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An Upstream Enhancer Regulating Brown-Fat-Specific Expression of the Mitochondrial Uncoupling Protein Gene

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Previous studies on the regulation of a *Ucp* minigene in transgenic mice demonstrated that the sequences necessary for brown-fat-specific expression and inducibility by norepinephrine were located in the 5′ flanking region between 1 and 2.8 kb from the transcriptional start site. We have investigated this region in more detail in cultured mouse brown adipocyte tumor cells. Deletion analysis of two types of chloramphenicol acetyltransferase reporter gene constructs under control of either the *Ucp* promoter or a heterologous herpes simplex virus-*tk* promoter defined an enhancer in a 220-bp *Hin*dIII-*Xba*I fragment which was essential for both brown fat specificity and norepinephrine inducibility. Site-directed mutagenesis of the reporter gene constructs established that independent mutations to a cyclic AMP-responsive element (CRE-2) or one of two TTCC motifs (BRE [brown fat regulatory element]), all within 17 bp, eliminated transient expression. Competitive DNA mobility shift assays with probes of the CRE and BRE motifs indicate that nuclear proteins interact with these motifs in a cooperative, synergistic manner. While these CRE-BRE probes do not show changes in binding which is dependent on norepinephrine treatment, a probe containing a third TTCC motif located 130 bp downstream of BRE-1 does show this dependency. The results indicate that a complex interaction of the CRE and BRE motifs, which cannot be functionally separated, control *Ucp* expression.

The mitochondrial uncoupling protein (UCP) is located in the inner mitochondrial membrane of brown fat, where it is an essential component of a mechanism to produce heat by uncoupling respiration from oxidative phosphorylation (24). The expression of the gene encoding UCP (*Ucp*), occurring only in brown fat, is induced in response to the thermal requirements of the animal (17). The specific needs for heat which are contributed to by brown fat thermogenesis include exposure to cold, particularly in the newborn mammal; arousal from hibernation; and possibly the control of energy balance (28, 29). The latter need, although unproven, could be important in the control of obesity. To understand the role of brown fat thermogenesis in energy balance, it is important to understand the mechanisms which control the content of brown fat in an animal and the level of UCP in the brown adipocyte.

Induction of the *Ucp* gene is controlled centrally from the hypothalamus via the sympathetic nervous system (11). Evidence from the study of rodents with in vivo and in vitro systems suggest that norepinephrine binds to β1 and β3 adrenergic receptors to initiate a cyclic AMP (cAMP) signal transduction pathway to activate *Ucp* transcription (1, 2, 19, 26). It has been shown that in transgenic mice, the regulatory signals for brown-fat-specific expression and cold inducibility are present in a 1.8-kb region of 5′ flanking DNA located approximately 1 kb upstream from the *Ucp* transcriptional start site (4). Within this fragment of DNA, near a DNase I-hypersensitive site at 2.8 kb, are consensus sequences for cAMP-responsive elements (CREs). These results identified a region of DNA which has properties required for the brown-fat-specific regulation of *Ucp* in the animal during cold exposure. Recently, Cassard-Doulcier et al. (5), using transgenic mice and transient expression assays, also determined that a region controlling brown fat expression and norepinephrine induction of the rat *Ucp* gene was located −2.5 kb from the *Ucp* transcriptional start site. In this study, we have used transient expression assays, site-directed mutagenesis, and gel shift assays for nuclear proteins to precisely define the regulatory motifs required for *Ucp* expression in brown fat and induction by norepinephrine.

MATERIALS AND METHODS

Cell cultures and transfection. The majority of the transient expression assays were carried out with primary brown fat adipocyte tumor (BAT) cells before a brown adipocyte cell line was developed. Following the development of the B-7 line, the results on transient expression were confirmed in the cell line. All nuclear extracts for DNA mobility gel shift assays were prepared from the B-7 line.

For transient expression assays in primary cultures, BAT cells were cultured in standard medium plus 10% calf serum as described previously (19). Twenty-four hours after seeding, cultures were washed extensively to remove nonadherent cells. On the second day, the medium was changed 3 to 4 h prior to transfection. The protocol for calcium phosphate-mediated transfection was as described by Wigler et al. (33). Ten to twenty micrograms of DNA was added to each 10-cm-diameter plate. After 4 h of incubation, cells were shocked with 3 ml of 15% glycerol for 2 min, washed with 10 ml of medium, and fed with 10 ml of fresh medium. The medium was replaced after 24 h, and norepinephrine was added where required. Twelve hours later, the cells were harvested and lysates were prepared for chloramphenicol acetyltransferase (CAT) assays as described by Gorman et al. (12). CAT activity was determined by incubating 50 μl of cell extract with 0.25 μCi of [^{14}C]chloramphenicol–0.5

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mM acetyl coenzyme A in 0.25 M Tris (pH 7.8) at 37°C for 60 to 180 min. The amount of substrate acetylated was directly quantified with a radioimaging device (AMBIS). The data reported represent the means and standard deviations of two or three replicate plates from a single experiment. Each result was confirmed by at least two experiments.

For experiments with the brown adipocyte cell line B-7, an ampoule of frozen cells was resuscitated, plated, and expanded in standard medium containing 10% fetal calf serum and 1 μM norepinephrine. Two passages prior to transfection or isolation of nuclear proteins, medium was changed to standard medium containing 10% calf serum, 5×10^{-10} M T3, and 5×10^{-8} M insulin. PC-12 cells were cultured in Dulbecco's modified Eagle medium plus 10% fetal calf serum; 3T3-L1 and 3T3-442A cells were cultured in growth medium prior to confluency and then in differentiation medium as described previously (14). For transient expression assays, 24 h prior to transfection, 1.5×10^6 cells were seeded into 10-cm-diameter dishes. Cells for the isolation of nuclear proteins were grown to approximately 90% confluency.

