Mechanism of ubiquitous expression of mouse uncoupling protein 2 mRNA: control by *cis*-acting DNA element in 5'-flanking region

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Uncoupling protein (UCP) 2 is a member of the uncoupling-protein family, and it appears to function as an uncoupler of oxidative phosphorylation. To identify *cis*-acting regulatory elements controlling this gene's expression, we cloned an approx. 6.2-kb region upstream from the translation-initiation site of the mouse *UCP2* gene and analysed its transcription activity using chimaeric mouse *UCP2* promoter-placental-alkaline-phosphatase (PLAP) reporter-gene constructs. Sequence analysis showed that the 5'-flanking region of the mouse *UCP2* gene was not similar to those of mouse *UCP1* or *UCP3*. For the mouse *UCP2*, the region near the transcription-initiation site lacked the typical TATA box, but was GC-rich, resulting in presence of several potential specificity protein 1 (Sp-1), activator protein (AP)-1 and AP-2 binding sites. The putative regulatory motifs for muscle-regulatory protein (MyoD), brown-fat regulatory element, CCAAT box, cAMP-response element and Y box were also found in the mouse *UCP2* promoter region by computer-assisted analysis. From the results of Northern-blot analysis and transient expression assay, we found that the mouse *UCP2* gene responded to the cAMP-dependent protein kinase α -catalytic

subunit signal activation at the transcription level. Additionally, deletion analysis of the *UCP2* promoter-PLAP constructs indicated that the minimal region exhibiting the promoter activity was located between nt -33 and +100, and that a strong enhancer was present within 601 bp of the 5'-promoter region. In particular, the region from nt -233 to -34 significantly induced PLAP activity in the cell lines derived from various tissues and in the primary culture cells of rat brown adipose tissue, suggesting that this region is most important for the ubiquitous expression of mouse *UCP2* mRNA. Furthermore, it was shown that two silencer elements were involved in the mouse *UCP2* gene; one was located between nt -2746 and -602, and the other was identified in intron 1. These regions deprived the enhancer of the ability to induce PLAP activity. This study shows a fundamental role for positive and negative *cis*-acting DNA elements in regulating the basal and cAMP-induced transcription activity of the mouse *UCP2* gene.

Key words: enhancer, promoter analysis, reporter assay, silencer, transcription regulation.

INTRODUCTION

Non-shivering thermogenesis is a major component of energy expenditure to control body weight and metabolism. It contributes not only to maintenance of body temperature, but also to consumption of calories ingested in excess. Brown adipose tissue (BAT) is regarded as an important tissue for non-shivering thermogenesis in rodents [1–3] and the young of larger mammals [4], and it contains uncoupling proteins (UCPs) in mitochondria. UCPs are thought to transport fatty acid anions from an inner surface to an outer surface of the inner mitochondrial membrane to release stored energy as heat without coupling to other energy-consuming process [5–7]. The heat production generated mainly from BAT and skeletal muscle in rodents plays important roles, especially in newborn mammals, and in arousal from hibernation [1,8]. These facts suggest that UCPs contribute to controlling energy balance.

Uncoupling protein (UCP) 1, the first uncoupling protein to be identified [9–11], is expressed exclusively in the BAT. The transcription of the *UCP1* gene is controlled by the hypothalamus

via the sympathetic nervous system [12], and noradrenaline (norepinephrine) binds to β_1 - and β_3 -adrenergic receptors to initiate a cAMP signal-transduction pathway to induce *UCP1* mRNA expression [13–16]. A 211-bp enhancer called the 'activator element' located upstream in the *UCP1* promoter is most important for a tissue-specific and β -adrenergic regulation of the *UCP1* gene, as described previously [17,18]. However, because the amount of the BAT in humans is limited, the role of the *UCP1* gene in controlling energy balance in humans and the effect of β_3 -adrenergic receptor agonists in human obesity and diabetes are still uncertain.

UCP2, reported recently [19,20], seems to function as an uncoupler of oxidative phosphorylation [19]. The tissue distribution of UCP2 mRNA is different from that of UCP1 and UCP3. UCP1 mRNA expression is restricted tightly to the BAT, and UCP3 is expressed strongly in the skeletal muscle and heart in rodents. On the other hand, UCP2 mRNA is expressed ubiquitously, including in sites not thought to mediate adaptive thermogenesis, such as spleen, lymph node, thymus and gastrointestinal tract [19,20]. However, mice lacking UCP1 are not

Abbreviations used: UCP, uncoupling protein; UTR, untranslated region; BAT, brown adipose tissue; CRE, cAMP-response element; PLAP, placental alkaline phosphatase; PKA, cAMP-dependent protein kinase α -catalytic subunit; dibutyl-*o*-dibutyl cAMP, *N*⁶,2'-*o*-dibutyl cAMP; 8-Br-cAMP, 8-bromo cAMP; IBMX, 3-isobutyl-1-methylxanthine; AP, activator protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPDH, glycerol-3-phosphate dehydrogenase; Sp-1, specificity protein 1; MyoD, muscle-regulatory protein; Cap site, 7-methylguanosine 5'-triphosphate site; NF-Y, CCAAT-box-binding factor.

