Transgenic mice were generated with a transgene containing the 211-base pair (bp) enhancer and 0.4 kilobase pairs of 5′-flanking DNA of the uncoupling protein (ucp) gene. Expression of this transgene was restricted to brown adipose tissue and was inducible by cold exposure or treatment of transgenic mice by norepinephrine, retinoic acid (RA), or CL-316,243 β3-adrenoreceptor agonist. A search for retinoic acid response elements in the ucp gene enhancer was undertaken using mutagenesis and transfection of cultured cells with chloramphenicol acetyltransferase constructs. Deletion or mutations of several putative retinoic acid response elements were ineffective. Mutations of a TGAATCA region dramatically decreased the transcriptional activity in the presence of RA. In vitro this region was able to bind a complex containing proteins recognized by antibodies against Jun or Fos. Mutations of an adjacent region related to an inverted repeat of type 2 also markedly decreased RA effect. This region was able to bind in vitro retinoid X receptor α and retinoic acid receptor β.

The two regions form an activating region between bp –2421 and –2402 (referred to as the ucp gene-activating region), which has an enhancer activity but cannot confer RA response to a promoter. This response was obtained with a larger DNA fragment (bp –2489 to –2398) constituting a complex RA response domain.

Transcription of the uncoupling protein (ucp) gene is restricted to brown adipocytes (1, 2). ucp gene transcription is quickly activated following exposure of rodents to the cold (3–6) and is positively regulated by norepinephrine and cAMP (1, 2, 7–11), thyroid hormones (12, 13), and retinoic acid (RA) (14, 15). Data from this laboratory and Kozak’s laboratory pointed out the presence of a strong upstream enhancer that is located between bp –2494 and –2283 in the rat ucp gene 5′-flanking region (16) and between bp –2530 and –2310 in the mouse ucp gene (17). The presence of this positive regulatory region in rat ucp gene was recently confirmed by two other studies (15, 18).

Although the importance of this enhancer in the brown adipose tissue (BAT) specificity is debated, it is clear that it plays a significant role in transcriptional regulation of the ucp gene (16, 17). An analysis of the rat ucp enhancer based on DNase footprint analysis and electrophoretic mobility shift assays, identified two footprints and revealed that retinoid X receptor (RXR) and thyroid hormone receptors (TRs) were putative transactivators of the ucp gene (14). More precisely, RXR binding was proposed to be located between bp –2348 and –2334 (14), a sequence related to a direct type 3 repeat (DR3 repeat), which represents a potential binding site of nuclear receptors (19, 20). In the course of the present work, Alvarez et al. (15) reported that a large deletion of the rat enhancer abolished the RA activation of transcription of a chloramphenical acetyltransferase (CAT) construct, and mutagenesis experiments allowed Silva and colleagues (18) to prove that the DR3 element participates in the activation of ucp gene transcription by thyroid hormones.

The present work, based on studies of transgenic mice and in vitro analysis of the effect of mutations in the rat ucp enhancer, was undertaken in order to investigate the importance of the enhancer and to test the significance of in vitro interaction analysis of RA effect (14). A minigene bearing the 211-bp enhancer was shown to be able to drive a specific and regulated expression of a reporter gene in mouse BAT. Deletion and site-directed mutagenesis of CAT-reporter gene constructs tested in cultured cells demonstrated that the DR3 repeat mentioned above does not mediate the transcriptional activation of ucp by RA. The search for other cis-acting elements mediating RA activation led to the discovery that mutations crippling the response to RA cluster in a region located at the 3′-boundary of the FP1 footprint. This 20-bp region, referred to as the ucp gene-activating region (UAR) is made of a short domain resembling the AP-1 binding site, attached to an inverted repeat of type 2 (IR2). UAR has an enhancer activity,
but its response to RA requires other element(s) located at the 5′-end of the enhancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes were purchased from Appligene (Illkirch) and New England Biolabs (Ozyme, Montigny-Le-Bretonneux, France); [α-32P]dATP, [α-32P]dCTP, [γ-32P]ATP and d-threo-(dichloroacetyl-1-C)chloramphenicol were obtained from Amersham Corp. and ICN. All-trans-retinoic acid was obtained from Sigma. 9-cis-Retinoic acid was a generous gift from Dr. Gutknecht (Hoffman La Roche, Basel). Purified oligonucleotides were purchased from Eurogentec (Seraing, Belgium) or Institut Pasteur (Paris).

**Cell Culture, Western Analysis, RNA Analysis, and Cellular Transfections**—Primary cultures of rat brown adipocytes were carried out as described previously (10). Western analysis of ucp in mitochondria from cultured brown adipocytes was performed as described (9). An immortalized cell line, termed IB8, was cloned from the same tumor used to derive the HIB 1B cell line (21, 22) and was used in the present work. In the absence of cAMP, adrenergic agonist, or RA, this cell line does not derive the HIB 1B cell line (21, 22) and was used in the present work. In the absence of cAMP, adrenergic agonist, or RA, this cell line does not derive the HIB 1B cell line (21, 22) and was used in the present work.