Plasmids. The plasmid constructs for transient expression assays to identify regulatory motifs controlling *Ucp* expression utilized pBLCAT5, which contains a polylinker site, the thymidine kinase (*tk*) promoter, and the CAT gene, and pBLCAT6, which is similar to pBLCAT5 but promoterless (21). The 3.85-kb *Bgl*I fragment from +109 to −3740 bp was isolated from a genomic *Ucp* clone in the pGEM vector (18) and blunt-end ligated into the *Sph*I site of the polylinker region of pBLCAT5, upstream from the *tk* promoter. Plasmid BBtkCAT, with 5′-3′ orientation of the *Bgl*I fragment, and the plasmid 3′BBtkCAT, with opposite orientation of the insert, were obtained. Deletions in the construct were generated by cutting BBtkCAT at restriction enzyme sites in the polylinker region and within the *Ucp* insert and religating directly if compatible cohesive ends were generated or by filling in the ends with either Klenow fragment or T4 polymerase and then religating when noncohesive ends were generated. To define the regulatory regions in 5′ flanking DNA of *Ucp* when the CAT gene is under control of the *Ucp* promoter, plasmid pBLCAT6 (21) was used. The same 3.85-kb *Bgl*I fragment as specified above was isolated, the *Bgl*I sites at the ends of the fragment were replaced by *Bam*HI sites with a synthetic linker, and the fragment was ligated into the *Bam*HI site in the polylinker region of plasmid pBLCAT6, upstream from the CAT gene. Plasmid BBCAT, with 5′-3′ orientation of the *Bgl*I fragment, and plasmid 3′BBCAT, with opposite orientation of the insert, were obtained. Deletions from the 5′ end of the *Bgl*I-*Bgl*I fragment were generated by using known restriction enzyme sites in the polylinker region and *Ucp* insert as described above for the pBLCAT5 series. Plasmids were extensively characterized by restriction enzyme mapping and purified initially by two centrifugations through cesium chloride and then later with Qiagen midiprep kits.

Site-directed mutagenesis. The transformer site-directed mutagenesis kit of Clontech was used to introduce mutations directly into the double-stranded BBCAT vector (8). The procedure depends on conversion of the *Pvu*I site in the pBLCAT vector sequence to a *Bsi*WI site with the oligonucleotide TCCTCCGTACGTTGTCAGAAG; neither site is present in the *Ucp* region. Oligonucleotides used to mutagenize sites in the *Ucp* sequence are listed in the figure legends. Reverse mutations to CRE-4 in *Ucp* depended on reconversion of the *Bsi*WI site to the *Pvu*I site. All mutations were characterized by dideoxynucleotide sequencing, using the Sequenase kit from U.S. Biochemical.

Nuclear extracts and DNA mobility shift assay. Nuclear extracts for DNA mobility shift assays were prepared as described previously (9). Buffers for the preparation of nuclear extracts contained the protease inhibitors phenymethylsulfonyl fluoride (0.5 mM), 10 μM α-*p*-tosyl-L-lysinechloromethylketone (10 μM), aprotinin (8 μl/ml), leupeptin (5 μg/ml), and pepstatin, antipain, and chymostatin (0.5 μg/ml each), and the phosphatase inhibitors dibasic sodium phosphate (20 mM), sodium fluoride (5 mM), and ammonium molybdate (0.2 mM). End labelling of double-stranded oligonucleotides was carried out with [^{32}P]dCTP, using T4 DNA polymerase. Protein-DNA binding reactions were performed in a volume of 20 μl containing 20 mM Tris-HCl (pH 7.6), 75 mM NaCl, 5% glycerol, 0.1 mM EDTA, 1.0 mM dithiothreitol, 1.5 μg of poly(dI-dC), and 5 to 8 μg of nuclear protein extract. After 10 min of preincubation at room temperature, approximately 30,000 cpm of labelled probe (0.3 to 1.0 ng) was added, and the reaction mixture was incubated for 20 min at room temperature. For competition assays, poly(dI-dC) and unlabelled competitors were incubated at room temperature and then incubated for 20 min after addition of the labelled probe. After the inclusion of 2 ml of loading dye, the samples were electrophoresed on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer for 2 h at 150 V. Gels were dried and autoradiographed overnight.

RESULTS

The analysis of *Ucp* expression in transgenic mice provided three important pieces of information: (i) the minigene contained regulatory information for brown-fat-specific expression and induction by cold; (ii) an element or elements critical to specific expression was located in the 5′ flanking region between −1 and −2.9 kb from the transcription start site; and (iii) a minigene with only 1 kb of the 5′ flanking region was silent even though a CRE (CRE-1) was located in the region, suggesting that a silencer or negative element was uncovered upon removal of the 1.8 kb of DNA located further upstream. Locations of the regulatory functions in the *Ucp* gene are summarized in Fig. 1.

Deletion analysis in transient expression assays. To define more precisely the locations, sequences, and functions of positive and negative regulatory elements controlling *Ucp* expression, we have developed transient expression assays utilizing a CAT expression vector with and without the *tk* promoter (pBLCAT5 and pBLCAT6, respectively). Plasmid constructs carrying deletions of the 5′ flanking region were transfected into primary cultures of BAT cells. The characteristics of these cells, including the induction of *Ucp* expression by β1 agonists, have recently been described (19). High CAT activity occurred with constructs extending from a *Bgl*I site (+110) within the first exon to a *Bgl*I site at −3740 (BBCAT; Fig. 2). Norepinephrine-induced activity was higher with an *Sph*I-*Bgl*I CAT (SpHCAT) construct but then dropped off rapidly with the *Xba*I-*Bgl*I CAT (XBCAT) construct. The ratio of CAT activity in the presence versus the absence of norepinephrine was maximal with BBCAT (65-fold), was 24-fold with SpHCAT, and then dropped to 5-fold with XBCAT and remained at that ratio through the remainder of the deletion series. The analysis indicated that a positive regulatory element was located in a region of 630 bp from −2.9 to −2.3 kb from the transcription start site. The deletion series also showed that the lowest level of CAT activity occurred with *Hin*dIII-*Bgl*I (HBCAT); the recovery

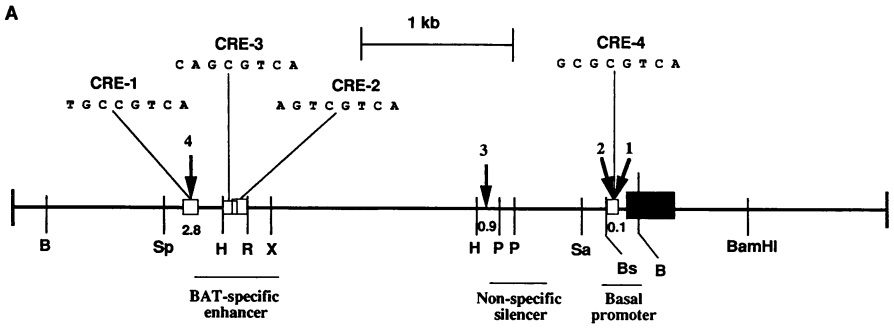

FIG. 1. (A) Map of the 5′ flanking region of the mouse *Ucp* gene showing the locations and sequences of the four CREs. The CRE consensus sequence is TGACGTCA. The numbered arrows refer to DNase I-hypersensitive sites previously mapped at approximately 0.1, 0.9, and 2.8 kb from the transcriptional start site. The solid box marks the location of exon I. Restriction enzyme sites: B, *Bgl*I; Sp, *Sph*I; H, *Hin*dIII; R, *Eco*RV; X, *Xba*I; P, *Pst*I; Sa, *Sac*II; Bs, *Bst*XI; *Bam*HI. (B) DNA sequence of the *Hin*dIII-*Xba*I fragment.

of a basal level of expression with additional deletions suggest the presence of a repressor element between −272 and −950. These in vitro results are consistent with those from the in vivo transgenic experiment (4).