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obese [21], and UCP2 mRNA expression is increased in the white adipose tissue of A/J mice that are resistant to a high-food diet, but is not increased in the white adipose tissue of C57BL/6N mice, which gain body weight under the same conditions [22]. These findings suggest that UCP2 also contributes to controlling energy balance. Therefore, ubiquitous UCP2 mRNA expression could be important for basal energy consumption and regulation of metabolism. Very recently, the mouse *UCP2* gene structure, composed of eight exons and seven introns, was reported [23], but the mechanism of its transcription regulation is not well understood.

In this study, we isolated the 5'-flanking region of the mouse *UCP2* gene as well as its introns 1 and 2, and used the transient expression assay utilizing the placental-alkaline-phosphatase (PLAP) reporter vector to define the regulatory elements required for the ubiquitous expression of the mouse UCP2 mRNA. We found several potential transcription-regulatory motifs in the mouse UCP2 promoter, in which the region between nt -233 and -34 may be most important for its ubiquitous expression.

EXPERIMENTAL

Chemicals

($\pm$)-5-($-$)-{ p -($-$)-[2-(5-Ethyl-2-pyridyl)ethoxy]benzyl}thiazolidine-2,4-dione (pioglitazone), a high-affinity ligand for peroxisome-proliferator-activated receptor γ_2 , was synthesized in the Tsukuba Research Laboratories of Eisai Co., Ibaraki, Japan (purity > 95%).

*N*⁶-2'-*o*-Dibutyl cAMP (dibutyl cAMP), 8-bromo cAMP (8-Br-cAMP), forskolin, D,L-isoproterenol hydrochloride and 3,3',5'-tri-iodo-L-thyronine (T_3) were purchased from Wako (Osaka, Japan). Dexamethasone and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma (St. Louis, MO, U.S.A.).

Cloning of 5'-flanking regions and introns of mouse *UCP2* gene

We initially determined the 5'-untranslated region (UTR) of the mouse UCP2 gene by 5'-RACE (rapid amplification of cDNA 5' ends) methodology using the Marathon Ready cDNA library from 17-day mouse embryo (Clontech, Palo Alto, CA, U.S.A.) as a template. The following primers were employed: adaptor primer 1 (AP1) and mUcp2-3R (5'-CATCTGTGGCCT-TGAAACCAAC-3', antisense, nt 4-25 relative to the first nucleotide of the translation-initiation codon) in the first PCR; and AP2 and mUcp2-4R (5'-TGATTTCCTGCTACCTCCC-AGA-3', antisense, nt -27 to -6) in the nested PCR. PCR-amplification conditions were 45 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C, with a final extension of 4 min at 72 °C in the first PCR, and 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C, with an additional extension of 4 min at 72 °C in the nested PCR. The amplified fragment was cloned into the pT7Blue (R) vector (Novagen, Madison, WI, U.S.A.) and sequenced using T7, U19 and gene-specific primers with an ABI Prism 377 DNA sequencer (Perkin Elmer-Applied Biosystems, Foster City, CA, U.S.A.). The PCR parameter for sequencing was 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C.

To determine the precise transcription-initiation site(s) of the mouse *UCP2* gene, we next carried out 7-methylguanosine 5'-triphosphate (Cap)-site hunting using Cap-site cDNA [24,25] (Nippon GENE, Tokyo, Japan) as a template. The primers utilized for this reaction were as follows: 1RC (where RC stands for ribonucleic acid-complementary primer) and mUcp2-3R in the first PCR; 2RC and mUcp2-4R in the nested PCR. PCR-amplification conditions were 40 cycles of 30 s at 94 °C, 1 min at

55 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C in the first PCR, and 25 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C in the nested PCR. The amplified fragment was cloned into the pT7Blue (R) vector and the transcription-initiation site(s) were determined with sequence analysis in eight independent clones.

We also subcloned the 5'-flanking region of the mouse *UCP2* gene using GenomeWalker kit for the mouse (Clontech) according to the manufacturer's manual. Primers used were: AP1 and gene-specific primer 1 in the first PCR, and AP2 and gene-specific primer 2 in the nested PCR. The gene-specific primers are summarized in Table 1. PCR parameters were: 7 cycles of 25 s at 94 °C and 4 min at 72 °C, 32 cycles of 25 s at 94 °C and 4 min at 67 °C, with a final extension of 4 min at 67 °C in the first PCR; and 7 cycles of 25 s at 94 °C and 4 min at 72 °C, 30 cycles of 25 s at 94 °C and 4 min at 67 °C, with a final extension of 4 min at 67 °C in the nested PCR. The PCR product was cloned into the pT7Blue (R) vector and subjected to sequencing.