**Construction of the ucp-CAT Transgenic Mice**—Construction of the −400-UCP AA−CAT plasmid containing the 211-bp ucpII-ApoI (enhancer) fragment attached to the first 400 bp located upstream of the transcriptional start site has been described earlier (16). The fragment containing the 211-bp enhancer, 400 bp of the promoter, and the CAT sequence was separated from vector sequences by digestion with BglII and XhoI followed by preparative electrophoresis through agarose gel and electroelution. All microinjection and oviduct transfer procedures were carried out as described (25).

Fertilized eggs were recovered from mating between B6D2F1 hybrids, microinjected with approximately 500 copies of DNA, and transferred to oviducts of C57 BL/6 x CBA-F1 pseudopregnant females. The presence of the transgene in founder animals was checked by Southern blot analysis of tail DNA (26).

**Plasmids, Deletions, and Mutagenesis**—The parent 4551-CAT plasmid contains the 4551 bp of the 5′-flanking region (GenBank™ number X12925) plus the first 110 bp of the transcription unit of the rat ucp gene inserted in the EcoRI and KpnI sites of plasmid pSP73; then the CAT gene was added using KpnI and PvuII sites (16). The D0-CAT construct was made free of the −451/+110 sequence by digestion of the parent 4551-CAT plasmid with KpnI and BglII. The pUCP AA−CAT plasmid contains the minimal promoter of the rat ucp gene (bp −157 to +110) fused to the 211-bp ucpII-ApoI enhancer (bp −2494 to −2283) of the rat ucp gene (16); the same enhancer had been cloned into the unique HindIII restriction site of the pTK CAT plasmid in which CAT expression is under the control of the Herpes simplex virus thymidine kinase (TK) promoter to generate pTK AA− (16). Deletion of a fragment containing a large part of the enhancer (bp −2469 to −2283) from the 4551-CAT construct was made by digestion with BglI and ApoI and religation; this plasmid was named 4551 B/A del. The CAT constructs were subjected to deletion or site-directed mutagenesis using procedure...
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**RESULTS**

**Specific and Inducible Expression of −400-AA′−CAT DNA in Brown Adipose Tissue of Transgenic Mice**—Eight positive founder mice bearing the −400-AA′−CAT DNA (Fig. 1A), as identified by Southern analysis, were outbred to generate heterozygous lines. Four lines of transgenic mice out of six analyzed expressed CAT activity. In the four lines, a low CAT activity was detected in interscapular brown adipose tissue but was undetectable in heart, liver, or brain (Fig. 1B). A higher level of CAT activity was observed in brown adipose tissue when mice were either exposed to a cold environment for 16 h (Fig. 1B) or kept at room temperature but injected with norepinephrine, the β3-adrenoreceptor agonist CL-316,243 (28) or all-trans-RA (Fig. 1, C and D). No such CAT induction was observed in liver, brain, or muscle of these mice. In some mice, treatment by norepinephrine or CL-316,243 also induced CAT activity in white adipose tissue (data not shown). We attribute this induction to the presence of a small number of brown adipocytes as well as dormant brown adipocytes in white fat depots (29). The addition of all-trans-RA to *in vitro* incubated brown fat fragments from transgenic mouse increased CAT activity (data not shown). The data obtained from −400-UCA′-CAT transgenic mice demonstrated that a DNA fragment made of the 211 bp enhancer fused to 400 bp of the proximal promoter contains sequences that can confer both specific transcription in brown fat and induction or activation by the cold, adrenergic agents or RA. Although the promoter alone linked to the CAT gene was not tested in transgenic mice (see “Discussion”), these data strengthened the interest in analyzing mutants of the ucp gene enhancer using cell transfection experiments.

