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Interaction of Nuclear Factors with the cAMP Response Elements of the Human β_3-Adrenoceptor Gene

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Four potential cyclic adenosine 3',5'-monophosphate (cAMP) response elements (CREs), each having at most two mismatches from the classical canonical sequence, have been identified in the 5'UTR of the human β_3-adrenoceptor gene by Liggett and Schwinn (1991). Recently, three of these CREs were shown to confer responsiveness to cAMP when cloned into a CAT reporter vector (Thomas et al., 1992). In this study, in vitro gel-retardation assays have shown that recombinant human CRE binding protein-1 (CREB-1) or activating transcription factor-1 (ATF-1) can interact specifically with these four putative CREs (termed β_3CRE1–4), although with different affinities. Nuclear extracts from human brown or white adipose tissue contain proteins interacting with β_3CRE3 and β_3CRE2. These adipose nuclear factors were shown by competition assays and the use of antibodies to differ from CREB-1 or ATF-1. The nuclear factor(s) interacting with β_3CRE2 was found in human and rat brown and white adipose tissues, but not in the other nonadipose tissues examined, i.e., rat lung, liver, kidney, and heart, suggesting an adipose tissue-specific DNA binding or expression pattern. β_3CRE2 is found to constitute a hexameric element that is highly homologous to the binding site for members of the nuclear hormone receptor superfamily, and a competition assay using this site has provided evidence that an adipose tissue-specific orphan member of this superfamily may bind to β_3CRE2. Reporter gene assays have indicated that β_3CRE2 and β_3CRE3 slightly repress the basal level of transcription and that β_3CRE2 confers cAMP responsiveness, whereas β_3CRE3 does not.

Key Words: β_3-adrenoceptor; cAMP; transcription; CREB; adipose tissue.

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Introduction

The β_3-adrenoceptor (β_3-AR) gene is known to be strongly expressed in rodent brown and white adipose tissues. Its stimulation is the major determinant of increased lipolysis and thermogenesis, and may therefore contribute to prevention of the development of obesity. In fact, in hereditarily obese animals, the expression of the β_3-AR gene in brown and white adipose tissues was found to be decreased compared with that in lean controls (Giacobino, 1995; references therein). Furthermore, β_3-adrenergic agonists, used as thermogenic drugs, were found to decrease body weight gain in hereditarily obese rodents (Arch et al., 1984; Howe, 1993).

In contrast to rodents, the level of expression of the β_3-AR gene is much less in human brown adipose tissue, where its mRNA amounts to 9% of the total β-AR mRNAs (Deng et al., 1996), and in human white adipose tissue, where its mRNA can be detected only by RT-PCR (Revelli et al., 1993). However, the possibility that it could be used as a target for antiobesity drugs in humans is currently under study. It is therefore important to study the mechanism of the tissue- and species-specific expression of the β_3-AR gene.

Transcription factors play an important role in modulating the specificity and efficiency of transcription initiation by RNA polymerase II. These factors mediate their effects by interacting with specific DNA sequences or with target proteins (Dynan and Tjian, 1982). Sequence analysis of the human β_3-AR gene revealed the existence of multiple cis-elements, including four potential cAMP response elements (CREs) (Liggett and Schwinn, 1991). Three of the four potential CREs were shown to mediate cyclic adenosine 3',5'-monophosphate (cAMP) responsiveness in VERO cells in the chloramphenicol acetyltransferase (CAT) reporter gene system (Thomas et al., 1992). One CRE has also been described in the 5'UTR of the mouse β_3-AR gene (Van Spronsen et al., 1993), whereas none have been found in the rat gene (Brown and Machida, 1994).

CRE binding protein (CREB) and activating transcription factor-1 (ATF-1) are members of the large bZIP super-

family of transcription factors (reviewed by Meyer and Habener, 1993). They both bind to the CRE element, which consists of a palindromic sequence (5'TGACGTCA3') that is found in cAMP-inducible gene promoters. Transcriptional activation by cAMP is mediated by cAMP-dependent protein kinase (PKA), which acts by phosphorylating CREB. Phosphorylation of the PKA phosphoacceptor site of CREB is required for transcriptional activation.