Introns 1 and 2 of the mouse *UCP2* gene were amplified by PCR methodology using the following primers: mUcp2-7 (5'-TTCTGCACTCCTGTGTTCTCCT-3', sense, nt -289 to -268) and mUcp2-5R for intron 1; and mUcp2-13 (5'-CTAT-GGAAAATCGAGGGGACTG-3', sense, nt -213 to -192) and mUcp2-3R for intron 2. C57BL/6J genome DNA purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) was used as a template. PCR conditions were: 45 cycles of 30 s at 94 °C, 30 s at 60 °C and 4 min at 72 °C, with a final extension of 10 min at 72 °C for intron 1; and 7 cycles of 25 s at 95 °C and 4 min at 72 °C, 38 cycles of 25 s at 95 °C and 4 min at 67 °C, with an additional 4 min at 67 °C for intron 2. The computer analysis for potential transcription-regulatory motifs was carried out using GENETEX software (Software Development, Tokyo, Japan).

Plasmid constructions

The plasmids for the transient expression assay to identify transcription-regulatory elements in the mouse *UCP2* gene were constructed utilizing the PLAP reporter-gene basic vector, which was described essentially by Goto et al. [26]. The following primers with appropriate restriction sites were used to amplify the promoter regions: mUcp2.p-1F, 5'-CCGCGGTACCCCA-CTATTCAAACACATCA-3', mUcp2.p-5F, 5'-GGTACCCG-ATCCAGAACAGCTGGCCAGTCA-3', and mUcp2.p-6F, 5'-GGTACCCTAGACTTAAGTTCTGTAGGCGCG-3', where the underlined sequences indicate *KpnI* sites; and mUcp2.p-2R, 5'-ATGAACGCGTGAGAACACAGGAGTGCAGAA-3', mUcp2.p-3R, 5'-ATTACGCGTCTTTGAGGTCTCAGGCT-GAG-3', and mUcp2.p-4R, 5'-AATGACGCGTATTTCCTGC-TACCTCCAGA-3', where the underlined sequences indicate *MluI* sites. Primer combinations were: mUcp2.p-6F and mUcp2.p-2R (6F-2R; $\approx$ 130 bp, nt -33 to +100 relative to the transcription-initiation site); mUcp2.p-5F and mUcp2.p-2R (5F-2R; $\approx$ 330 bp, nt -233 to +100); mUcp2.p-1F and mUcp2.p-2R (1F-2R; $\approx$ 2.85 kb, nt -2746 to +100); mUcp2.p-1F and mUcp2.p-3R (1F-3R; $\approx$ 3.75 kb, nt -2746 to +1008); and mUcp2.p-1F and mUcp2.p-4R (1F-4R; $\approx$ 6.20 kb, nt -2746 to +3457). PCR condition was 35 cycles of 30 s at 94 °C and 5 min at 68 °C with a final extension of 15 min at 68 °C. Each amplified fragment was ligated into the *KpnI*/*MluI* sites of the polylinker region of the PLAP basic vector. The PLAP vector with a $\approx$ 0.7 kb fragment (4F-2R; nt -601 to +100) was generated by removing a *KpnI*/*ApaI* fragment from the construct 1F-2R. The ends of the restriction-endonuclease-treated 1F-2R construct

Table 1 Primers used for genome walking to subclone 5'-flanking regions of the mouse *UCP2* gene

The subcloning was done using the GenomeWalker kit for the mouse, including *EcoRV*, *ScaI*, *DraI*, *PvuII* and *SspI* libraries. All primers listed are antisense. Nucleotide positions in the cDNA are numbered from the first residue of the initiation codon. Gene-specific primers 1 were used in the first PCR, gene-specific primers 2 in the nested PCR.

Gene-specific primer	Primer sequence	Position in cDNA	PCR product size	Origin of library
Primer 1, mUcp2-3R	CATCTGTGGCCTTGAACCAAC	4 to 25	≈ 1.0 kb	<i>DraI</i>
Primer 2, mUcp2-4R	TGATTCCTGCTACCTCCCAGA	−27 to −6		
Primer 1, mUcp2-5R	TGTCTCGTTCTTCAAAGTGCC	−176 to −155	≈ 600 bp	<i>ScaI</i>
Primer 2, mUcp2-6R	CGATCCCTCGATTTTCCATAG	−213 to −192		
Primer 1, mUcp2-8R	AAGGGCGTATTAGGAGGAAAG		≈ 500 bp	<i>PvuII</i>
Primer 2, mmUcp2-9R	AGAGAGGGCAAATTAGGACTCC			
Primer 1, mUcp2-10R	AGAGGCTCCAGTCTCAGATCTT		≈ 1.2 kb	<i>ScaI</i>
Primer 2, mUcp2-9R	TTCTATTCCCGGGGAGACAAT			
Primer 1, mUcp2-21R	ACTATCAAGTGGCAAACCCCA		≈ 1.4 kb	<i>PvuII</i>
Primer 2, mUcp2-22R	TAGCTGGTGGTGGATAGTGAT