Expression assays with plasmid constructs containing parts of the 5′ flanking region ligated to the heterologous *tk* promoter confirm and extended the results obtained with CAT transcribed from the endogenous *Ucp* promoter (Fig. 3). The region between −2410 and −3740 bp is capable of stimulating transcription from the heterologous *tk* promoter in brown fat when norepinephrine is added to the cultures. This result together with data in Fig. 2, which show that XBCAT (−2310 to +110) has lost expression, establishes the presence of a strong positive element from −3740 to −2410. An additional finding from these *tk*-CAT constructs is that the construct with the *Hin*dIII-*Bgl*I (−950 to +110) fragment is inactive, suggesting the presence of a strong negative element in this region which is overcome by the positive upstream elements.

A pattern of transient expression, consistent with the in vivo transgenic experiments, was observed only when the plasmids were transfected into brown adipocyte cultures. The plasmids described in Fig. 2 and 3 were also assayed in both differentiated and undifferentiated 3T3-442A preadipocytes. No activity could be detected in either the presence or absence of forskolin (data not shown). In addition, when the plasmids were transfected into PC-12 cells, forskolin stimulated transcription nonspecifically and promiscuously (data not shown). The exception to this nonspecific activity was that the HBCAT construct also carried an element which was inhibitory in PC-12 cells as it was in the brown fat cells.

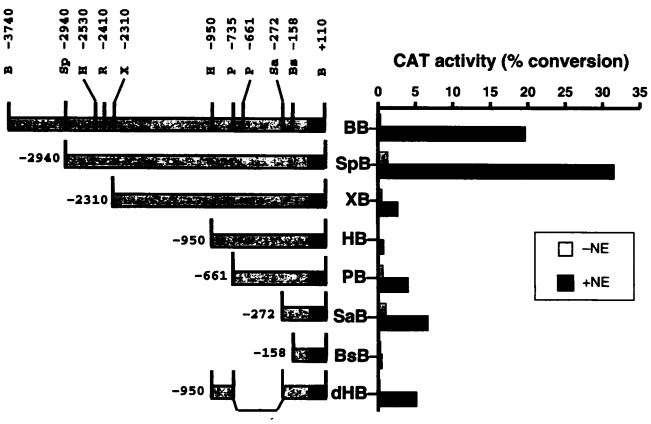

FIG. 2. Transient expression assays of pBLCAT6-derived plasmids, using the *Ucp* promoter and various lengths of the 5′ flanking region to drive transcription of CAT. Restriction enzyme sites used to construct the plasmids are defined in the legend to Fig. 1. Plasmids were transfected into primary BAT cells cultured in the absence or presence of 1 μM norepinephrine (NE). Data are based on duplicate assays of two separate experiments.

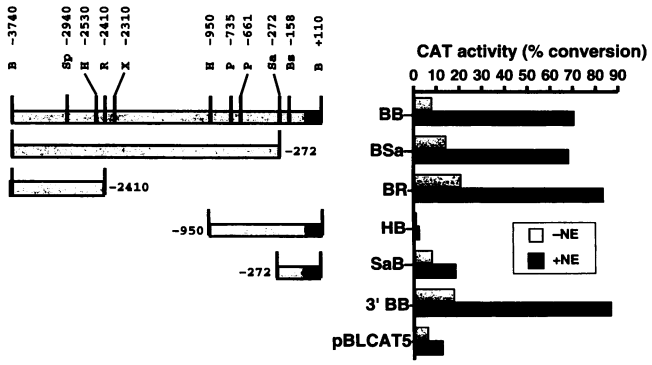

FIG. 3. Transient expression assays of pBLCAT5 plasmids which contain a herpes simplex virus *tk* promoter in front of the CAT gene. Data are based on duplicate assays. NE, norepinephrine.

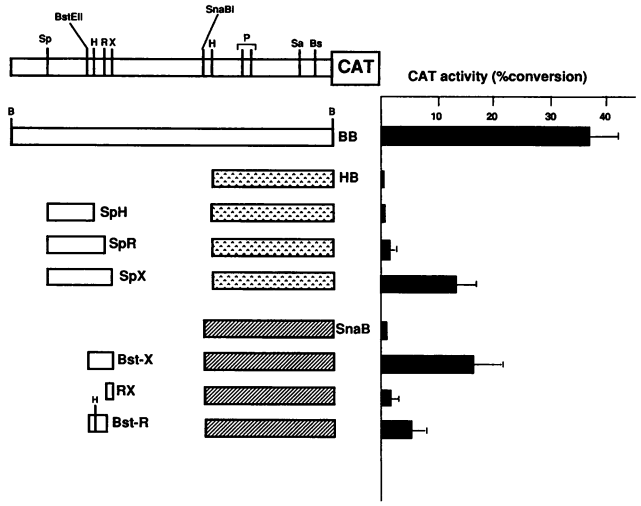

FIG. 4. Transient expression assays to define the enhancer region controlling induction of pBLCAT6 plasmids by norepinephrine in brown fat cells. The initial constructs tested the ability of upstream fragments to overcome repression by the *Hin*dIII-*Bgl*I fragment. Subsequently, in order to construct the more defined Bst-X, RX, and Bst-R plasmids, it was necessary to use the *Sna*BI-*Bgl*I fragment for the silencer and promoter regions. This fragment was as effective as the *Hin*dIII-*Bgl*I fragment in repressing CAT expression. All constructs were tested in the presence and absence of norepinephrine, but only data with norepinephrine are presented. In no case did the CAT activity in the absence of norepinephrine exceed 2% of BBCAT activity levels. Data are presented as the means and standard deviations of three determinations.

