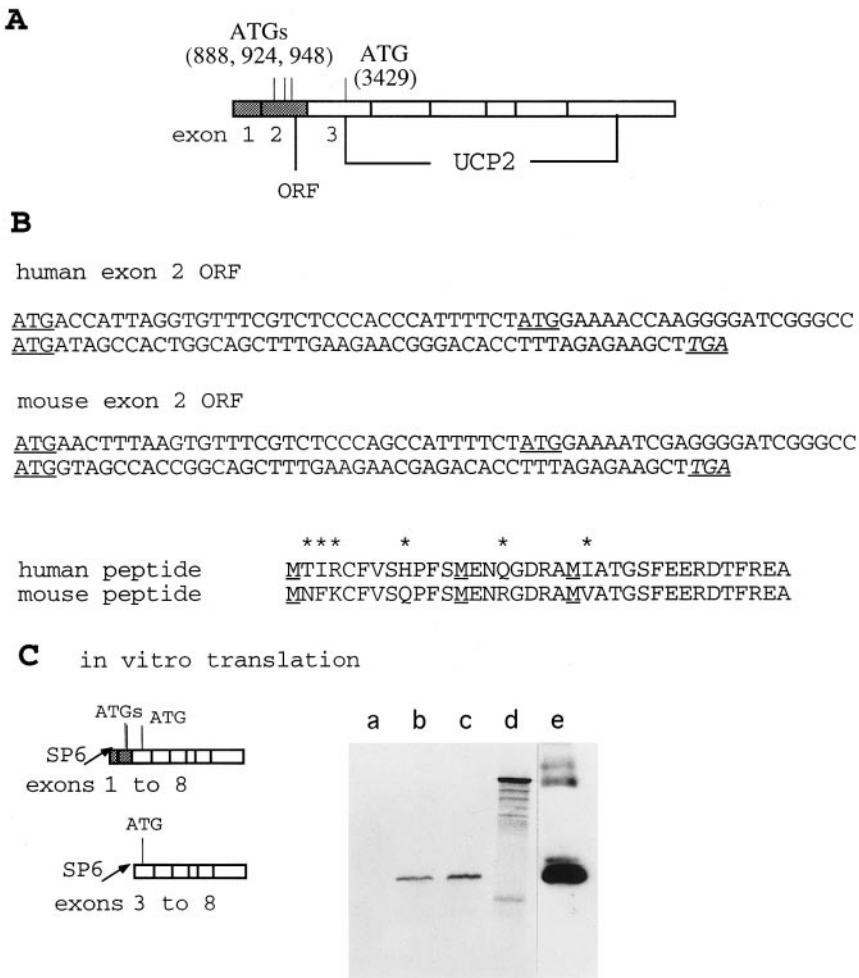

FIG. 4. Demonstration that the ATG codon in exon III is the initiation codon. of UCP2. (A) Schematic organization of human or mouse UCP2 transcript. The numbers refer to positions of ATGs in the human gene relatively to the transcriptional start site. ORF, open reading frame; UCP2, UCP2 open reading frame. (B) Nucleotide sequence of human and mouse ORF present in exon 2 and predicted amino acid sequences. ATGs and methionines are underlined. Stars indicate different amino acid residues. (C) *In vitro* translation experiment. UCP2 cDNAs containing or not exons 1 and 2 were inserted in pSELECT vectors and were transcribed using SP6 promoter. After translation in reticulocyte lysate containing radioactive methionine, the products were analyzed by gel electrophoresis. a, empty vector; b, UCP2 plasmid with 8 exons; c, UCP2 plasmid lacking exons 1 and 2; d, control experiment using luciferase cDNA (main band of Mol. Weight 61,000 Da); e, immunodetection of mouse UCP2 expressed in yeast used as a control of molecular weight of translation products (antibodies against residues 1–69 of mouse UCP2 were used, Miroux and Ricquier, unpublished data). Molecular weight markers were used in electrophoresis (not shown).