Figure 1 Constructs of the mouse *UCP2* promoter-PLAP plasmids

The numbers show the nucleotide positions relative to the transcription-initiation site numbered as +1. The top diagram denotes the partial genomic structure of the mouse *UCP2* gene. Boxes represent exons, in which white and black parts indicate untranslated and translated regions, respectively. The dotted lines represent introns. Potential binding sites for the transcription factors are indicated as follows: MyoD, black rectangle; CRE, white oval; CCAAT-enhancer-binding protein, white triangle; CCAAT box, black oval; brown-fat regulatory element, white rectangle; and Y box, black triangle.

were blunt-end ligated with T4 DNA polymerase (Takara, Kyoto, Japan) and then re-ligated. The plasmid constructs are depicted in Figure 1.

Determination of basal promoter activity of the mouse *UCP2* gene

To examine basal promoter activity of the mouse *UCP2* gene, we carried out a transient expression assay of the *UCP2* promoter-PLAP constructs in 3T3-L1, C₂C₁₂, L6, HIT-T15, Hep-G2 and NIH/3T3 cell lines, which were derived from mouse preadipocytes, mouse muscle myoblasts, rat skeletal-muscle myoblasts, Syrian hamster β cells, human hepatocellular carcinoma and mouse embryonic fibroblasts, respectively. We also used the primary culture cells of rat BAT for this assay. The interscapular BAT was taken from newborn rats and treated as described by Shimizu et al. [27]. The cells were plated at a density of 1×10^5 cells in a collagen-coated 35 mm dish and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

fetal calf serum (FCS)/100 units/ml penicillin/100 μ g/ml streptomycin. After the seeding (24 h), the culture was washed extensively to remove non-adherent cells and the medium was replaced. On the second day, transfection was carried out with FuGENE 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the manual. The plasmid (1 μ g) consisting of the mouse *UCP2* promoter region fused to the PLAP reporter gene and 1 μ g of the pSV- β -galactosidase control vector (Promega, Madison, WI, U.S.A.) were co-transfected into the cells. After the transfection (24 h), the medium was replaced and the culture was continued for an additional 24 h. The culture supernatant was drawn from each sample and the alkaline phosphatase activity was determined as described previously [26]. Briefly, the accumulated activity of the alkaline phosphatase secreted into the medium for 24 h was measured with the use of a chemiluminescent substrate, 4-methoxy-4-(3-phosphatophenyl)spiro(1,2-dioxetane-3,2'-adamantane) (Lumi-stain reagent, Sumitomo Kinzoku, Osaka, Japan). The sample

was heated at 65 °C for 30 min to inactivate non-specific alkaline phosphatase contained in the medium from the FCS, and a 10 μ l aliquot of heat-treated sample was mixed with 50 μ l of assay buffer, which was followed by the addition of 50 μ l of chemiluminescent substrate. The mixture was incubated at room temperature for 60 min, and then the steady-state chemiluminescence was measured with a microplate luminometer (Wallac-EG&G, Turku, Finland). Data represented are the means $\pm$ S.D. from three to eight independent experiments.

Cell culture

The 3T3-L1 pre-adipocytes were plated at a density of 1×10^4 cells per cm^2 and cultured in DMEM supplemented with 10% FCS/100 units/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin. After the confluence, the cells were treated with 10 $\mu\text{g}/\text{ml}$ bovine insulin/0.25 mM dexamethasone/0.5 mM IBMX in DMEM containing 10% FCS during the first 3 days to trigger the adipose differentiation, and maintained for a further 18 days in DMEM supplemented with 10% FCS/17 nM bovine insulin/1 nM 3,3', 5-tri-iodo-L-thyronine (T_3). Medium was changed at least every 48 h. Before adding various compounds, the differentiated triacylglycerol-containing 3T3-L1 adipocytes (> 70% differentiation, determined visually) were incubated in the serum-free medium composed of DMEM supplemented with 5% lipid-free BSA/17 nM bovine insulin/10 $\mu\text{g}/\text{ml}$ human transferrin for 12 h, and then cultured in the presence of the compounds described in the Results section for 12 or 24 h. The compound-treated cells were washed with PBS twice, and harvested to prepare total RNA for Northern-blot analysis.

