In Vitro Interactions between Nuclear Proteins and Uncoupling Protein Gene Promoter Reveal Several Putative Transactivating Factors Including Ets1, Retinoid X Receptor, Thyroid Hormone Receptor, and a CACC Box-binding Protein*

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Previous studies of rat ucp (uncoupling protein) gene organization carried out in this laboratory identified regulatory sequences located in the 5'-flanking region. In this work, DNase I footprint analysis of the enhancer revealed two domains at base pairs (bp) -2444 to -2423 and bp -2352 to -2319. The former domain can bind in vitro, in a cooperative manner, factors related to nuclear factor 1 and Ets1; the latter domain contains a type 3 directly repeated sequence that was shown to be able to bind the retinoid X and triiodothyronine receptors. Moreover, a positive effect of retinoic acid on ucp mRNA levels in immortalized brown adipocytes was observed.

DNase I footprint analysis identified two hypersensitive regions, A and B, at bp -509 to -472 and bp -403 to -350, respectively; region A contains a repeated CACC box, and region B can bind protein related to Ets1. The A box differentially binds liver and brown adipose tissue nuclear proteins and could be involved in uncoupling protein induction. Further analysis showed three footprinted boxes, C–E, at bp -182 to -159, -147 to -120, and -111 to -85, able to bind in vitro proteins related to nuclear factor 1, CAMP response element-binding protein, and Sp1, respectively.

The salient feature of the mitochondrial uncoupling protein (UCP) is its unique expression in brown adipocytes, where it provokes the dissipation of oxidation energy as heat. The ucp gene is mainly regulated at the transcriptional level and is positively regulated by norepinephrine acting though β1- and β3-adrenoceptors and subsequent cAMP production. Other hormones such as T3 and insulin also activate ucp gene transcription (1, 2).

Following the isolation of mouse (3), rat (4), and human (5) ucp genes, the first studies of the ucp gene promoter in transgenic mice demonstrated that both tissue-specific and β-adrenergic elements are present in 3.8 or 4.5 kilobases of DNA upstream of the start site of transcription of the mouse (6) or rat (7) ucp gene, respectively. Using transgenic mice bearing ucp minigenes, Boyer and Kozak (6) proposed that a sequence between kilobases -1.2 and -3 of the 5'-start site flanking region was involved in the control of mouse UCP expression. Another study of the rat ucp promoter based on transfection of cultured brown adipocytes by CAT-DNA constructs established the importance of a strong 211-bp enhancer element (regulatory region 1 (R1)) located between bp -2494 and -2283 (7). In the same study, a second region (regulatory region 2 (R2)) located between bp -400 and -157 was suspected to contain negative cis-acting elements; a minimal promoter was identified between bp -157 and -57 (7). Very recently, Kozak et al. (8) identified regions of the mouse ucp gene homologous to the R1 and R2 elements of the rat gene as well as a TTCC motif in R1 and several cAMP response elements (CREs) involved in UCP inducibility. In this study, we have examined in detail the DNA elements present in the promoter of the rat ucp gene and tried to identify proteins binding in vitro to these elements.

MATERIALS AND METHODS

Cell Culture Conditions and Transfections—Primary cultures of brown adipocytes were carried out as described previously (9). 1B8 cells correspond to a cell line derived from a brown fat tumor of a transgenic mouse previously used for the isolation of HIB 1B cells (10, 11); this cell line, when stimulated by catecholamines or cAMP, expresses brown fat-specific mitochondrial UCP. 1B8 and HIB 1B cells were grown and differentiated as described (10). Northern analysis of ucp mRNA in 1B8 cells was carried out as described previously for primary cultures (9). CHO K1 cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum and 10% glucose.