The existence of CRE sites in the human β_3-AR gene suggests that the β_3-AR expression might be positively or negatively regulated by the end product of the cAMP, PKA, CREB signal transduction pathway. Indeed, exposure to β-adrenergic agonists has been shown to regulate the β_3-AR expression through the cAMP pathway. However, the results are conflicting. Thomas et al. (1992) reported an upregulation, whereas we and others (Granneman and Lahners, 1992; Revelli et al., 1992) reported a downregulation. The mechanisms of how cAMP acts in controlling the β_3-AR gene expression needs to be elucidated.

To our knowledge, an analysis of the binding of CREB/ATF family proteins with the human β_3-AR gene has not been performed. We have therefore examined the possibility that CREB-1 or ATF-1 binds to the potential CREs of the 5'-flanking region of the human β_3-AR in vitro. Furthermore, we provide evidence that two nuclear factors from human adipose tissues that are distinct from CREB-1 or ATF-1 interact with the aberrant CREs of the human β_3-AR gene. We also studied the effect of these nuclear factors on transcription in the reporter gene assay. This work represents the first attempt to analyze the protein binding characteristics of the β_3-AR CREs that might play an important role in the regulation of the human β_3-AR gene expression.

Results

ATF-1 or CREB-1 Interacts with the Putative CREs of the Human β_3-AR Gene

Sequence analysis of the human β_3-AR promoter region revealed the existence of four putative CRE sites at positions 518-525, 622-629, 955-962, and 1125-1132 upstream from the initiation codon (Liggett and Schwinn, 1991). However, none of them perfectly matches the consensus palindromic CRE sequence TGACGTCA (Table 1). It was therefore of interest to test whether these putative CRE sites of the human β_3-AR gene could interact with ATF-1 or CREB-1 protein. To answer this question, in vitro gel-retardation assays were performed using the purified recombinant human ATF-1 (full-length) or CREB-1 bZIP domain and double-stranded ^{32}P radiolabeled oligonucleotides as probes. As shown in Fig. 1A, strong ATF-1-dependent DNA–protein complex signals were observed with the consensus CRE and with the β_3CRE3 probes. A weak signal was observed with the β_3CRE4, and very faint but still detectable signals were observed with the β_3CRE1 and β_3CRE2 probes. As shown in Fig. 1B, CREB-1-dependent signals were observed with the consensus CRE and with the β_3CRE1, β_3CRE3 and β_3CRE4 probes. A weaker signal was observed with the β_3CRE2 probe. These experiments (Fig. 1 panels A,B) were performed under an identical condition. In both experiments, no signal was observed with H2B octamer oligonucleotide, which was used as a negative control. Altogether these experiments show that ATF-1 or CREB-1 can bind to the four putative β_3CRE sites in vitro, although with different affinities.

Table 1
The Nucleotide Sequences of the Canonical CRE and of the Putative Human β_3-AR CREs[a]

Canonical CRE	TGACGTCA
β_3CRE1	TGAC**tc**CA
β_3CRE2	TGA**g**GTCt
β_3CRE3	TcAC**a**TCA
β_3CRE4	cGA**g**GTCA

[a]The mismatches are shown with small, bold letters.

In order to assess the specificity of the DNA–protein complexes formed between ATF-1 or CREB-1 and the four putative β_3CRE sites, competition experiments were carried out. The radiolabeled consensus CRE oligonucleotide was incubated with ATF-1 (Fig. 2A) or CREB-1 (Fig. 2B) protein in the absence or presence of an increasing molar excess of unlabeled CRE, β_3CRE1–4, or H2B octamer as competitor. As shown in Fig. 2A and B, the DNA binding (arrow) was displaced in a dose-dependent manner by the competitors. In experiments using ATF-1 (Fig. 2A), CRE had the highest competition efficiency (self: 10- and 20-fold excess). Higher amounts of β_3CRE3 (25- and 50-fold excess), β_3CRE4 (100- and 200-fold excess), and β_3CRE1 or β_3CRE2 (200- and 400-fold excess) were required to achieve a similar level of competition. The faster migrating band indicated with an asterisk could be a proteolytic fragment that shows a similar competition pattern as the band indicated with the arrow.