**Retinoic Acid Activates ucp Gene Transcription in 1B8 Cells**—1B8 cells were used to analyze mutants of the ucp gene enhancer. The 1B8 immortalized cells do not express the ucp gene unless they are activated by norepinephrine or RA (14). Fig. 2A shows the time course of ucp mRNA induction in 1B8 cells treated with 1 μM all-trans-RA for 6–30 h. RA also induced ucp mRNA induction in 1B8 cells (Fig. 2A). As soon as 2 h after the RA addition, ucp mRNA induction was observed in 1B8 cells. This early response, most logically explained by transcriptional activation, was prevented by the addition of actinomycin D (Fig. 2B). Moreover, in agreement with Alvarez et al. (15), we also observed RA stimulation in the presence of cycloheximide (at 60 μM); this effect must be independent of protein synthesis (data not shown). High concentrations of all-trans-RA or 9-cis-retinoic acid induces ucp gene expression in 1B8 cells and increases the ucp mRNA level in primary cultures of rat brown adipose tissue. Transient expression of 4551-CAT plasmid in 1B8 cells is stimulated by RA in comparison with isoprenaline or norepinephrine. A. Differentiated 1B8 cells (immortalized brown adipocytes) were treated with 10−6 M all-trans-RA (RA) for 6–30 h. Cells were harvested and both RNA and mitochondria were isolated. ucp mRNA was analyzed by Northern blotting (20 μg of total RNA in every lane was hybridized with a rat ucp cDNA); ucp was detected by Western blotting (10 μg of mitochondrial protein/lane). B. Differentiated 1B8 cells were treated with 10−6 M all-trans-RA for 2–8 h in the presence or absence of 0.4 μM actinomycin D. ucp mRNA (20 μg of RNA/lane) was detected by Northern blotting; equivalent amounts of intact RNA were run in each lane as indicated by hybridization to a probe specific for 18S RNA.

![Image](https://example.com/image.jpg)
RA were necessary to raise ucp mRNA (Fig. 2C). The quantitative effects of RA or isopropenol on ucp mRNA in 1B8 cells were similar (Fig. 2D). RA also increased ucp mRNA in primary cultures of rat brown adipocytes; in this system RA was as potent as CL-316,243 (Fig. 2D, inset). A dose effect of all-trans-RA similar to that observed on the endogenous gene was observed when the 4551-CAT plasmid was transfected in 1B8 cells; RA induced higher CAT expression than norepinephrine (Fig. 2E).

**The Region between bp −2469 and −2283 in the ucp Gene Enhancer Mediates the Positive Effects of Retinoic Acid**—The CAT vector containing no ucp promoter or only the minimal promoter (bp −157 to +110) of the rat ucp gene (pUCP-AA−CAT plasmid) or in the pTK-CAT vector containing the promoter of the herpes simplex thymidine kinase gene (pTK AA−CAT plasmid). 4551 B/A del corresponds to a BclI (bp −2469/Apal (bp −2283) deletion of the 4551-CAT construct. The CAT constructs were or were not cotransfected with RXR or RAR pSG5 expression vectors (no response to retinoic acid was obtained when empty pSG5 vectors were used; data not shown). Cells were treated or not treated with 10−6 M all-trans-RA for 20 h. Each bar corresponds to the mean ± S.E. of four independent experiments, each value being obtained from three (1B8 cells) or two (CHO cells) dishes. *, significant stimulation by RA, p < 0.01. The numbers above the bar representing the +RA data denote the -fold induction of CAT activity (with RA/without RA).

**Fig. 3. Deletion of bp −2469 to −2283 in the rat ucp gene abolishes retinoic acid induction of 4551-CAT plasmid.** Transient transfection experiments of 1B8 cells or CHO cells with CAT constructs made of the entire (4551-CAT) or partial 5′-flanking region of the ucp gene are shown. D0-CAT is a control plasmid lacking the ucp promoter. The AarII-Apal 211-bp enhancer element (AA+, bp −2494 to −2283, see sequence in Fig. 4) was cloned in the sense orientation either in a CAT vector containing the minimal promoter (pUCP-CAT, bp −157 to +110) of the rat ucp gene (pUCP-AA−CAT plasmid) or in the pTK-CAT vector containing the promoter of the herpes simplex thymidine kinase gene (pTK AA−CAT plasmid). 4551 B/A del corresponds to a BclI (bp −2469/Apal (bp −2283) deletion of the 4551-CAT construct. The CAT constructs were or were not cotransfected with RXR or RAR pSG5 expression vectors (no response to retinoic acid was obtained when empty pSG5 vectors were used; data not shown). Cells were treated or not treated with 10−6 M all-trans-RA for 20 h. Each bar corresponds to the mean ± S.E. of four independent experiments, each value being obtained from three (1B8 cells) or two (CHO cells) dishes. *, significant stimulation by RA, p < 0.01. The numbers above the bar representing the +RA data denote the -fold induction of CAT activity (with RA/without RA).