Nuclear Extracts and Protein Purification—Nuclear extracts from Siberian hamster brown adipose tissue, rat liver, and rat kidney were prepared as described (12) in the presence of 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml pepstatin. Nuclear extracts from cells were prepared according to Shapiro et al. (13) using the dialysis buffer described by Gorski et al. (12). For protein purification, nuclear extracts were resuspended in 20 mM Hepes (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 5% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride. Expression in Escherichia coli and purification of GST-βZ protein were achieved according to Tsujiyama and Niwa (14).
labeled by filling in with the Klenow fragment of DNA polymerase I in the presence of \([\alpha-32P]dGTP\), and cut with SpeI (at bp -173 of the promoter). The labeled fragment was isolated after polyacrylamide gel electrophoresis. For analysis of R2, a -241-CAT plasmid (7) was digested with BglII and SacI (position +63) to label one strand or was digested with BglII and BamH1 (position +92) to label the other strand. A 231-BP fragment was obtained by digestion of a -111-CAT construct (7) with BglII and BamHI, subcloned in pUC18 in the two orientations using repaired SacI and SphI sites, cut with EcoRI, and labeled with \([\alpha-32P]dATP\). Binding reactions were carried out for 20 min on ice in a final volume of 50 \(\mu\)l containing 0.5 ng of labeled probe (15,000-20,000 Cerenkov cpm) at a final buffer concentration of 20 mm Hepes (pH 7.6), 100 mm KCl, 0.5 mm EDTA, 1 mM dithiothreitol, 5 mm MgCl2, 2 \(\mu\)g of poly(dI-dC), 2% polyvinyl alcohol, and 10% glycerol. After incubation, DNase I (1 \(\mu\)l, 50-500 ng/\(\mu\)l) was added, and incubation was continued for 1 min at 20 °C. A stock solution of DNase (2 mg/ml in 0.15 M NaCl and 50% glycerol, stored at -80 °C) was freshly diluted in 25 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 100 \(\mu\)g/ml bovine serum albumin, and 10% glycerol (15). The reaction was stopped by the addition of 150 \(\mu\)l of 120 mM NaCl, 0.6% sodium dodecyl sulfate, 12 mM EDTA, 150 \(\mu\)g/ml tRNA, and 90 \(\mu\)g/ml proteinase K, incubated for 1 h at 50 °C; extracted with phenol/chloroform (1:1) and precipitated with ethanol. Dried DNA extracts prior to the addition of the DNA probe; antiserum against DR1 and DR2 were used to confirm the binding of the DNA probes to the DR1 and DR2 elements, respectively.

**RESULTS**

**DNase I Protection Analysis of R1:** Delineation of Boxes FP1 and FP2—We delineated earlier a 211-bp enhancer region (R1, bp -2494 to -2283) using transfection of cultured differentiated brown adipocytes (7); however, this enhancer was shown to be able to activate the minimal promoter of the ucp gene or of a heterologous promoter both in brown adipocytes and in cell lines that do not express UCP. To dissect R1 further, nuclear extracts prepared from either Siberian hamster brown adipose tissue (BAT) or rat liver were used for DNase footprint analysis with a DNA fragment including the 211 nucleotides of R1. Two footprints were observed on both strands of DNA. These footprints, termed FP1 and FP2, span 21 nucleotides from bp -2444 to -2423 (FP1) and 39 nucleotides from bp -2352 to -2219 (FP2) in the coding strand (Fig. 1). A similar picture of FP1 and FP2 was obtained using proteins extracted from rat kidney nuclei (data not shown). Box FP1 includes potential binding sites for NF-1 (17) and Ets1 (18) factors. When footprint analysis was performed in the presence of oligonucleotides corresponding to the binding sites for these factors, competition was observed with the NF-1 oligonucleotide; this competition was abolished when using a FP1 nucleotide containing a mutation of 2 base pairs essential to NF-1 binding, FP1 mut NF-1 (Fig. 1B). In the presence of the Ets1 oligonucleotide, no competition was seen (data not shown).

**Gel Shift Analysis of Protein-DNA Interactions at Box FP1:** Participation of NF-1 and Ets1 Factors—The potential NF-1- and Ets1-binding sites present in the FP1 region were studied using band-shift experiments (Fig. 2). Retarded complexes were observed using a labeled FP1 probe and liver, CHO cell, 1B8 cell (immortalized brown adipocytes), or BAT protein extracts. In competition experiments, the FP2, AP1, C/EBP, and HNF-1 oligonucleotides were ineffective, whereas the FP1, APFP4 (which contains an NF-1-binding site and a C/EBP-binding site), and NF-1 oligonucleotides inhibited the formation of complexes. These data are in agreement with the footprint experiment shown in Fig. 1 and strongly suggest that a protein related to NF-1 binds in vitro to the FP1 box. Although an Ets1 probe was unable to compete with the FP1 probe in footprint experiments, competition was noticed in the band-shift analysis with BAT or liver proteins (low molecular weight complex in Fig. 2, C and D); mutations of 2 essential bases in the Ets1-binding site (Ets1 mut) suppressed the competition (Fig. 2D). Interestingly, a better competition effect of the NF-1 or Ets1 oligonucleotide was obtained when both oligonucleotides were present, suggesting a cooperative effect of the two factors. To demonstrate binding of Ets1 to FP1, purified Ets1 protein was added to the labeled Ets1 probe; under these conditions, the FP1 oligonucleotide behaved as a competitor, and mutations of the Ets1-binding site of FP1 (FP1 mut Ets1) prevented this competition (Fig. 2E).