In experiments using CREB-1 (Fig. 2B), similarly, CRE had the highest competition efficiency (self: 10- and 20-fold excess). Higher dose of β_3CRE3 (25- and 50-fold excess), β_3CRE1 or β_3CRE4 (50- and 100-fold excess), or β_3CRE2 (100- and 200-fold excess) were needed to achieve a similar level of competition. In both cases, H2B octamer did not compete with the DNA binding activity of ATF-1 or CREB-1, suggesting that the competition by the β_3CREs is specific. The results shown in Fig. 2A and B confirm those of Fig. 1 with orders of binding potency of the various oligonucleotides tested being similar. It can also be seen in Fig. 2A and B that the affinity of ATF-1 is weaker than that of CREB-1 for the β_3CREs with the exception of β_3CRE3.

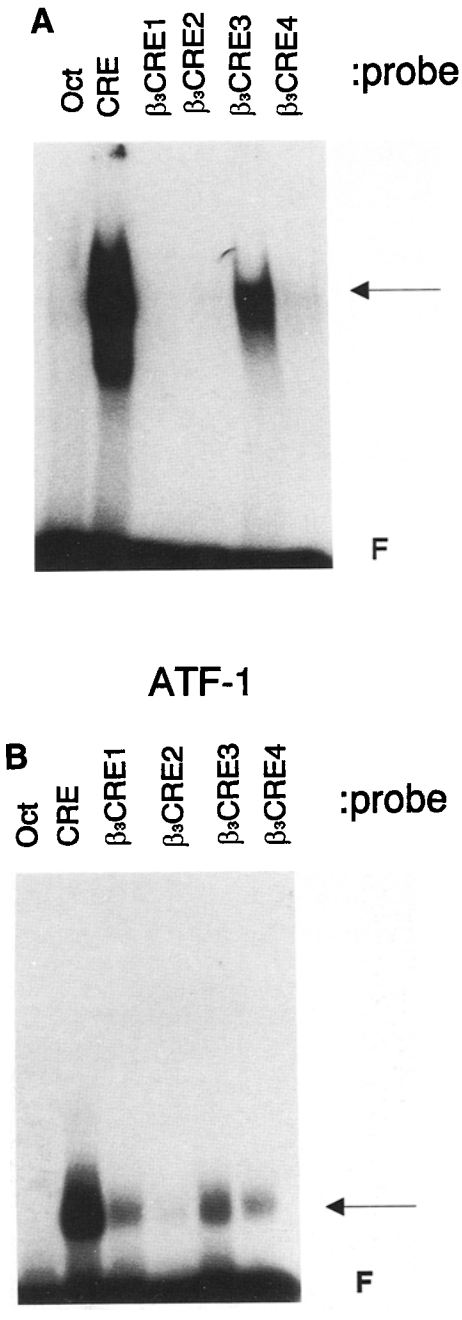

Fig. 1. ATF-1 or CREB-1 binds to the β_3-AR CREs. ^{32}P-labeled double-stranded canonical CRE or putative β_3-AR CRE oligonucleotides were incubated with 50 ng of purified ATF-1 **(A)** or CREB-1 protein **(B)**, and the DNA binding was analyzed by gel-retardation assay as described in Materials and Methods. The ATF-1- or CREB-1-dependent DNA–protein complex is indicated with an arrow. Free probes are shown at the bottom of the gel (F). Histone H2B octamer (Oct) was used as a control.