made to investigate the promoter activity of DNA upstream of the transcriptional start site of human UCP2 gene (Figure 5). A significant CAT activity was recorded when a short CAT construct made of 218 bp (positions bp −125 to + 93) was transfected in differentiated adipocytes; a higher activity was recorded when the construct was extended to position −1383. The deletion of nucleotides surrounding the transcriptional start site (bp −48 to +93) in plasmid bearing the TK promoter decreased the CAT activity despite the presence of TK promoter. A significant activity was measured with this construct in normal or inverted sense, suggesting an enhancer activity. Similar data were obtained when the constructs were transfected into HeLa cells (data not shown). Yamada et al. measured a significant activity in the mouse UCP2 gene using 1250 bp of promoter region (12).

The data reported here demonstrate that regulatory elements allowing a high level of transcription are located in 1.4 kb of DNA upstream of human UCP2 transcriptional start site. A large number of situations or factors such as hyperlipidic diet, starvation, obesity, free fatty acids, leptin, thiazolidinediones, thyroid hormones, endotoxin to phorbol ester modulate UCP2 gene transcription or expression in various tissues or cell types (5, 5, 8, 23–35). These data support a role for UCP2 in metabolic regulations, diet-induced thermogenesis, or fever. The present characterization of a

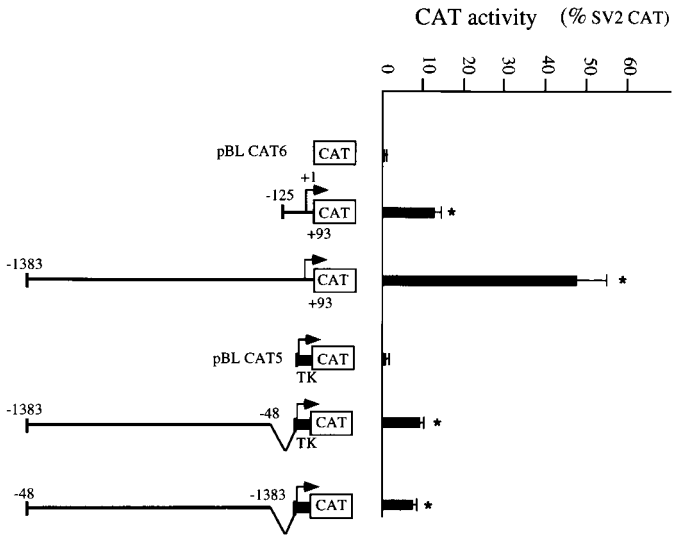

FIG. 5. Characterization of human UCP2 promoter. Transient transfection experiments of 1B8 cells with CAT constructs made of short (bp −125) or long (bp −1383) 5′-flanking region of the human UCP2 gene are shown. Plasmids containing regions bp −1383 to + 93 or bp −125 to +93 were cloned in pCLCAT6 plasmid. The DNA from bp −1383 to bp −48 was cloned in the sense or the antisense orientation in pBLCAT5 vector which contains the TK promoter. CAT activity is given as % of value obtained with SV2CAT plasmid and were normalized relatively to β-galactosidase activity. Data represent mean ± S.E. of three independent experiments. *, significant statistical difference ($p<0.01$) relatively to the activity of pBLCAT6 or pBLCAT5 plasmid. Very similar data were obtained when HeLa cells were transfected (not shown).

genomic region controlling UCP2 gene transcription will allow further characterization of regulatory elements mediating the response of UCP2 gene to particular signals.