**Gel Shift Analysis of Protein-DNA Interactions at Box FP2:** Binding of Factors Belonging to Superfamily of Nuclear Hormone Receptors and Related to RXX and T\(_3\) Receptor—A FP2 probe incubated with proteins from several tissues or cells was retarded during gel migration (Fig. 3). Competition was only observed with the FP2 oligonucleotide; an unrelated oligonucleotide such as FP1 did not compete. A FP4 probe, shorter than the FP2 probe, was also retarded (Fig. 3). In fact, both the FP2 and FP4 sequences contain a 5'-AGGTCATA-3' element that has the P box consensus sequence of the first zinc finger of the steroid hormone receptor superfamily, which also includes thyroid hormone receptors, vitamin D3 receptors, retinoic acid receptors, and peroxisome proliferation-activated receptors (19); this element is itself included in the sequence CAAGGTCA, which is a binding site for ELP (16), and is included in a DR3
**Fig. 2.** Gel shift analysis of box FP1; binding of proteins related to NF-1 and Ets families. Labeled probes are indicated by asterisks. Gel-purified double-stranded synthetic oligonucleotide -2444 to -2423 (FP1 box) was end-labeled (FP1*) and incubated with liver (3 µg), CHO cell (1.8 µg), 1B8 cell (4 µg), or BAT (1.8 µg) nuclear extract (A-D). An Ets1 probe and purified Ets1 protein were also used (E). Competition experiments were performed using excess unlabeled double-stranded oligonucleotides as indicated (x-fold mass excess). Nuclear extraction and band-shift assays were carried out as described under "Materials and Methods." The synthetic oligonucleotides are as follows: FP1, AATTCCWCCACGCTTCCTGCCAGAGCATGAG; AP1, AGCTTGATGAGTCAGCCG; CEBP, AAWCAATTGGGCAATCAGG; AFP4, TGCTGTTAATTATTGGCAAATTGCCTAACTTC; HNF-1, GTGTGGTTAATGATCTACAGTTA; NF-1, GATCTTA'MTTGGATTGAAGCCAATATGATA; Ets1, TCGGGCTCGAGATM-CAGGAAGTGGTC; Ets1 mut, TCGGGCTCGAGATAAACACCAAGTGGTGGTC; FP1 mut NF-1, AATTCCWCCACGCWCCTGCGTGAGCATGAG and FP1 mut Ets1, AATTCCCTTCACGCTTGCAGCGAGCATGAG.

element (20), 5'-AGGGCA/GCA/AGGTCA (see Fig. 3). A double point mutation of AGGTCA (FP8 oligonucleotide) (Fig. 3) did not compete with FPA for the formation of the FP4-protein complex, indicating that the AGGTCA consensus sequence is essential. This was the first indication of binding of a nuclear receptor to the FP2 region of the UCP enhancer. When a double mutation (FP7 oligonucleotide) (Fig. 3) of the first element of the putative DR3 sequence was used, competition with the FP4 oligonucleotide, although weaker, was also observed. We also observed that when the FP7 oligonucleotide was used as a probe, the complex obtained with the FP2 or FP4 oligonucleotide was partially destabilized (data not shown). We concluded that the first element of the presumed DR3 sequence was also involved in protein binding. Several nuclear receptors bind to genomic regulatory elements by forming a heterodimer with RXR (20–23). To test the hypothesis of binding of the T3 receptor to the DR3 sequence, which, in fact, is not a consensus sequence for the thyroid hormone-binding site, the FP4 probe was incubated with recombinant v-ErbA protein (Fig. 5). This experiment demonstrated that FP4 can bind in vitro the thyroid hormone receptor. The addition of anti-T3 receptor antibodies to BAT nuclear proteins incubated with the labeled FP4 probe induced a supershifted complex and confirmed that the FP4 region of the ucp promoter contains a binding site for the T3 receptor (Fig. 5).