**Fig. 4. Sequence and map of the whole rat ucp enhancer from bp −2494 (AarII site) to bp −2283 (ApaI site).** FP1 and FP2 correspond to in vitro footprinted regions in DNase protection experiments (14). Binding sites of Ets1, nuclear factor 1 (NF1), RXR, or TR (DR3 element) were derived from electrophoretic mobility shift experiments (14). A sequence related to an AP-1 binding site is underlined. tat-ind-HIV-LTR identifies a sequence identical to that implicated in Tat-induced activation of the HIV-LTR (32). The UAR is made of a domain related to AP-1 binding site and a downstream inverted repeat of type 5. TREs were identified between bp −2381 and −2278 and between bp −2348 and −2234 (18). Horizontal arrows identify repeated elements. The complete sequence (GenBank™ number X12925) of the gene, including the 5′-flanking region containing the enhancer, was published by Bouillaud et al. (30).
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Fig. 5. Effects of deletions or mutations in the ucp enhancer on responsiveness to RA. Transient transfection experiments of 1B8 cells with CAT constructs made of the entire (4551-CAT) or the deleted or mutated 5'-flanking region of the ucp gene are shown. The DR3 motif (AGGGCGCAAGGTCA from bp -2348 to -2334) was deleted to create the 4551 DR3 del plasmid. A longer deletion (4551 FP2 del) was also made in the basic construct in order to eliminate the FP2 region (from bp -2357 to -2319), which encompasses the DR3 element. The 3 C residues at positions -2389, -2388, and -2387 belonging to a TRE (18) were mutated into T, T, and A, respectively, to create the 4551 TRE mut. This plasmid was mutated in the DR3 region (the 2 G residues of the half-site changed to 2 A residues) to generate the 4551 TRE/DR3 mut. Deletions of bp -2420 to -2319 and bp -2457 to -2421 were made to obtain 4551 3' del and 4551 5' del constructs, respectively. The plasmids were transiently expressed in 1B8 cells before the CAT assay. Data represent mean ± S.E. (four experiments). **, significant stimulation by all-trans-RA (p < 0.01); *, significant stimulation by all-trans-RA (p < 0.05).

UCP-CAT construct (data not shown). The 211-bp enhancer, attached either to the minimal promoter of the ucp gene (pUCP-AA plasmid) or to the promoter of the TK gene (pTK-A plasmid), conferred inducibility by RA to the reporter gene when transfected into 1B8 cells, even in the absence of cotransfection with a plasmid expressing high levels of RAR or RXR (Fig. 3); similar data were obtained with the -400-UCP-AA plasmid construct (data not shown). RA strongly stimulated expression of the basic 4551-CAT plasmid in 1B8 cells; this effect was not enhanced when RARs or RXRs were overexpressed, suggesting that 1B8 cells contain endogenous RA receptors. Deletion of bp -2469 to -2283 (4551-CAT BA del plasmid), which encompasses the DR3 element (bp -2348 to -2334) previously located by electrophoretic mobility shift assay analysis (14), abolished the RA effect, even when RXRs or RARs were overexpressed (Fig. 3).

These data indicated that bp -2469 to -2283 mediate RA effect. In transfected CHO cells, the same data were obtained, but stimulation by RA was weaker, and the CAT activity was one-tenth of the activity recorded in 1B8 cells. Moreover, RA stimulation of CHO cells was higher in the presence of exogenous RXRs (Fig. 3). In all subsequent experiments with CHO cells, an RXR expression vector was added.

None of the Putative Retinoic Acid Response Elements (RAREs) Present in the Rat ucp Enhancer Mediates RA Response: Effect of Large Deletions on Response to RA—Fig. 4 gives the sequence (30) and organization of the AsnII/ApaI domain (bp -2494 to -2283) forming the whole rat ucp enhancer (16). In gel shift analysis of rat ucp enhancer, we previously identified a 15-bp region AGGGCGCAAGGTCA (bp -2348 to -2334 in Fig. 4), referred to as the RXR/TR DR3 element, that was able to bind RXR or TR in vitro in such a way that the retarded complex could be supershifted by anti-RXR or anti-TR antibodies (14). Moreover, mutagenesis of the two G residues in the downstream half-site completely inhibited RXR binding (14). Therefore, the contribution of DR3 to RA stimulation was tested in 1B8 cells by transiently transfecting CAT constructs (Fig. 5). In fact, deletion of 15 bp forming the DR3 motif did not impair RA responsiveness (4551 DR3 del in Fig. 5), indicating that the DR3 element by itself was not responsible for the RA effect. The sequence of the enhancer shows that the DR3 element is bound on the 5'-side with the sequence AGGCTC (from bp -2356 to -2351, Fig. 4) that may constitute a DR2 element in association with the upstream half-site of the DR3 element (see Fig. 4). Such a DR2 was putatively able to mediate RA effect. Actually, this hypothesis was ruled out by the analysis of the FP2 deletion (bp -2357 to -2319 were deleted) in the parent 4551-CAT plasmid that showed no inhibition of RA stimulation (Fig. 5).