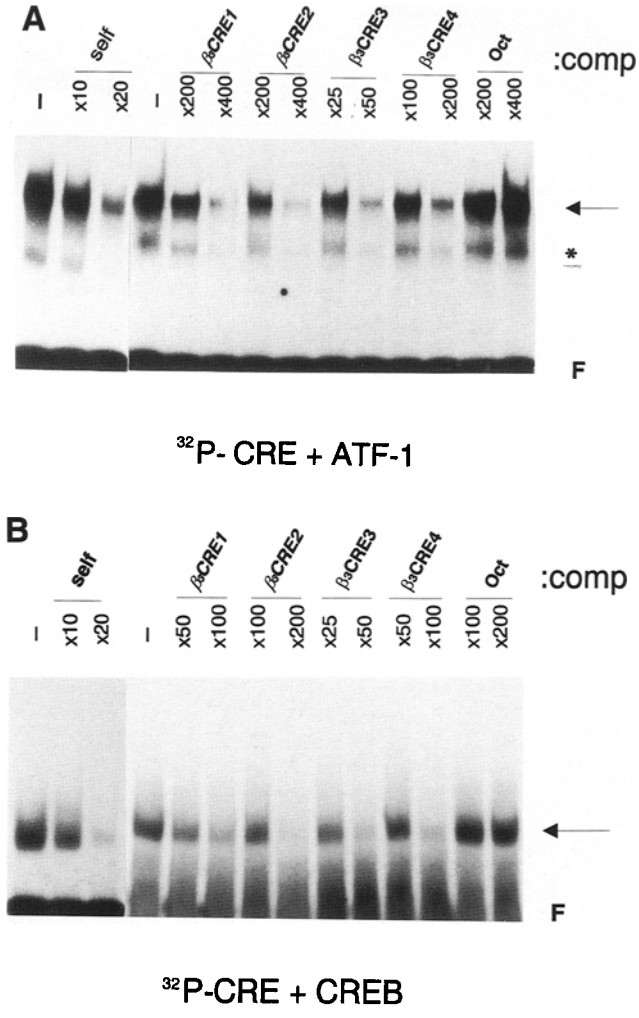

Fig. 2. Specificity of the complexes formed between the β_3-AR CREs and ATF-1 or CREB-1 protein. Radiolabeled canonical CRE was incubated with 50 ng of purified ATF-1 **(A)** or CREB-1 protein **(B)** in the absence (–) or presence of increasing concentrations of the unlabeled consensus CRE (self) and β_3CREs as competitors (comp) to analyze the specificity of the DNA–protein complex. The DNA binding was analyzed by gel-retardation assay. The molar excess of competitors is indicated. The ATF-1- (A) or CREB-1- (B) dependent DNA–protein complex is indicated with an arrow. Histone H2B octamer (Oct) was used as a control. (A) * indicates a possible proteolytic fragment.

Nuclear Factor(s) Interacts with β_3CREs

β_3-AR expression is higher in human brown than white adipose tissue (Deng et al., 1996). The question could then be addressed concerning whether nuclear factors from human brown or white adipose tissue could interact with the β_3-AR putative CREs. As shown in Fig. 3A, when nuclear extracts from human brown adipose tissue were incubated with the radiolabeled probes, DNA–protein complexes were formed with H2B octamer (arrow a), CRE (arrow b), and with β_3CRE3 (arrow c). The faster migrating band shown with an asterisk is probably nonspecific, since it is not observed in some experiments (*see* Fig. 3B and 4A); such nonspecific bands were described by others (Alksnis et al., 1991). On longer exposure of the film, a band could be observed with β_3CRE2 at the position of arrow a (*see* Fig. 5). As shown in Fig. 3B, when the nuclear extracts from human white adipose tissue were used, DNA–protein