UCP2 gene is adjacent to UCP3. The UCP2 gene was originally mapped to mouse chromosome 7 and human chromosome 11 (4). It was reported that the human UCP3 gene was located within 100 kb of the UCP2 gene (8, 9). We previously reported that the mouse UCP2 gene was 8 kb downstream of the UCP3 gene (17). In the present work, we sequenced the whole DNA corresponding to this intergenic region and found that it is 8249 bp long (Figure 6). In order to analyze UCP2 and UCP3 localization in human, we used a genomic clone containing 4.3 kb of DNA upstream of the transcriptional start site of the human UCP2 gene and containing no UCP3 exon. A human genomic fragment containing exon 6, intron 6 and exon 7 of the UCP3 gene was amplified by PCR, sequenced and used as a probe to screen a human genomic library. We isolated a DNA containing the human UCP3 gene and 1.8 kb of DNA downstream of the 3′ end of exon 7 of UCP3 gene. This region was sequenced and used to design a 5′-GCTTCCCTGCTCCTACCCCTCCAG oligonucleotide to sequence the DNA of a human P1 clone (ref. 324-H6) in the 3′ sense. A more 3′ oligonucleotide (5′-GTTTCCGGTTGGTGCAGTGG) was designed. We then used this sense oligonucleotide and an antisense 5′-TCTCCCCAACTTCTTAGAGC oligonucleotide corresponding to the 5′ extremity of U2GL2 to amplify a DNA fragment from the P1 clone and obtained a 600 bp product which was sequenced. This strategy proved that the human UCP2 gene is 3′ to the UCP3 gene and identified the complete sequence of the DNA between the 2 genes. The exact sequence between the TGA codon of the human UCP3 gene and the transcriptional start site of the UCP2 gene is 6987 bp long. The organization of the UCP3/UCP2 locus probably results from

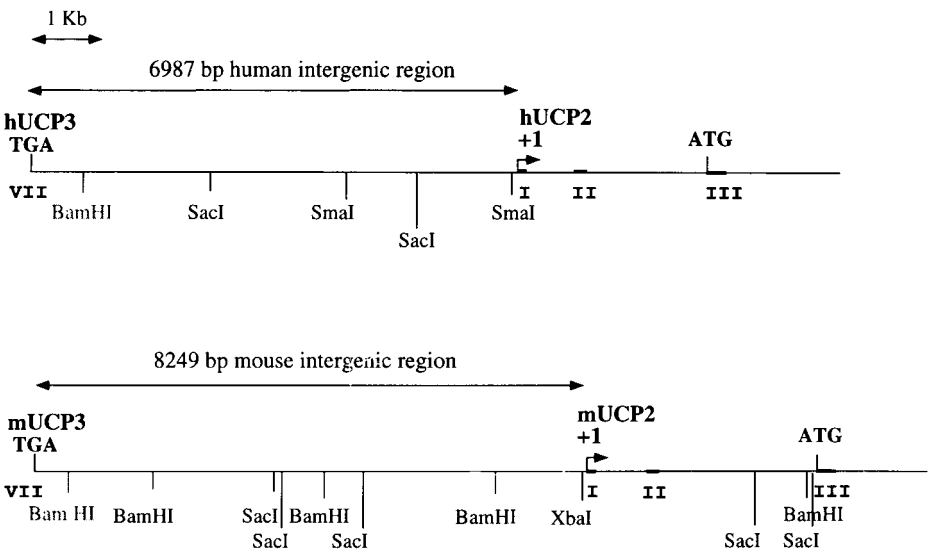

FIG. 6. Analysis of human and mouse UCP3/UCP2 locus, evidence for juxtaposed genes. The intergenic region is shown from the stop codon (TGA) in exon 7 of UCP3 gene, to the transcriptional start site (+1) of UCP2. Roman numerals indicate the exons; restriction sites are shown. Human and mouse intergenic region is rich in repetitive DNA.

a duplication event and is shown in Figure 6. This co-localization is particularly interesting since it was demonstrated that this locus is markedly genetically linked to resting metabolic rate in human (36).

In conclusion, human or mouse UCP2 gene is made of 8 exons of which exons 1 and 2 are untranslated. Transcriptional start sites were identified in exon 1, and translation initiation site in exon 3. Human and mouse UCP2 genes are highly similar and are located 7 or 8.2 kb downstream of UCP3 gene, respectively. CAT-constructs and cell transfection experiments identified a very active promoter in the 5′ side of the human UCP2 gene. These data will facilitate the identification of regulatory elements and transcriptional factors controlling UCP2 gene expression. They will also help in understanding the biological importance of UCP2, its medical relevance and pharmacological potential towards obesity, diabetes and inflammatory processes.

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