Reexamination of the enhancer sequence revealed two other putative RAREs located between bp -2398 and -2376 (see Fig. 4). Nucleotides -2398 to -2386 may form a DR1 element on the opposite strand (CGGCTCTACCCCT), and nucleotides -2391 to -2376 may constitute an inverted repeat of type 4 (ACCCACTAGGCAA). Interestingly, Rabelo et al. (18) have shown that this latter region, termed upstream thyroid hormone response element (TRE) by the authors, contributes to responsiveness of the ucp gene to triiodothyronine. RA responsiveness of the region between bp -2398 and -2376 was investigated. The three C residues at positions -2389 to -2387, known to be protected from methylation in the presence of purified triiodothyronine Rβ (18), were mutated into TTA in the parent CAT construct (Fig. 5). This triple mutation did not alter the responsiveness of the 4551-CAT construct (4551-CAT mut TRE) to RA in 1B8 cells (Fig. 5). Moreover, the same triple mutation was introduced in a 4551-CAT construct also mutated at the level of the two G residues of the downstream half-site of the RXR/TR DR3 element to give the 4551 TRE/DR3
Mutations in a TGAATCA Region Inhibit RA Responsiveness—Fig. 6A shows that the two large deletions analyzed in Fig. 5 split the bp –2421 to –2415 TGAATCA motif, a sequence resembling the consensus AP-1 binding site (TGA(C/G)TCA; Ref. 31). This motif is located just at the 3’-end of the footprinted FP1 region and is bound to a sequence previously identified as tat-ind-HIV-LTR (32), which is itself inserted in a domain resembling an inverted repeat of type 2 referred to as IR2 (see Figs. 4 and 6A). To further analyze the possible role of this putative AP-1 binding site, we made two types of mutation, preventing Jun and Fos binding (33). The first mutation (4551 AP-1 mut 1) was introduced either in the 4551 CAT DNA or in the 4551 FP2 del plasmid. In the presence of RA, the CAT activity generated from the 4551 AP-1 mut 1 plasmid or the 4551 AP-1 mut 1/FP2 plasmid was strongly decreased in 1B8 cells (Fig. 6B). The second mutation (4551 AP-1 mut 2) also decreased RA responsiveness (Fig. 6B). In CHO cells, a similar inhibition of RA effect on mutants was observed. These experiments demonstrated that integrity of a TGAATCA site, located between bp –2421 and –2415, was required for enhancer activity in the presence of RA.

Mutation of Nucleotides Downstream of TGAATCA Also Inhibits RA Responsiveness: Delineation of UAR, an Activating Region—We were surprised by the finding that mutations of the element related to AP-1 markedly decreased RA responsiveness, since a role for AP-1 factors in transcriptional activation by RA had never been described. A hypothesis explaining the function of TGAATCA in RA activation of ucp transcription is that the RA effect is mediated by an unidentified RARE, which may be inhibited when the AP-1-type site is mutated. This element could be the sequence immediately downstream of the TGAATCA domain: a DNA stretch that is similar to a sequence important in Tat-induced activation of HIV-LTR (31) and that is contained in IR2 (Fig. 6A). Another repeated region related to a nuclear receptor binding site is also present in FP1, upstream of the TGAATCA domain. In other respects, the comparison of mouse (17) and rat (30) enhancer sequences shows that the AP-1-type TGAATCA sequence of the rat gene is replaced by a GAAATCA sequence in the mouse gene. This mouse genomic fragment is not an AP-1 motif and did not compete in a gel shift experiment (see use of mutant oligonucleotide shown in Fig. 7A, right part). However, the sequences that are on both sides of the (T/G)(G/A)AATCA region are highly conserved in the two species. This suggests that these regions contain important cis-acting elements.

In order to delineate cis-acting elements close to the TGAATCA domain, several mutations were made upstream of this domain at position –2427, –2429, –2433, –2435, –2441, –2443, –2451, or –2457 in CAT constructs. RA responsiveness of these CAT constructs was not impaired (data not shown). Then we mutated bp –2407 to –2404 in the IR2 element to create the 4551 IR2 mut CAT DNA (Fig. 6B). The IR2 mutation was also introduced in the 4551 CAT DNA already mutated at the level of the putative AP-1 binding site to give the 4551 AP-1/IR2 mut. The RA responsiveness of 4551 CAT, 4551 AP-1 mut 1, 4551 IR2 mut, and 4551 AP-1/IR2 mut was compared in transfection experiments. Fig. 6B shows that mutation of the IR2 domain decreased RA responsiveness by 80%, indicating that this mutation was almost as effective as mutation of the TGAATCA domain; moreover, mutation of both TGAATCA and IR2 domains decreased RA responsiveness by 85–90%. TGAATCA and IR2 domains form a ucp gene-activating region referred to as UAR (see Fig. 6A).