Northern-blot analysis

Total RNA was extracted by RNAzol B (Tel-Test, Friendwood, TX, U.S.A.) according to the manual. Total RNA (15 μg) was applied per lane on 1.2% agarose gel containing 2.2 M formaldehyde, and electrophoresis was carried out. RNA was then transferred on to nylon membranes (GeneScreen Plus, DuPont, Boston, MA, U.S.A.) for 20 h and crosslinked by UV irradiation. It was confirmed that total RNA was loaded equally for all lanes by hybridization of human 28S probe (Clontech). The probes of mouse *UCP2*, mouse fatty acid-binding protein (aP2) and mouse glycerol-3-phosphate dehydrogenase (GPDH), corresponding to each gene's entire open reading frame, respectively, were generated with PCR methodology and labelled by random priming in the presence of [α - ^{32}P]dCTP by BcaBEST random-priming kit (Takara). Hybridization was performed at 68 °C for 2–5 h in ExpressHyb Hybridization solution (Clontech), followed by washing twice for 10–30 min at room temperature with $2 \times \text{SSC}$ (where $1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate)/0.05% SDS, twice for 15 min at 50 °C with $2 \times \text{SSC}/0.05\%$ SDS, and finally twice for 5–30 min at 50 °C with $0.1 \times \text{SSC}/0.1\%$ SDS. The filter was exposed to X-ray film (Konica, Tokyo, Japan) at –80 °C for 5–48 h, and each band intensity was quantified with a Bioimaging analyser, BAS2000 system (Fuji Photofilm, Tokyo, Japan).

Effects of cAMP-dependent protein kinase α -catalytic subunit (PKA) activators on mouse *UCP2* gene transcription

To determine the effects of PKA activation on mouse *UCP2* gene transcription, we examined the transient expression assay of the *UCP2* promoter–PLAP reporter plasmids in 3T3-L1 pre-adipocytes. The cells were plated at a density of 2×10^5 cells in a 35 mm dish and cultured in DMEM supplemented with 10% FCS/100 units/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin. After the

seeding (24 h), transfection was performed with FuGENE 6 transfection reagent. The plasmid (1 μg) consisting of the mouse *UCP2* promoter linked to the PLAP reporter gene and 1 μg of the pSV- β -galactosidase control vector were co-transfected into the cells. After the transfection (24 h), the medium was replaced and the compound was added to the medium. The accumulated activity of the alkaline phosphatase secreted into the medium for 24 h was measured as described above. Data are shown as means determined in two independent experiments.

RESULTS

Cloning of 5'-flanking region and introns of mouse *UCP2*

Analysis of the Marathon cDNA library showed that the mouse *UCP2* gene had a 346-bp length of 5'-UTR, while the Cap-site hunting indicated that a single transcription-initiation site was located 369-bp upstream from the translation-initiation site. The existence of two introns upstream from the translation-initiation codon was revealed by PCR methodology. Sizes of the exons and introns were as follows: exon 1, 110 bp; intron 1, 746 bp; exon 2, 162 bp; and intron 2, 2349 bp. The AG/GT splice-site consensus sequences were conserved at the junctions. Repeating PCR allowed us to obtain a total ≈ 2.8 -kb in length of the mouse *UCP2* promoter region. The sequence of the promoter region was not similar among mouse *UCP1* (GenBank/EMBL accession number U63418), *UCP2* (AB018416) or *UCP3* (AB011070) (Figure 2, upper panel). For instance, the positions of potential transcription-regulatory motifs, such as TATA box, CCAAT box, Y box, cAMP-response element (CRE), activator protein (AP)-1, AP-2 and specificity protein 1 (Sp-1), were different among the mouse UCPs, as determined by the computer-assisted analysis of their presumed promoter regions. The region near the transcription-initiation site of the mouse *UCP2* gene lacked a typical TATA box, but was GC-rich, resulting in formation of several potential Sp-1, AP-1 and AP-2 motifs (Figure 2, lower panel).

Analysis of mouse *UCP2* basal promoter activity

To examine the basal transcription activity of the mouse *UCP2* promoter, we constructed plasmids consisting of the mouse *UCP2* promoter fused to the PLAP reporter gene (Figure 1). These constructs were transiently transfected into the cell lines derived from various tissues and into the rat BAT primary culture cells. The results of the transient expression assay in 3T3-L1 pre-adipocytes and rat brown adipocytes are shown in Figure 3. The shortest construct, 6F-2R, from nt –33 to +100 of the mouse *UCP2* gene (nt –33/+100 PLAP, numbered from the transcription-initiation site as +1), exhibited PLAP activity at least 10-fold higher than that of the promoterless construct (PLAP basic vector), implying that this region can be regarded as a minimal promoter. Expression of PLAP activity by the construct 1F-2R (nt –2746/+100 PLAP) was 6.28 ± 3.28 -fold higher than that of the construct 6F-2R. Deletion of the sequences between nt –2746 and –602 in the 1F-2R (construct 4F-2R, nt –601/+100 PLAP) showed greatly increased basal promoter activity (21.47 ± 3.15 -fold), and further deletion from nt –601 to –234 did not significantly alter this enhancement (5F-2R, 15.85 ± 4.36 -fold). These findings indicated not only that a strong enhancer element of basal promoter activity is located between nt –233 and –34 in the mouse *UCP2* promoter, but also that a *cis*-acting negative regulatory element is located within the region from nt –2746 to –602. Furthermore, the region between nt +101 and +1008, which corresponds to intron 1, completely lowered the enhanced basal promoter activity to the level of the