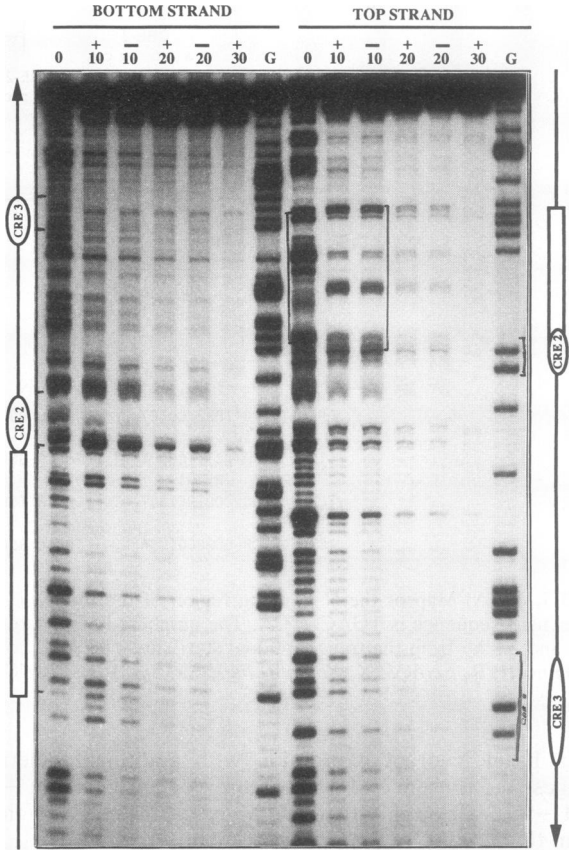

FIG. 5. DNase I footprint analysis of the *Hin*dIII-*Eco*RV fragment. Fragments were end labelled with [γ-^{32}P]ATP and polynucleotide kinase. DNA probes were incubated under conditions used for the gel shift assays with nuclear extracts isolated from BAT cells, plus (+) or minus (−) treatment with 1 μM norepinephrine. The numbers indicate the amounts of nuclear protein (in micrograms) in the incubation mixture. Each reaction mixture was incubated with 12 μg of DNase I for 2 min on ice, the reaction was terminated, and the products were analyzed on sequencing gels.

The positive upstream regulatory region. The next series of experiments focused on elucidating elements in the positive region between an *Xba*I site at −2310 and the *Sph*I site at −2940. This region was analyzed by ligating DNA fragments to vectors containing the downstream region from either the *Hin*dIII-to-*Bgl*I sites (−950 to +110) or from the *Sna*BI-to-*Bgl*I sites (−966 to +110), both of which have strong repressive activity (Fig. 4). Our strategy was designed to determine the characteristics of the regulatory motifs in the *Sph*I-to-*Xba*I fragment which were necessary to overcome repression in the downstream silencer region. We selected this strategy rather than use of a heterologous promoter in the event that cell-specific regulatory interactions may not be evident if a heterologous promoter is used. Near-maximal activity is measured when the complete *Sph*I-*Xba*I fragment is included in the construct. Since this fragment is also active in the reverse orientation, it has the characteristics predicted for an enhancer (data not shown). The 5′ end of this region, from *Sph*I to *Hin*dIII (SpH), was completely inactive. Inclusion of an additional 120 bp from *Hin*dIII to *Eco*RV (SpR) resulted in partial recovery of the activity. However, this region by itself (Bst-R) had very low activity. Addition of the next 100 bp from *Eco*RV to *Xba*I, which by itself was essentially inactive (RX), greatly enhanced CAT activity. The effects of this *Eco*RV-*Xba*I (−2410 to −2310) fragment were not evident in the *tk*-CAT constructs (Fig. 3) since the *Bgl*I-*Eco*RV (−3740 to −2410) *tk*-CAT construct had full activity. This result suggests that the effects of the *Eco*RV-*Xba*I fragment on transcription become evident only with the *Ucp* promoter and not with a heterologous promoter. Thus, separable regulatory elements, located within 220 bp between −2310 and −2530, act synergistically to regulate *Ucp* expression.

DNA footprint analysis. The region between *Hin*dIII and *Eco*RV (−2530 to 2410) contains two CREs at −2460 and −2500 and a DNase I footprint just downstream of CRE-2 at −2460 (Fig. 5). No DNase I footprint was associated with either of the CREs, nor was the presence of a DNase I footprint dependent on whether the cells had been treated with norepinephrine. Within this footprint is a sequence with similarity to the adipocyte response element, ARE-6, which is involved in controlling expression of the adipocyte-specific aP2 gene (13).

Site-specific mutagenesis. A mutation analysis was undertaken to evaluate the contributions of CRE-2 and CRE-3 to the enhancer activity of the *Hin*dIII-*Eco*RV region to determine whether CRE-1 and CRE-4 were involved in *Ucp* expression and to identify the regulatory motifs in the DNase I footprint of the *Hin*dIII-*Eco*RV region that were required for *Ucp* expression. All mutations were introduced into plasmid BBCAT, which carries 3.7 kb of 5′ flanking region and is the strongest and most specific plasmid that we have constructed. The same mutation, which changed CGTCA to TGTCA, was introduced into all four CREs. This mutation

TABLE 1. CRE mutants versus CAT expression

Mutant	% BBCAT[a]
CRE-1 cl C3	82.6 ± 52.5 (2)a
CRE-1 cl C4	125.7 ± 5.7 (3)a
CRE-2	8.2 ± 1.5 (6)b
CRE-3 cl G1	54.3 ± 12.9 (3)a
CRE-3 cl G3	84.4 ± 30.3 (3)a
CRE-4 cl 2	9.4 ± 2.9 (3)b
CRE-4 cl 5	3.7 ± 1.1 (2)b
CRE-4 cl m2	6.0 ± 6.0 (3)b
CRE-4 rev mA8	46.7 ± 12.4 (6)a
CRE-4 rev mB12	66.7 ± 12.4 (3)a

[a] Data groups with an a or b are not different statistically; a differs from b statistically at a 95% confidence level.

has been shown by Bokar et al. (3) to be critical for the CRE enhancer activity of the genes for the α subunit of glycoprotein hormones and phosphoenolpyruvate carboxykinase. Except for CRE-2, more than one independent mutant clone was isolated and analyzed. Furthermore, for CRE-4, the mutated plasmid was back mutated to regenerate the wild-type plasmid. This procedure allowed us to evaluate whether the methods for generating the mutations may have resulted in random mutations which abolished CAT expression. Mutations to CRE-1, the most upstream element, caused no detectable reductions in CAT activity, mutations to CRE-3 resulted in a 15 to 50% reductions in CAT activity, and mutations to CRE-2 and CRE-4 reduced CAT activity 90 to 95% of the wild-type plasmid level (Table 1). It was expected that mutations to CRE-2, and possibly CRE-3, would have strong effects because of their location in the enhancer region; however, the strong effect of mutations to CRE-4 was unexpected because CAT constructs which contained CRE-4 but not CRE-2 or CRE-3 (e.g., BsBCAT; Fig. 2) had very low basal activity and were very weakly induced by norepinephrine. In contrast, plasmids carrying CRE-2 and CRE-3 but not CRE-4 were very active when attached to the *tk* promoter. To verify that the mutations to CRE-4 were specific, three independent clones were analyzed. Each showed similar reductions; in addition, two clones of the reverted CRE-4 mutation (rev mA8 and rev mB12) restored CAT expression to levels close to that of the wild-type plasmid. The results support the conclusion that the mutations were specific to CRE-4 and that they result in almost complete loss of CAT expression.