In other experiments, since a consensus sequence for ELP (which is a member of the superfamily of nuclear receptors) binding is also present in FP2, a FP3 oligonucleotide with a double point mutation at positions known to be necessary for the specific recognition of ELP (16) was used; this oligonucleotide mRNA in 1B8 cells; in addition, retinoic acid promoted the effect of adrenergic agonists on UCP (Fig. 4). No effect of retinoic acid on α-glycerophosphate dehydrogenase, a marker of adipocyte differentiation, was observed. The same observations were made with primary cultures of brown adipocytes. T3 receptors are known to form heterodimers with RXR (20, 23). To test the hypothesis of binding of the T3 receptor to the DR3 sequence, which, in fact, is not a consensus sequence for the thyroid hormone-binding site, the FP4 probe was incubated with recombinant v-ErbA protein (Fig. 5). This experiment demonstrated that FP4 can bind in vitro the thyroid hormone receptor. The addition of anti-T3 receptor antibodies to BAT nuclear proteins incubated with the labeled FP4 probe induced a supershifted complex and confirmed that the FP4 region of the ucp promoter contains a binding site for the T3 receptor (Fig. 5).

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2 S. Pavelka, O. Champigny, and D. Ricquier, unpublished data.
FP2 TCTGAGGCCAGCAAGTCAGCCCTTTTTTGGGA
FP4 GAGGGCAGCAAGGTACCCCT
FP7 GAAAGCAGCAAGTCAGCCT
FP8 GAGGGCAGCAATCAGCCT

![Image](image_url)
The labeled FP4 oligonucleotide (sequence is given in Fig. 3) was incubated with liver nuclear extracts, BAT nuclear extracts, or recombinant v-ErbA protein expressed in baculovirus (1 µg of baculovirus extract) in the presence or absence (+) or absence (−) of antibodies against v-ErbA protein.

Competition with the FP2 probe is shown. The third box identified between these two regions and another region identified in DNase footprinting of the UCP 5' flanking region was confirmed using band-shift analysis (Fig. 10). This type of analysis also revealed an Sp1-binding site in the bp −111/−85 box. The third box identified between these two regions and located between bp −147 and −120 contains a TGACG sequence closely related to that of CRE. Use of an oligonucleotide corresponding to the CREB-binding site in band-shift experiments confirmed the presence of a CRE between bp −147 and −120 of the ucp gene minimal promoter (Fig. 10).

**DISCUSSION**

This work was based on the previous identification (7) of three regions essential to ucp promoter activity: a 211-bp enhancer located at kilobase −2.4 (R1), a more proximal region containing a silencing element (R2), and a minimal promoter (bp −157 to −57). Similar regions were also recently identified in the murine ucp gene (8). The main goal of this work was a detailed in vitro analysis of R1, R2, and regions flanking R2 to delineate short DNA regions of interest and to identify putative transactivating proteins on which future studies will be based.

**Role for Ets1, RXR, and T_{3} Receptor at Level of R1 Enhancer Element**—A partial alignment of the rat R1 enhancer (4) with...
FIG. 8. Gel shift analysis of bp -509/-472 and -403/-350 boxes; correlation with UCP induction. A, the labeled double-stranded bp -509/-472 DNA fragment was incubated with liver, 1B8 cell, or BAT nuclear extracts. In the case of 1B8 cells, nuclear extracts were prepared from control cells or from cells treated with norepinephrine (NE) for 2 or 4 h. Competition was carried out using a 100-fold mass excess of either the homologous oligonucleotide (homol) or a shortened oligonucleotide (CACCC). B, the double-stranded bp -403/-350 fragment was labeled and used as a probe. A 200-fold mass excess of homologous competitor oligonucleotides (comp) was used. Nuclear extraction and band-shift assays were carried out as described under "Materials and Methods." The nucleotide sequences of labeled or unlabeled oligonucleotides are as follows: bp -509/-472 probe, AATTCCTCCCCCCTCCACCCCCACCCCGCCGCGCCCATCG (direct and inverted CACCC repeated boxes are underlined); oligonucleotide CACCC, ATTTCCTCCCCCCTCCACCCCCACCCCGCCGCGCCCATCCTCCCTAG. The sequences of other oligonucleotides are given in the legend of Fig. 2.