Figure 2 Schematic comparison of the 5'-flanking regions of mouse *UCP1*, *UCP2* and *UCP3* genes

(Upper panel) The transcription-initiation sites are aligned vertically by a dotted line. Boxes represent exons, in which white and black parts indicate untranslated and translated regions, respectively. The dotted lines represent introns. Potential transcription regulatory motifs are shown as follows: TATA box, small black box; CCAAT box, black oval; CCAAT-enhancer-binding protein (C/EBP), white triangle; Sp-1, downward arrow; AP-1, small white box; AP-2, upward arrow; brown-fat regulatory element, hatched triangle; CRE, white oval; MyoD, black triangle; Y box, white diamond. The sequences of the 5'-flanking regions of mouse *UCP1*, *UCP2* and *UCP3* are derived from the sequence data with GenBank/EMBL accession numbers, U63418, AB018416 and AB011070, respectively. (Lower panel) Nucleotide sequence of the GC-rich region spanning from -700 to +100 in the mouse *UCP2* promoter. A transcription-initiation site (+1) is shown by an asterisk. Putative transcription-regulatory motifs are underlined.

minimal promoter activity (21.47 ± 3.15 -fold for 4F-2R versus 1.45 ± 0.16 -fold for 1F-3R). The construct 1F-4R showed the PLAP activity equal to that of the construct 1F-3R.

We next examined whether the above-mentioned enhancer element contributes to the ubiquitous mouse *UCP2* mRNA expression. Plasmids of the 4F-2R, 5F-2R or 6F-2R constructs were transiently transfected into 3T3-L1, NIH/3T3, L6, C₂C₁₂, HIT-15T and Hep-G2 cell lines and into rat BAT primary culture cells. As shown in Figure 4, both 4F-2R and 5F-2R constructs markedly increased basal promoter activity in various

cell types. Especially, the PLAP activities of the 4F-2R and 5F-2R constructs in the Hep-G2 cell line were at least 100-fold higher than that of the 6F-2R construct.

Effects of cAMP on mouse *UCP2* mRNA expression in differentiated 3T3-L1 adipocytes

The mouse *UCP2* gene has three putative CREs; two are present in the 5'-flanking region and one is located within intron 1. In order to study the effects of PKA activation on mouse *UCP2*

Figure 3 Transient expression assay to define the regulatory element controlling basal promoter activity of the mouse UCP2 promoter in 3T3-L1 pre-adipocytes and brown adipocytes

The UCP2 promoter–PLAP reporter plasmids were co-transfected into the 3T3-L1 pre-adipocytes or into the primary culture of rat brown adipocytes with the pSV- β -galactosidase control plasmid. PLAP activity was normalized for the level of β -galactosidase activity as an internal control and expressed as -fold activity of the 6F-2R construct. The data are expressed as the means $\pm$ S.D. of four to eight independent transfection experiments.

Figure 4 Effects of the enhancer element on basal promoter activity of the mouse UCP2 gene

Each of 4F-2R (black bars), 5F-2R (hatched bars) and 6F-2R (white bars, top of the three in each cell type) plasmids were transiently transfected into the indicated cell lines and into the primary culture of rat brown adipocytes with the pSV- β -galactosidase control plasmid. PLAP activity was corrected for the level of β -galactosidase activity and expressed as -fold activity of the 6F-2R construct. The data are represented as the means $\pm$ S.D. of three independent transfection experiments.

mRNA expression, differentiated triacylglycerol-containing 3T3-L1 adipocytes were treated for 12 h in serum-free conditions, and then cultured in the presence of various stimulators for 12 or 24 h. As shown in Figure 5, the differentiated 3T3-L1 adipocytes were able to respond rapidly (within 12 h) to dibutyl-cAMP/IBMX or 8-Br-cAMP/IBMX with a maximal 5-fold increase. The mouse UCP2 mRNA was detected weakly in the DMSO-treated control, but it was significantly increased 12 h after adding the cAMP analogues, and slightly decreased at 24 h. Pioglitazone, a thiazolidinedione that promotes the adipose differentiation, was also able to induce mouse UCP2 mRNA expression in the differentiated triacylglycerol-containing 3T3-L1 adipocytes as reported previously [28]. The expression of

Figure 5 Effects of cAMP analogues, isoproterenol and pioglitazone on mouse UCP2 mRNA expression in differentiated 3T3-L1 adipocytes

(Upper panel) The differentiated triacylglycerol-containing 3T3-L1 adipocytes were treated with the lipid-free medium for 12 h, and cultured in the presence of the indicated compounds for 12 or 24 h. Total RNA was then extracted and analysed by Northern-blot methodology as described in the Experimental section. Lanes 1 and 6, 0.1% DMSO-treated control; lanes 2 and 7, 0.5 mM dibutyl-cAMP (Di-cAMP)/0.2 mM IBMX; lanes 3 and 8, 0.5 mM 8-Br-cAMP/0.2 mM IBMX; lanes 4 and 9, 2 μ M isoproterenol; and lanes 5 and 10, 10 μ M pioglitazone. (Lower panel) Quantification of mouse UCP2 mRNA expression is shown as white bars for 12 h treatment and as black bars for 24 h treatment, expressed as -fold density of each DMSO-treated control. The data are represented as the means $\pm$ S.D.s of three independent experiments.