We next mutated the DNase I footprint downstream of CRE-2 to establish a functional map for an enhancer element. The oligonucleotides used to generate the mutations are listed in the legend to Fig. 6. The most critical mutations are within the DNase I footprint, particularly mutations 8 and 10, which alter two contiguous TTCC motifs (Fig. 6). These mutations resulted in 90 to 95% reductions in CAT expression. Mutations further downstream of the footprint are less effective; mutation 3 had a minimal effect on expression, while the others were intermediate in their effects. We tested the region downstream of the footprint for a functional role because the region covered by mutations from 10 to 5 has the structure of a palindrome of two inverted repeats, TTCC on the left of the palindrome and TTCT on the right (Fig. 6). In addition, the sequence CCAGAAGAG covered by mutations 7, 2, and 3 has significant similarity to ARE-6, an enhancer which is involved in controlling the fat-specific expression of the aP2 gene (13). The mutation analysis suggests that the left-hand side of the palindromic

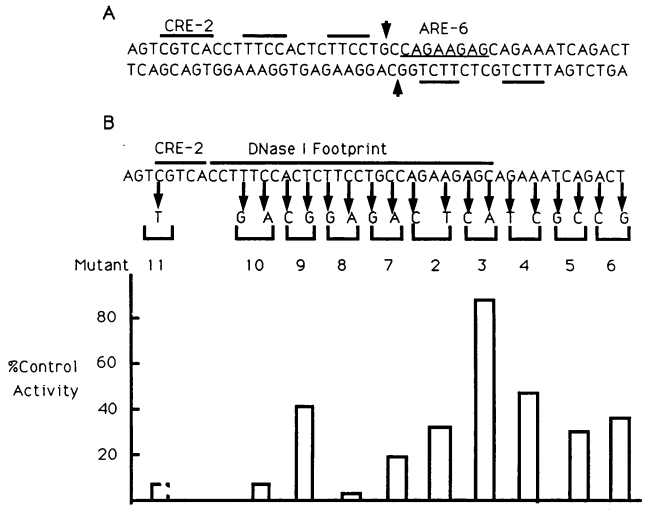

FIG. 6. (A) The sequence of the region submitted to mutagenesis shows dyad symmetry with its axis indicated by the arrows. (B) The synthetic oligonucleotides used to generate the mutated BBCAT plasmids are as follows: BRE-M2, CTCTTCCTGCCCGATGAGCA GAAATG; BRE-M3, CCTGCCAGAAGCGAAGAAATCAGACT; BRE-M4, GCCAGAAGAGCATACATCAGACTCTC; BRE-M5, AAGAGCAGAAAGCCGACTCTCTGGGG; BRE-M6, AGCAGAA ATCAGCCGCTCTGGGGATA; BRE-M7, TTCCACTCTTCCGGA CAGAAGAGCAG; BRE-M8, ACCTTTCCACTCGTACTGCCAG AAGA; BRE-M9, CGTCACCTTTCCCCGCTTCCTGCCAG; BRE-M10, TAGTCGTCACCTGTACACTCTTCCTG; and CRE-M11, CACTGAACTAGTTGTCACCTTTCCAC. The activity of each mutant construct is presented as the percentage of CAT activity of the unmutated BBCAT vector. Data are presented as simple means. Each bar is based on a minimum of six assays in two separate experiments except those for mutants 4, 5, and 6, which were from one experiment done in triplicate.

region is critical for control of *Ucp* expression while the right-hand side of the palindrome is less important. The region with similarity to ARE-6 also seems to be less significant. Given the essential role for the sequence covered by mutations 10 and 8 in *Ucp* expression, we are naming this region brown adipocyte regulatory element 1 (BRE-1).

The CRE-BRE motifs interact cooperatively with nuclear proteins. The *Ucp* enhancer region was investigated by gel shift assays to identify nuclear proteins which interact with the CRE-2 and BRE-1 regulatory motifs. Three synthetic double-stranded oligonucleotide probes were synthesized: a 40-mer which includes both CRE-2 and BRE-1; a 15-mer CRE-2 probe; and a 24-mer BRE-1 probe. The sequences covered by these probes are given in the legend to Fig. 7, and their nuclear protein binding activity is illustrated in Fig. 7. The composite CRE-BRE probe showed strong binding to crude nuclear protein extracts, the BRE-1 probe was moderately strong, and the CRE-2 probe bound nuclear proteins very weakly. We then determined whether CRE-2 and BRE-1 individually could compete with the composite 40-mer probe for binding to the nuclear proteins (Fig. 8). The addition of a 50-fold molar excess of unlabelled CRE-BRE probe completely eliminated binding of the corresponding labelled probe. On the other hand, a 200-fold molar excess of BRE-1 was required before competition with the composite CRE-BRE probe was observed, and CRE-2 was ineffective at even a 200-fold molar excess (Fig. 8A). In contrast, a mixture of CRE-2 and BRE-1 was very effective in competing with the labelled 40-mer at a 50-fold molar excess of each

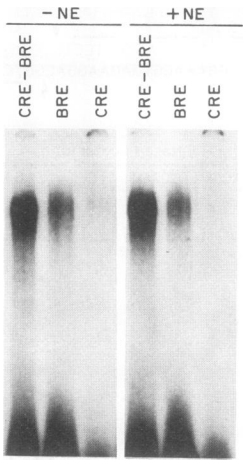

FIG. 7. DNA mobility shift binding assay of probes for regions covering CRE-2 plus BRE-1. All probes for binding assays were double-stranded, but only the top strand is shown. Sequences of probes are as follows: CRE-BRE, CTAGTCGTCACCTTTCCAC TCTTCCTGCCAGAAGAGCAGA (nucleotides 61 to 100 in Fig. 1B); BRE-1, CCTTTCCACTCTTCCTGCCAGAAGA (nucleotides 71 to 95 in Fig. 1B); and CRE-2, CTGAACTAGTCGTCA (nucleotides 56 to 70 in Fig. 1B). Nuclear extracts were prepared from the B-9 brown fat cell line cultured in differentiation medium plus calf serum in the absence of norepinephrine (−NE) or after a 1-h treatment with norepinephrine (+NE). Lanes labelled BRE and CRE refer to the BRE-1 and CRE-2 probes, respectively.

oligomer, a concentration similar to that observed with cold 40-mer. To evaluate further whether this nuclear binding was relevant to *Ucp* expression, we synthesized mutant CRE-2 and BRE-1 probes which carried the same mutations as those introduced into the mutant 11 CRE and mutant 8 BRE (Fig. 6). These probes lost their ability to bind nuclear proteins (data not shown) and, in addition, were unable to compete synergistically with the 40-mer probe (Fig. 8B).