DNA Mobility Shift Binding Activity of TGAATCA in the ucp Enhancer Reveals Binding of Factors Related to Jun and Fos—The ability of bp –2422 to –2416 of the ucp gene enhancer to bind nuclear factors was analyzed in vitro using an electrophoretic mobility shift assay (Fig. 7). A probe referred to as probe 24 and corresponding to bp –2424 to –2407 was synthesized (see Fig. 4). Probe 24 was labeled with 32P and incubated with nuclear proteins from various tissues. Whereas a very
CREB (20-, 50-, and 100-fold mass excess). Labeled probe 24 was competed with increasing concentrations of either competitor oligonucleotide 24 (20- and 50-fold mass excess) or AP-1 or NIH serum-treated 1B8 cells (50-fold excess) are shown. In the left part, the probe 24, encompassing the putative AP-1 binding site of the enhancer, was incubated with nuclear factors from rat liver (10 μg), control 1B8 cells (1B8c; 5 μg), or BAT (2 and 5 μg). In the right part, the probe 24 was incubated with nuclear factors from 1B8 cells treated with 10−6 M norepinephrine for 4 h (1B8 + NE; 5 μg), or 1B8 cells treated with 10−4 M all-trans-RA for 2 or 4 h (1B8 + RA; 5 μg). Mu oligonucleotide competitor corresponds to the mouse ucp gene fragment located at a position equivalent to the AP-1 binding site of rat gene when enhancers of both species are aligned. In the left and right parts, competitor oligonucleotides were used at a 50-fold mass excess. In the middle part, binding of 1B8 nuclear factors to labeled probe 24 was competed with increasing concentrations of either competitor oligonucleotide 24 (20- and 50-fold mass excess) or AP-1 or CREB (20-, 50-, and 100-fold mass excess). B, probe 24 or AP-1 was incubated with nuclear factors prepared from NIH 3T3 cells untreated (NIH), treated by serum (NIHs), or transformed by Ras (NIHr) NIH 3T3 cells. The same probes were also incubated with nuclear proteins prepared from control (1B8c) or norepinephrine-treated 1B8 cells (1B8 + NE). Competition with cold 24 or AP-1 probe is shown. Antibodies used were broadly reactive against Jun family, Fos family, or CREB family (Atf) or specific for RXRα or RXRβ (Rx) or RARα or RARβ (Ra). On the right, the arrow indicates a supershifted complex. Nuclear extraction and band shift assays as well as details of use of antibodies were as described under “Experimental Procedures.” The sequences of synthetic oligonucleotides were as follows: 24, AATTCATGAAATCAGGCTCTCTG; AP-1, AGCTTGATGAGTCAGGCTCTCTG; IR2, AATTCAGGCTCTCTGGGGATACCG; 24, AATCATGAAATCAGGCTCTCTG; AP-1, AGCTTGATGAGTCAGGCTCTCTG; IR2, AATTCAGGCTCTCTG.

**Fig. 7.** Gel shift analysis of the putative AP-1 binding site in the ucp gene enhancer. A, in the left part, the labeled probe 24, encompassing the putative AP-1 binding site of the enhancer, was incubated with nuclear factors from rat liver (10 μg), control 1B8 cells (1B8c; 5 μg), or BAT (2 and 5 μg). In the right part, the probe 24 was incubated with nuclear factors from 1B8 cells treated with 10−6 M norepinephrine for 4 h (1B8 + NE; 5 μg), or 1B8 cells treated with 10−4 M all-trans-RA for 2 or 4 h (1B8 + RA; 5 μg). Mu oligonucleotide competitor corresponds to the mouse ucp gene fragment located at a position equivalent to the AP-1 binding site of rat gene when enhancers of both species are aligned. In the left and right parts, competitor oligonucleotides were used at a 50-fold mass excess. In the middle part, binding of 1B8 nuclear factors to labeled probe 24 was competed with increasing concentrations of either competitor oligonucleotide 24 (20- and 50-fold mass excess) or AP-1 or CREB (20-, 50-, and 100-fold mass excess). B, probe 24 or AP-1 was incubated with nuclear factors prepared from NIH 3T3 cells untreated (NIH), treated by serum (NIHs), or transformed by Ras (NIHr) NIH 3T3 cells. The same probes were also incubated with nuclear proteins prepared from control (1B8c) or norepinephrine-treated 1B8 cells (1B8 + NE). Competition with cold 24 or AP-1 probe is shown. Antibodies used were broadly reactive against Jun family, Fos family, or CREB family (Atf) or specific for RXRα or RXRβ (Rx) or RARα or RARβ (Ra). On the right, the arrow indicates a supershifted complex. Nuclear extraction and band shift assays as well as details of use of antibodies were as described under “Experimental Procedures.” The sequences of synthetic oligonucleotides were as follows: 24, AATTCATGAAATCAGGCTCTCTG; AP-1, AGCTTGATGAGTCAGGCTCTCTG; IR2, AATTCAGGCTCTCTGGGGATACCG; 24, AATCATGAAATCAGGCTCTCTG; AP-1, AGCTTGATGAGTCAGGCTCTCTG; IR2, AATTCAGGCTCTCTG.