GPDH and aP2 mRNAs, both of which are known as markers of adipose differentiation, were not increased by cAMP analogues, but induced by pioglitazone. However, when the cells were exposed to isoproterenol, no increase of the mouse UCP2 mRNA expression was noticed.

To assess whether these PKA activators respond to the mouse UCP2 promoter region, we performed a transient expression assay using the UCP2 promoter–PLAP plasmids (the 4F-2R, 5F-2R and 6F-2R constructs) in 3T3-L1 pre-adipocytes. A 3–4-fold

Figure 6 Effect of 5'-deletion mutation on basal promoter activity and PKA activator-inducibility of the mouse *UCP2* gene promoter

Each *UCP2* promoter–PLAP plasmid was transiently transfected into 3T3-L1 pre-adipocytes with the pSV- β -galactosidase control vector, and the PLAP activity of each plate of cells was measured as described in the Experimental section. Cross-hatched bars, 0.1% DMSO; white bars, 2 μ M isoproterenol; hatched bars, 0.5 mM 8-Br-cAMP/0.2 mM IBMX; black bar, 100 μ M forskolin (for each construct, treatments are displayed, top to bottom, in the order shown in the key). PLAP activity was corrected for the level of β -galactosidase activity and normalized to the basal activity of the 6F-2R construct for each experiment. The basal and PKA activator-treated PLAP activities of each 5'-deletion construct are shown as means determined in two independent experiments.

increase of PLAP activity in response to 8-Br-cAMP/IBMX was noted in the constructs that contained at least 233 bp of the 5'-flanking sequence (Figure 6). In addition, these constructs were also able to respond to forskolin with a $\approx$ 3-fold increase. On the other hand, isoproterenol did not enhance PLAP activity in the 3T3-L1 pre-adipocytes.

DISCUSSION

To study the transcription regulation of the mouse *UCP2* gene and to identify *cis*-acting DNA elements important for ubiquitous *UCP2* mRNA expression, an approx. 6.2-kb-long region upstream from the translation-initiation site was cloned using PCR methodology. Several PCR reactions were run and five independent clones were used for sequence determination. As a result, we found that the 5'-UTR of the mouse *UCP2* gene was interrupted by two introns, which is different from that of mouse *UCP1*. The promoter region of the mouse *UCP2* gene lacked a typical TATA box, but was GC-rich, resulting in the presence of several Sp-1, AP-1 and AP-2 motifs near the transcription-initiation site (Figure 2). Computer-assisted sequence analysis of the promoter region revealed several potential consensus sequences for transcription factors thought to be important for the mouse *UCP2* mRNA expression. For instance, the consensus binding motifs for muscle-regulatory protein (MyoD), brown fat regulatory element, CCAAT box, CRE and Y box were found in the mouse *UCP2* promoter region (Figure 2, upper panel). It is proposed that the MyoD-binding site works as a muscle-specific enhancer of murine neurofilament light chain [29] and that the brown-fat regulatory element is an important element for a BAT-specific expression of the *UCP1* gene [17]. In addition, the Y-box element, which was originally defined as an inverted CCAAT motif in the MHC class-II genes, also plays an important role in tissue-specific expression [30]. The CCAAT-box-binding factor (NF-Y) stimulates gene transcription when it

binds to the Y box. Although the MHC class-II gene is expressed in a cell-specific pattern, NF-Y is expressed ubiquitously and binds to the Y-box motif in the non-class-II genes such as albumin, globin and β -actin [31–33]. Thus these transcription-regulatory motifs could be involved in control of the ubiquitous expression of mouse *UCP2* mRNA.

In order to define the *cis*-acting DNA elements necessary for ubiquitous *UCP2* mRNA expression, we subcloned the mouse *UCP2* gene in various-length fragments into the promoterless PLAP basic vector and constructed the plasmids consisting of the mouse *UCP2* gene fused to the PLAP reporter gene (Figure 1). These constructs were transiently transfected into six cell lines of different types and into the primary culture cells of rat BAT. Deletion analysis of the mouse *UCP2* gene identified three transcription-regulatory regions (Figures 3 and 4). (i) A minimal promoter extending from –33 to 100 bp with no TATA box or CAAT box. The constructs that included this region revealed low but obvious activity in the 3T3-L1, L6, C₂C₁₂, NIH/3T3, HIT-15T and Hep-G2 cell lines and in rat BAT primary culture cells. (ii) A strong enhancer was located between nt –233 and –34 from the transcription-initiation site. This enhancer region significantly increased PLAP activity in various cell types. (iii) The presence of two silencers was suggested. One was located within nt –2746 and –602 of the promoter region, and the other was identified in intron 1. The deletion of these region resulted in an increase of PLAP activity, indicating that the deleted regions were concerned with basal transcription regulation of the mouse *UCP2* gene. An inhibitory region has been also identified in other hormonally regulated promoters, including the *UCP1* promoter [18,34,35]. These results indicate that both positive and negative *cis*-acting elements might control the transcription regulation of the mouse *UCP2* gene.