The sequence in the region around mutations 2 and 3, while not critical in the control of transient expression of the BBCAT construct, nevertheless shows significant homology to ARE-6. In addition, the motifs on the opposing strand of mutations 10 and 8 show the AAG motif which is also found in ARE-6. It was important to evaluate whether the ARE-6 probe as described by Graves et al. (13) and those in the *Ucp* enhancer were interacting with the same nuclear proteins. The ARE-6 probe showed strong binding to nuclear extracts (data not shown), an expected result since the aP2 gene is expressed in brown fat. It was then tested for its ability to substitute for the BRE-1 oligomer and cooperate with CRE-2 in a competition assay against the labelled CRE-BRE probe. The data in Fig. 8C indicate that the ARE-6 oligomer could not substitute for the BRE-1 oligomer in the competitive gel shift assay. Accordingly, these two motifs interact with different nuclear proteins.

Interactions of the CRE-2–BRE-1 enhancer with a regulatory element in the *Eco*RV-*Xba*I fragment. The transient expression assays illustrated in Fig. 4 indicated that the *Hin*dIII-*Eco*RV fragment which carries the CRE-2–BRE-1 motif was only marginally active in stimulating CAT transcription. However, the addition of the contiguous *Eco*RV-*Xba*I fragment, which by itself had no activity, was able to restore expression. To search for the regulatory motifs responsible for this effect in the *Eco*RV-*Xba*I fragment, three overlapping synthetic double-stranded oligonucleotides, called R, M, and X, were prepared for gel shift assays.

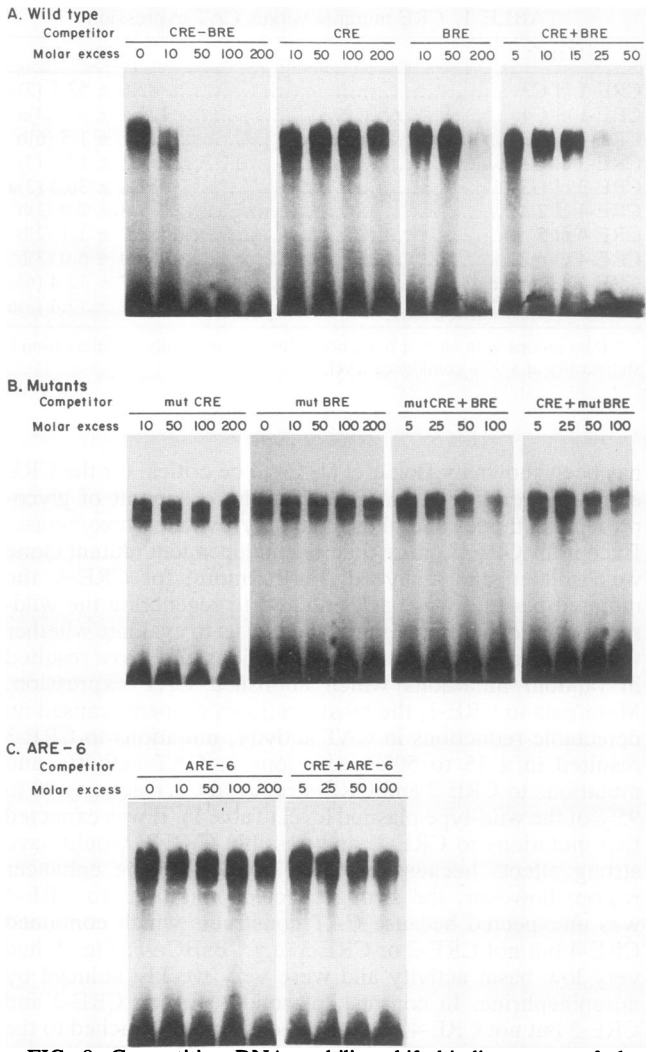

FIG. 8. Competitive DNA mobility shift binding assay of the ^{32}P-labelled CRE-BRE probe against wild-type unlabelled CRE-2 and BRE-1 competitor (A), mutant unlabelled CRE-2 and BRE-1 competitor (B), and unlabelled ARE-6 competitor (C). The sequences of the wild-type oligomer are shown in the legend to Fig. 7. The sequences of the mutant forms are the same as the wild-type sequence except that mutations which correspond to mutant 11 for CRE-2 and mutant 8 for BRE-1 shown in Fig. 6 have been introduced. The sequence of the top strand of the ARE-6 oligomer is CATTTCACCCAG AGAGAAGGGATT. Nuclear extracts were isolated from BAT cell cultures treated with 1 μM norepinephrine for 1 h.

These oligomers covered the entire *Eco*RV-*Xba*I fragment. Only oligomer X (closest to the *Xba*I site) showed strong specific gel shifts with nuclear proteins prepared from BAT cells (Fig. 9A). Inspection of the sequence of the X oligomer revealed a region with striking similarity to the CRE-2–BRE-1 element (legend to Fig. 9). Most interestingly, it possessed the TTCC motif which was shown by site-directed mutagenesis to be critical for the CRE-2–BRE-1 enhancer activity. This sequence similarity suggested that CRE-2–BRE-1 and the X oligomer could interact with related nuclear proteins, which was confirmed when we found that the CRE-2–BRE-1 probe was a strong competitor of the X oligomer in a gel shift assay (Fig. 9B). The CRE-2–BRE-1 and X probes were next compared for binding to nuclear proteins isolated from cells treated with norepinephrine for