**Fig. 8.** Gel shift analysis of IR2 in ucp gene enhancer. In the right panel, the labeled probe IR2 was incubated with nuclear factors from 1B8 cells (1B8; 5 μg); competition with cold IR2 or 24 probe (50-fold excess) is shown. In the left panel, the IR2 probe was incubated with nuclear factors from 1B8 cells (5 μg) or rat liver (L; 10 μg). Antibodies used were specifically reactive against RXRα, RXRβ, or RXRγ or broadly reactive against TRs, CREB family (Atf), or Jun family (Jun). The use of antibodies was as described under “Experimental Procedures.” The sequences of synthetic oligonucleotides were as follows: IR2, AATTCAGGCTCTCTGGGGATACCG; 24, AATCATGAAATCAGGCTCTCTG.

Faint retardation was observed when using liver factors, a sharp retardation was observed with 1B8 cell proteins; a similar retardation was also obtained with nuclear proteins of hamster brown adipose tissue. Oligonucleotides corresponding to footprinted FP1 and FP2 did not compete with the probe 24. Competition was observed in the presence of an excess of 24, AP-1, or cAMP response element-binding protein (CREB) oligonucleotides (Fig. 7A, left). AP-1 oligonucleotide containing the motif TGAGTCA was a better competitor than CREB oligonucleotide containing the sequence TGAGTCA (Fig. 7A, middle). Moreover, a higher amount of probe was retained by proteins prepared from norepinephrine-stimulated 1B8 cells, whereas treatment of cells by RA did not increase retardation (Fig. 7A, right). Such an increase in binding with factors from norepinephrine-stimulated 1B8 cells was not obtained when using other probes such as Sp1 and CREB probes (data not shown). In order to approach the identity of protein(s) retained by the 24 DNA, this fragment was tested in the presence of nuclear proteins extracted from NIH 3T3 cells stimulated by fetal calf serum or transformed by RAS; both treatments induce AP-1 binding factors (34). Retardation was increased when the 24 probe was incubated with factors from activated NIH cells. A 50-fold excess of 24 oligonucleotide competed effectively (Fig. 7B, left). The band obtained with the 24 probe in the presence of extracts from 1B8 cells was also observed when using the AP-1 oligonucleotide as a probe; in that case, the complex was sensitive to the addition of antibodies against AP-1 factors (Fig. 7B, middle). Moreover, the complex obtained between the 24 probe and 1B8 extracts was inhibited in the presence of antibodies broadly reactive with c-Jun, Jun B, and Jun D proteins.
and was supershifted in the presence of antibodies broadly reactive with c-Fos, Fos-B, Fra-1, and Fra-2; in the same experiment, neither ATF-1 antibodies reactive with ATF-1, CREB-1, and CREM-1 proteins nor antibodies against RARα altered the shift (Fig. 7B, right); however, in the same experiment, antibodies against RXRa were slight inhibitors (Fig. 7B, right; see “Discussion”). The binding of 1B8 factors (sensitive to anti-ATF-1 antibodies) to a CREB probe was not stimulated by factors from norepinephrine-treated 1B8 cells, since it was observed with the 24 or AP-1 probe (data not shown).

DNA Mobility Shift Binding Activity of IR2 Reveals Binding of Factors Related to RXRa and RARβ—The ability of IR2 to bind nuclear factors was analyzed in vitro using an electrophoretic mobility shift assay (Fig. 8). A weak binding was obtained with liver nuclear factors, whereas a strong and specific complex was obtained with nuclear factors from 1B8 cells. The probe 24 corresponding to the putative AP-1 binding site of the rat ucp gene enhancer did not compete with IR2 probe. When using a series of antibodies against transcriptional factors, the complex retained by the IR2 probe was inhibited (and not supershifted) in the presence of antibodies against RXRa and RARβ. Antibodies against RXRa, RARγ, TR receptors, or members of CREB or Jun families were ineffective (Fig. 8). These data demonstrated that proteins related to RXRa and RARβ can bind to IR2. Moreover, treatment of brown adipocytes by RA induced the binding to the IR2 probe (data not shown).

Nucleotides Upstream of UAR and FP1 Are Required for RA Responsiveness: Enhancer Activity of UAR—Since several independent mutations in UAR strongly decreased the transcriptional activity of the ucp gene in presence of RA, transient transfection experiments were performed to test whether it was able to mediate a RA response in 1B8 cells. In fact, TGAATCA alone (data not shown), UAR (bp −2421 to −2402), or three tandem copies of UAR did not mediate RA effect (Fig. 9). The addition of FP1 (bp −2444 to −2423) to UAR did not confer RA responsiveness. We deleted nucleotides from the 5′-extremity of the enhancer (bp −2494) to the BclI site (bp −2469) and observed a significant but partial reduction of the RA effect, compared with the response of the pUCP-AA′-CAT construct (Fig. 9). These data indicated that nucleotides forming the BclI site or upstream of this site participate in the RA effect. It was decided to assay the RA response of a 92-bp DNA spanning the 5′-extremity of the enhancer to the 3′-end of the UAR. This 92-bp DNA conferred a strong response to RA both in the context of ucp gene minimal promoter and TK promoter. Similar data were obtained when transfecting CHO cells (data not shown).