FIG. 9. Footprint analysis of protein-DNA interactions at ucp gene R2 proximal domain, and minimal promoter reveals three boxes: bp -182 to -158, -147 to -120, and -111 to -85. DNase I footprinting of the UCP 5'-flanking proximal region was carried out with the bp -241/453 DNA fragment. The probe was incubated with nuclear extracts from rat liver and treated with DNase I under the conditions described under "Materials and Methods." A similar pattern of footprinting was obtained when using brown fat or kidney nuclear extracts. The same windows were observed when using the opposite strand of the probe. Numbers above the lanes correspond to micrograms of nuclear proteins. Competition experiments were carried out in the presence of a 200-fold mass excess of nonradioactive FP1, FP2, AP1, AP4, HNF-1, or AP1 oligonucleotide. Vertical bars delineate footprints. comp, competitor.

the mouse enhancer (8) is given in Fig. 11, which shows a high level of conservation (86% identity). In fact, the FP1 domain of the rat gene is homologous to the CRE-2/brown fat regulatory element 1 element in the mouse gene, and rat FP2 is homolo-
gous to the mouse EcoRV-Xbal region also termed theXBrown fat regulatory element 2 element (8). We have shown that the rat homolog of mouse brown fat regulatory element 1 can bind an Ets1-related protein; such binding had been previously speculated by Kozak et al. (8). Interestingly, mutagenesis of 2 nucleotides in the mouse homolog Ets1 region resulted in 95% reduction in CAT expression of the entire ucp promoter transfected into the nonadipocyte-stimulated brown adipocyte cell line B-7 (8). It can therefore be concluded that this Ets1-binding site plays a crucial role in ucp gene transcription. In the rat DNA region that is homologous to murine brown fat regulatory element 2 and that also contains a TTTG motif, we were unable to detect Ets1 binding. The Ets oncogene family encodes a class of sequence-specific DNA-binding proteins that are involved in cell growth and differentiation (18, 25).

We have attached either the multimerized FP1 or FP2 region to the minimal promoter of the rat ucp gene and to the cat gene. When each plasmid was transfected into brown adipocytes, only a weak CAT transcription could be measured, much lower than that observed with the entire 211-bp R1 enhancer (data not shown). A similar observation was made by Kozak et al. (8). Therefore, it can be concluded that the full activity of the enhancer requires both FP1 and FP2 elements.

The main feature of the 33-bp footprinted FP2 element is the presence of a canonical AGGTCA sequence common to the binding site of all members of the superfamily of nuclear hormone receptors. Although the FP2 probe interacted with a fusion protein containing one-third of ELP, experiments with anti-ELP antibodies did not confirm a possible role for ELP. FP2 contains a potential binding site for ELP; however, this site is unlikely to bind ELP since this protein is known to bind a DNA element that does not contain repeated units (16). In fact, FP2 contains a potential DR3 sequence, which is generally considered as a potential binding site for the receptor of vitamin D3 (19, 26). Utilization of various oligonucleotides and the strong positive effect of an anti-RXR monoclonal antibody in gel shift experiments confirmed that FP2 contains a binding site for a member of the nuclear receptor superfamily. Mader et al. (27) have recently reported that RARs bind with similar efficiency
FIG. 10. Gel shift analysis of bp -182/-158, -147/-120, and -111/-85 boxes; binding sites of NF-1, CREB, and Sp1 factors. Labeled probes were incubated with nuclear extracts from liver, BAT, CHO cells, or IB8 cells. Competitor oligonucleotides (compet) (200-fold mass excess) are indicated above the lanes. The amounts of protein used were 3, 1.8, 6, and 4 μg for liver, BAT, CHO cell, and IB8 cell extracts, respectively. The synthetic Sp1 oligonucleotide used as competitor (ATTCGATCGGGGCGGGCGAGC), although corresponding to a consensus sequence for Sp1, was different from the Sp1 consensus sequence present in the bp -111/-85 box. Nuclear extraction and band-shift assays were carried out as described under "Materials and Methods." The nucleotide sequences of other synthetic oligonucleotides are as follows: CREB, AGA GATTGCCTGACGTCAGAGAGCTAG; FP182, AATTCATCAGGAACTAGTGCAAGCAG; FP147, AATTCCAGGGTGAGTGACGCGTCTG; and FP111, AATTCTGGCCCAGGGCACGCCCTGCGGAATG.