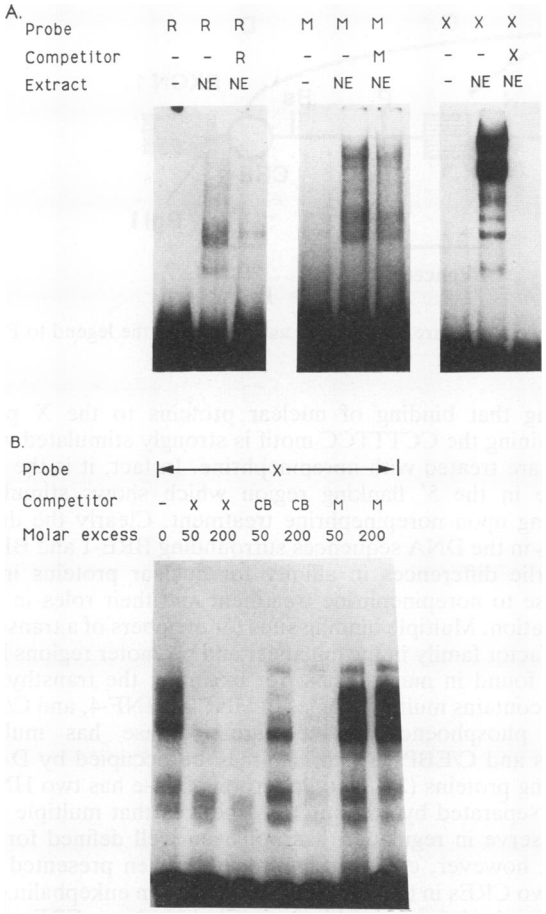

FIG. 9. (A) DNA mobility shift binding assay of overlapping probes prepared from the *Eco*RV-*Xba*I fragment. Reaction mixtures contained either no nuclear extract (−) or extract isolated from cells treated with norepinephrine (NE). The sequences of the oligomers beginning from the *Eco*RV restriction enzyme site are as follows: R, GGATATCAGCCTCACCCCTACTGCTCTCTCCATTATGAGGC (nucleotides 117 to 156 in Fig. 1B); M, CATTATGAGGCAAACTT TCTTTCACTTCCAGAGGCTCTGGGG (nucleotides 146 to 187), and X, GGCTCTGGGGGCAGCAAGGTCAACCCTTTCCTCAGA CTC (nucleotides 178 to 216). (B) The CB competitor refers to the CRE-2–BRE-1 oligomer described in the legend to Fig. 7. Nuclear extracts were described for Fig. 7.

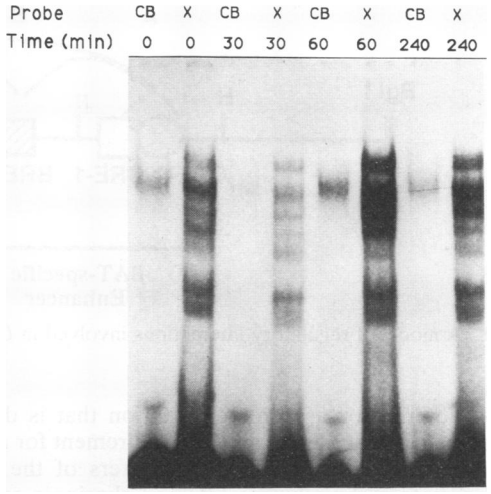

FIG. 10. Time course for induction of DNA mobility shift binding activity to the CRE-2–BRE-1 (CB) and X probes. BAT cell cultures were treated with 1 μM norepinephrine for the indicated times.

various lengths of time. As previously observed, the binding activity of the CRE-2–BRE-1 probe did not vary; however, the X probe showed weak binding in untreated cells and in cells treated for 30 min and then very strong binding in extracts from cells treated for 1 h (Fig. 10). After 4 h of treatment, the binding activity was reduced but still greater than in untreated cells. All proteins in the complex detected by the X probe seem to be induced by norepinephrine. These results indicate that the two motifs are probably binding to different proteins even though they share sequence similarities. On the basis of the sequence similarity, we shall refer to the BRE motif contiguous to CRE-2 as BRE-1 and the motif in the X probe as BRE-2.

DISCUSSION

Analysis of regulatory motifs in the 5′ flanking region of the murine *Ucp* gene indicates that the brown-fat-specific expression and induction by norepinephrine involves a complex interaction of positive and negative elements spread over a minimum of 3 kb. There is also the possibility that additional sites of regulation are located at DNase I-hypersensitive sites which were mapped several kilobases upstream of the region under investigation (4). Deletion analysis of the 5′ flanking region by transient expression assays identified three regions of regulation. (i) The basal promoter extends to approximately −272 bp and has a TATA box and CRE. CAT promoter constructs of this fragment have very low activity in BAT cells in the presence or absence of norepinephrine. (ii) A silencer is located between −272 and −900 bp. Its presence on the endogenous promoter or the heterologous *tk* promoter very strongly represses expression in BAT cells or PC-12 cells. (iii) A norepinephrine-responsive enhancer located at approximately −2600 bp is required for both brown fat specificity and induction by norepinephrine. This enhancer overrides the action of the downstream silencer. Similar locations for these elements controlling *Ucp* expression in rats have recently been described (5). A working model for the *Ucp* regulatory region involves a complex series of interactions between the three regions described above (Fig. 11).

The presence of several CREs in the 5′ flanking region of *Ucp* form a major hurdle in resolving the mechanisms controlling *Ucp* expression. Although multiple CREs have been found associated with many genes, the *Ucp* gene has more than most other genes. Cassard-Doulcier (5) recently reported that seven are present in the rat *Ucp* gene, and they indicated that all were active. We have identified four in the mouse *Ucp* 5′ flanking region. All four CREs have the same core sequence, CGTCAC, and they all deviate in the first three positions from the palindromic TGACGTCA consensus sequence. However, unlike the case for the rat gene, both transient expression analysis and mutation analysis of mouse *Ucp* indicate that CRE-1 has no regulatory function and that CRE-3 was only marginally significant. Accordingly, only two of the four CREs are important: CRE-2, which is critical for the enhancer function, and CRE-4, which is located near the TATA box promoter. The fact that mutations to CRE-4 completely knock out transient expression was a surprise because deletion constructs carrying CRE-4 without the CRE-2 have low basal activity and low inducibility by norepinephrine. This finding suggests that

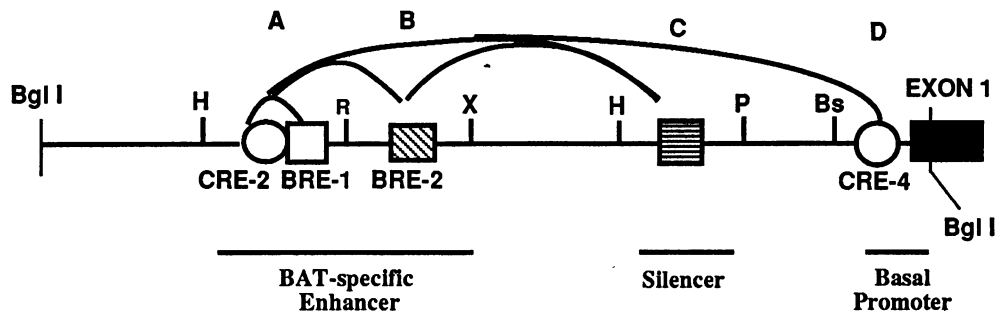

FIG. 11. A model of regulatory interactions involved in *Ucp* regulation. Restrictions are abbreviated as specified in the legend to Fig. 1.