In Fig. 9, it is shown that the basal activity of a plasmid bearing three tandem copies of UAR was 83% of the activity obtained with the whole 211-bp enhancer. These data point to the enhancer activity of UAR, at least in the context of the ucp gene minimal promoter.

DISCUSSION

We had shown earlier that cis-acting elements of the rat ucp gene are present in a 211-bp enhancer at −2.4 kilobase pairs (14). By performing cell transfections with constructs containing various deletions of the mouse ucp 5′-flanking region, Kozak et al. have proposed that the enhancer is essential for brown fat specificity (17). In order to further analyze the role of the rat enhancer in tissue specificity and to identify the cis-acting elements present inside this enhancer that are responsible for both brown fat specificity and regulation by hormonal factors such as retinoids, we have set up a strategy based both on creation of transgenic mice and transient expression of CAT constructs in cell lines.

Supershifted (in the presence of antibodies against RXR)
The specific and regulated expression of the −400-AA−CAT DNA in brown adipose tissue of several lines of transgenic mice allowed the location of essential cis-acting elements, since no transgene expression occurred in tissues other than BAT, even in animals treated with adrenergic compounds or RA. The marked effect of CL-316,243 on transgene transcription in brown fat agrees with the known ability of β3-adrenoreceptors to activate ucp gene transcription (36, 37). The rather weak stimulation by norepinephrine and RA observed in transgenic mice compared with the CL-316,243 effect may result from different degradation rates of the inducers or from dose effect.

The −400-AA− DNA is the smallest fragment known to be able to drive expression of a reporter gene specifically in brown adipose tissue. In fact, the present data belong to a detailed and not yet finished analysis of cis-acting elements of ucp gene in transgenic mice. Since we have no data yet with transgenic mice bearing the −400 bp promoter only, the type of construct used in −400-AA−CAT transgenic mice did not allow us to discriminate between the respective roles of the −400 bp promoter and the 211-bp enhancer located at −2.4 kilobase pairs. However, since Boyer and Kozak (35) were unable to detect any expression of a mouse ucp minigene in transgenic mice in which only 1.2 kilobase pairs of the 5′-flanking region was used, it may be concluded that the enhancer itself is essential for specific expression in brown adipose tissue. The creation of new transgenic mice containing either the enhancer alone attached to a minimal promoter or the 4551-bp DNA deleted from bp −2494 to −2283 will definitively establish the essential role of the ucp enhancer in tissue specificity.

Retinoids are positive effectors of adipose cell differentiation, and growing adipoblasts contain RAR and RXR mRNAs (38, 39). Moreover, differentiated adipocytes respond to RA with a rapid increase in S14 (40) and phosphoepinephrine carboxykinase gene transcription (41). A goal of the present work was a functional analysis in transfected cells of cis-acting elements present in rat ucp enhancer and involved in RA activation of ucp gene transcription.

Transfection of 1B8 or CHO cells with deleted and mutated CAT constructs demonstrated that neither the DR5 region nor the FP2 region mediates RA effect. This may be explained by the fact that the DR5 sequence differs from that of typical RARE. Moreover, it is known that RAR-RXR heterodimers and RXR homodimers strongly activate transcription from directly repeated RGGTCA separated by 1, 2, 5, 10, or more bp and weakly activate transcription from directly repeated RGGTCA separated by 3 bp (42–44). Our data also indicate that other putative RAREs do not mediate RA effect. Moreover, polymers of footprinted FP1 and FP2 regions (14) attached to a minimal promoter fused to the CAT gene and transfected in 1B8 cells responded very weakly to RA (data not shown).

UAR is the only region in which mutations strongly impaired RA responsiveness. This region can in vitro bind RXRs and RARs precisely at the IB2 level and probably constitutes a nonconventional site for RA effect in the ucp gene. Mutations of the TGAATCA sequence revealed that the integrity of this domain was required for high transcriptional activity in the presence of RA. Although electrophoretic mobility shift assays and use of various antibodies suggested that the TGAATCA region may be related to an AP-1 binding site, our data do not demonstrate that this domain present in the enhancer is a functional AP-1 binding site. Such a role for the AP-1 binding site in transcriptional activation by RA has never been reported. Moreover, we were unable to see any increase in ucp mRNA or 4551-CAT activity in 1B8 cells treated with phorbol esters (data not shown). Retinoid effects are mediated by the RARs and RXRs, both of which induce transcriptional activa-

Note Added in Proof—Data similar to those proteins present in this article were reported recently by Rabelo et al. (Rabelo, R., Reyes, C., Schifman, A., and Silva, J. E. (1996) Endocrinology 137, 3488–3496).

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3 M. Larose and D. Ricquier, unpublished data.