The region from nt –601 to +100 of the mouse *UCP2* promoter contains two potential CRE motifs. These CRE motifs had the same core sequence, CGTCA, and they all deviated in the first three nucleotides from a TGACGTCA consensus sequence. It is known that cAMP regulates the transcription rate of target genes through the phosphorylation of the CRE-binding protein by PKA activation [36–38]. To examine the effect of PKA activation on mouse *UCP2* mRNA expression, differentiated triacylglycerol-containing 3T3-L1 adipocytes were treated with cAMP analogues or pioglitazone, and the change in mouse *UCP2* mRNA expression was analysed by Northern-blot methodology. The results in Figure 5 show that both dibutyryl-cAMP/IBMX and 8-Br-cAMP/IBMX treatments were able to increase the mouse *UCP2* mRNA expression with a $\approx$ 5-fold increase. The expression of aP2 and GPDH mRNAs in the differentiated triacylglycerol-containing 3T3-L1 adipocytes was not increased by cAMP-analogue treatment in 24 h, whereas pioglitazone induced aP2 and GPDH mRNA expression in 24 h in the same experiment. These results show that the induction of mouse *UCP2* mRNA expression by cAMP analogues is not related to adipose differentiation. We also examined the effects of PKA activators on the *UCP2* promoter–PLAP constructs to study the function of potential CRE motifs in detail. We see from Figure 6 that the 4F-2R construct was able to respond to 8-Br-cAMP/IBMX or forskolin with a 3–4-fold increase and that a further deletion fragment, the 5F-2R construct, also responded to them with a maximal 4-fold increase in PLAP activity, suggesting that at least a 233-bp element of the 5'-flanking region is important for cAMP responsiveness. On the other hand, isoproterenol stimulation had no effect on the *UCP2* promoter–PLAP constructs. The same result was also obtained from Northern-blot analysis (Figure 5). The reason why isoproterenol stimulation was not able to induce the mouse *UCP2* mRNA

expression is uncertain from our study. One explanation for the absence of the effect of isoproterenol may be that the cells used in the present study do not express β -adrenergic receptors in appreciable amounts, since isoproterenol stimulation must be able to initiate a cAMP signal-transduction pathway to activate PKA in the same fashion as cAMP/IBMX and forskolin. In addition, although we did not examine the effect of PKA activation on mRNA stability, the agents that elevate intracellular cAMP levels are well known to regulate mRNA stability [39–42]. For instance, treatment with isoproterenol causes the destabilization of β_2 -adrenergic-receptor mRNA and increases the binding of both an A+U-rich element RNA-binding/degradation factor (AUF1)-related protein and a more specific β -adrenergic-receptor mRNA-binding protein to the 3'-UTR of β_2 -adrenergic-receptor mRNA in hamster smooth-muscle cells [43,44]. Therefore, the absence of the isoproterenol effect is possibly due to the deficiency of the β -adrenergic receptors' expression. However, we and others reported previously that no increment of the mouse UCP2 mRNA expression was observed in the white adipose tissue when mice were treated with a β_3 -adrenergic-receptor agonist [19,45]. In addition, little is known about the effect of the 3'-UTR of the mouse UCP2 gene on its mRNA stability. Accordingly, if we take this evidence into consideration, further studies will be required to elucidate the regulation of mouse UCP2 mRNA expression by PKA signal activation.

In conclusion, the enhancer spanning nt –601 to –34 in the mouse UCP2 promoter induced the basal promoter activity; the region located between nt –233 and –34 revealed especially strong enhancer activity. Hence, it is likely that at least 601 bp of the 5'-flanking region contribute to the ubiquitous expression of mouse UCP2 mRNA. Interestingly, this region is GC-rich, resulting in formation of several AP-1, AP-2 and Sp-1 motifs. A role for the AP-1-binding site in aP2 gene expression linked to the adipose differentiation has been proposed, and Sp-1 has been implicated in the transcription of house-keeping-gene promoters [46,47]. Other regulatory motifs such as CCAAT box, Y box and MyoD are also interesting when considering the mechanism of mouse UCP2 mRNA expression. Further experiments on these potential regulatory elements could clarify the detailed mechanism of mouse UCP2 mRNA expression.

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