CRE-4 is required for a promoter function that is distinct from an enhancer function. A similar requirement for a CRE was previously described for the promoters of the phosphoenolpyruvate carboxykinase (25) and chorionic gonadotropin (7) genes; however, the function of the CRE in these promoters is not well understood and must be investigated further.

Within region A, we have identified a functional CRE (CRE-2), one motif with the core sequence CCTTTCC (BRE-1), and a second with the sequence CTCTTCC which is contiguous to BRE-1 and may be a separate element or a part of BRE-1. Mutation analysis indicates that both motifs are essential for enhancer function. A second motif with the CCTTTCC sequence is located in region B. The other CRE in the region (CRE-3) plays only a minor role in the regulation of *Ucp*. With respect to CRE-2, it may be important that the CGTCA sequence is located within a 17-bp sequence which contains two TTCC motifs spaced so as to place the CGTCA and TTCC motifs on the same face on the DNA helix. Yamamoto et al. (34) demonstrated that the palindromic TGACGTCA sequence of the somatostatin gene was required to produce a transcriptionally active dimeric CRE-binding protein (CREB). Loss of the CGTCA on one of the DNA strands resulted in the binding of only a monomeric CREB and a concomitant loss of transcriptional activity. The CRE-2 of *Ucp* with the sequence AGTCGTCA has lost its palindromic structure and should be capable of interacting only with a monomeric CREB. However, there is now accumulating evidence that CREB as a member of the basic leucine zipper class of DNA-binding proteins is capable of forming transcriptionally active heterodimers with other members of this class (16, 20, 31). Our mutation analysis and gel shift data for the CRE-2 and CCTTTCC motifs indicate that these motifs in *Ucp* are acting synergistically. In one possible model, a heterodimeric transcription factor binds to the combined CRE-2–BRE-1 motif. One component of the heterodimer would bind to CRE-2 and could be a member of the rapidly expanding CREB/ATF/CEBP transcription family (6, 15), and the other member of the heterodimer would bind to BRE-1. Alternatively, one can postulate that a unique transcription factor binds in a cooperative manner with CRE-2 and BRE-1. These models cannot be distinguished until transcription factors which interact with these motifs are identified.

The second CCTTTCC motif, BRE-2, is located in the *Eco*RV-*Xba*I fragment, 122 bp downstream of BRE-1. This fragment is not capable of stimulating *Ucp* expression in transient expression assays but is nevertheless required for expression since it greatly enhances transient expression when combined with the *Hin*dIII-*Eco*RV fragment. Its potential importance in the control of *Ucp* also comes from the finding that binding of nuclear proteins to the X probe containing the CCTTTCC motif is strongly stimulated when cells are treated with norepinephrine. In fact, it is the only probe in the 5′ flanking region which shows stimulated binding upon norepinephrine treatment. Clearly the differences in the DNA sequences surrounding BRE-1 and BRE-2 underlie differences in affinity for nuclear proteins in response to norepinephrine treatment and their roles in *Ucp* regulation. Multiple binding sites for members of a transcription factor family in the enhancer and promoter regions have been found in many genes; for example, the transthyretin gene contains multiple copies of HNF-3, HNF-4, and C/EBP (23), phosphoenolpyruvate carboxykinase has multiple CREs and C/EBPs which also may be occupied by D-site-binding proteins (27), and lipoprotein lipase has two HNF-3 sites separated by 200 bp (10). The role that multiple sites may serve in regulation has not been well defined for any gene; however, evidence has recently been presented that the two CREs in the enhancer of the human enkephalin gene are required for transcription and that these CREs form stable hairpin loop structures that result in high-affinity binding to CREB (30).

The presence of regulatory motifs distributed over 2.5 kb of 5′ flanking region dictates that long-range interactions among these motifs are an essential feature of the regulation of *Ucp*. One clear and possibly the most important interaction involves BRE-2. Brown-fat-specific expression by *Ucp* appears to be determined by this motif. Three lines of evidence support this conclusion: (i) constructs in which CAT is driven by the *Ucp* promoter (e.g., pBLCAT6) are dependent on the presence of the *Eco*RV-*Xba*I fragment for expression in brown fat cells (Fig. 4), (ii) the *Eco*RV-*Xba*I fragment is not required for norepinephrine-induced expression when the heterologous promoter (pBLCAT5) is driving CAT expression (Fig. 3), and (iii) BRE-2 is the only region that binds to a nuclear protein which is induced by treating brown fat cells with norepinephrine (Fig. 10). CRE-2–BRE-1 is also essential for activity, but we have no evidence for induced DNA-protein interactions. It is possible that such interactions occur but are too unstable to be detected by our experiments. In the tyrosine aminotransferase gene, which resembles *Ucp* in having a CRE enhancer several kilobases upstream of the transcription start site, Weih et al. have demonstrated transient protein-DNA interactions in the CRE region with in vivo DNA footprinting (32). Independent of whether CRE-2–BRE-1 has inducible transcription factors which are interacting with it, the data can be interpreted to indicated that regions A and B in Fig. 11 interact with each other and with the silencer and promoter to control *Ucp* transcription.

Gel shift experiments suggest that transcription factors

similar, but not identical, in DNA binding affinity interact with CRE-2–BRE-1 and BRE-2. One interpretation would be that members of the same family of transcription factors, by themselves or in association with other proteins, mediate the functional properties of this enhancer region. We would like to speculate that one family of enhancer-binding proteins, the Elk/Ets family, could be involved here since the lower strands of both BRE-1 and BRE-2 show a great sequence similarity with binding sites known to bind to members of this family, including the core motif GGAA (22). Future efforts will be directed at resolving this matter.

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