FIG. 11. Schematic organization of rat ucp promoter: cis-elements and trans-factors; comparison with mouse ucp gene enhancer. Two footprints, FP1 and FP2, in the R1 enhancer region contain binding sites for factors related to NF-1, Ets1, RXR, and the triiodothyronine receptor; an alignment of the rat FP1 and FP2 domains (this work) with corresponding regions of the mouse ucp promoter (8) is given. A and B indicate hypersensitive zones particularly obvious during DNase I experiments in the presence of BAT nuclear factors; box A contains a repeated CACCC element previously identified in the β-globin promoter (29), and box B could bind protein related to Ets1. Footprinted boxes C–E can bind NF-1, CREB, and Sp1, respectively. Two C/EBP-binding sites were previously detected (24).

Two response elements containing directly repeated motifs separated by 2, 3, 4, or 5 bp, and they concluded that there is no simple rule defining the binding specificities of RXRs and RARs. RXRs are known to bind DNA by forming heterodimers with RARs, T3 receptors, vitamin D3 receptors, or peroxisome proliferation-activated receptors. On one hand, we report here that all-trans-retinoic acid stimulates ucp mRNA production in brown adipocytes.3 Thus, although the RXR-binding element present in FP2 has a DR3 structure that is a priori distinct from that of the T3 receptor-binding box, it was reasonable to investigate a role for the thyroid hormone receptor. Experiments described here (Fig. 5) establish that the T3 receptor can bind the ucp promoter in vitro, probably through forming a heterodimer with RXR.

Silencer R2 and Flanking Domains: Region Containing Repeated CACCC Elements Interacts Differentially in Vitro with...
Liver or BAT Nuclear Extracts and Could Be Involved in ucp Gene Transcription Induction—In addition to CREB-binding sites at bp -457 to -440 and bp -335 to -318 (24), this region was shown here to contain five potential regulatory elements termed A–E (Fig. 11). Boxes A and B, at bp -509 to -472 and bp -403 to -380, respectively, were detected as hypersensitive regions during DNase I footprint analysis and were shown to be able to bind proteins by using band-shift analysis. Analysis of sequence corresponding to the A and B boxes did not reveal any potential binding site, except for a CCAACCC sequence at bp -495 in region A previously described in the β-globin promoter (29). In fact, two copies of the CACC sequence plus one inverted repeat are present in region A, and it has been shown that such copies may function as important general transcription factors and may also interact with other transcription factors (30).

In other respects, the A box is the only region in the ucp promoter that did not bind liver and BAT factors in a similar manner. As previously observed with HIB 1B cells (10, 11), 1B8 cells do not transcribe the ucp gene, but the addition of norepinephrine strongly activates this transcription (see Fig. 4). Here it was shown that the induction of ucp gene transcription in 1B8 cells activates proteins able to interact with the A box (which does not contain CRE) in a manner similar to that observed with nuclear extracts from BAT. Therefore, a detailed analysis of the A box could constitute a strategy toward the understanding of the cell-specific transcription of the ucp gene.

In this work, we did not intend to identify and demonstrate the functional importance of CRE, as has been done recently for the mouse ucp gene (8). The CREB-binding site identified in the minimal promoter of the rat ucp gene at bp -147 to -120 (3'GCGCGTCA-5') is probably equivalent to CRE-4 identified in the minimal promoter of the murine gene (5'GCGCGTCA-3') and is considered to have more of a promoter function than an enhancer function (8).

In conclusion, several cis-elements and nuclear factors controlling the ucp promoter have been proposed. Two small regions, FP1 and FP2, were identified inside the R1 enhancer using DNase I footprint and band-shift assays. The FP1 element was shown to be able to bind in vitro factors related to NF-1 and Ets1 (in a cooperative manner), whereas FP2 can bind RXR and thyroid hormone receptors. Two DNase I-hypersensitive sites, A and B, particularly obvious in the presence of the pGST-EZ and pRV-ELP plasmids and ELP antisemur, Dr. L. P. Kozak for the gift of the pGPD-1 plasmid, Drs. A. Dejean and J. Janssen and Drs. Giralt and Villarroya for valuable discussions, Dr. M. Garabedian for the gift of 1,25-hydroxycholecalciferol, and D. Vacher for technical assistance. We also express our gratitude to Dr. P. Dijan for critical reading of the manuscript.

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