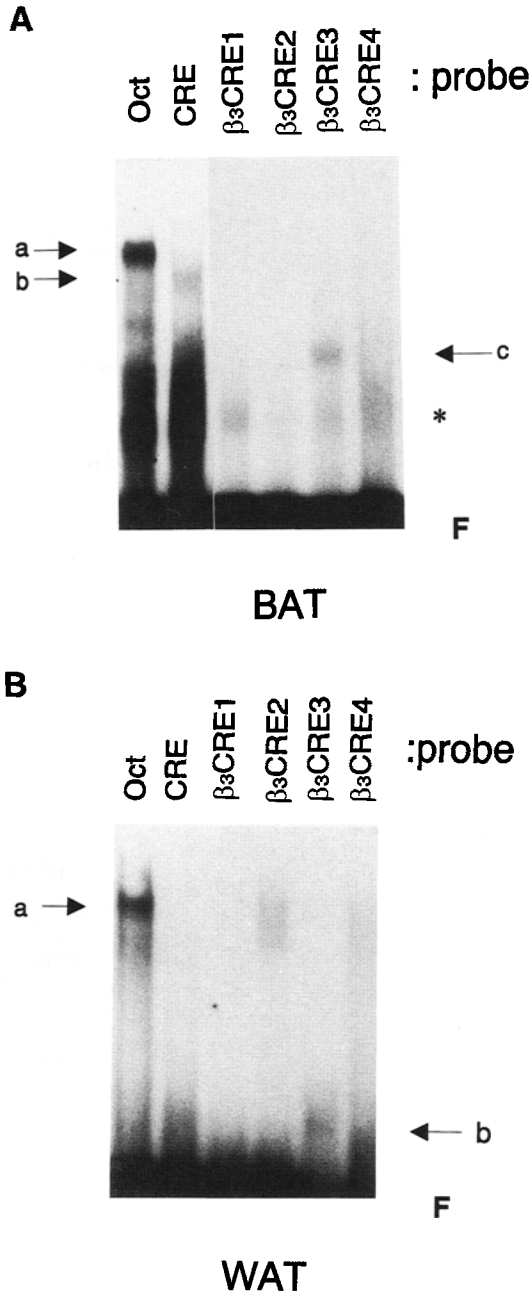

Fig. 3. Nuclear factor(s) from adipose tissue bind(s) to the β_3-AR CREs. Radiolabeled canonical CRE or β_3CREs were incubated with approx 5 μg of nuclear extract proteins from human brown adipose tissue (**A**, BAT) or human white adipose tissue (**B**, WAT). The DNA binding was analyzed by gel-retardation assay. Histone H2B octamer (Oct) was used as a control. (A) The DNA–protein complex of Oct DNA is indicated with arrow a, that of the consensus CRE with arrow b, and that of β_3CRE3 with arrow c. * Indicates a possible nonspecific binding. (B) The DNA–protein complexes of Oct DNA and β_3CRE2 are indicated with arrow a and the DNA–protein complex of β_3CRE3 with arrow b.

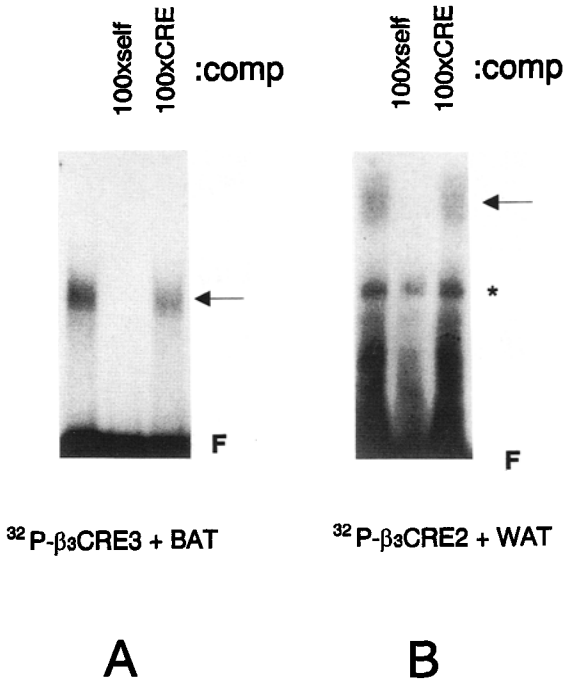

Fig. 4. Specificity of the DNA–protein complexes between the β_3CRE2 and β_3CRE3 and nuclear factors from human adipose tissue. Panel **A:** Radiolabeled β_3CRE3 was incubated with approx 5 μg of nuclear extract proteins from human brown adipose tissue (BAT) in the absence (–) or presence of a 100-fold molar excess of unlabeled β_3CRE3 (self) or the consensus CRE as competitors (comp). Panel **B:** Radiolabeled β_3CRE2 was incubated with approx 5 μg of nuclear extract proteins from human white adipose tissue (WAT) in the absence (–) or presence of a 100-fold molar excess of unlabeled β_3CRE2 (self) or the consensus CRE as competitors. The DNA binding was analyzed by gel-retardation assay. The arrow indicates the formed DNA–protein complex and * a nonspecific binding.

complexes were formed with H2B octamer and β_3CRE2 (arrow a) and β_3CRE3 (arrow b). These results show that nuclear factors from brown or white adipose tissue can interact with β_3CRE3 and β_3CRE2, respectively. On longer exposure of the film, a band could be observed with CRE (*see* Fig. 5). In comparison with the constitutively expressed octamer binding protein (OTF-1), CRE-dependent DNA binding activity is higher in brown than in white adipose tissue.

To assess the specificity of the interaction between the human adipose tissue nuclear factors and β_3CRE3 and β_3CRE2, competition assays were performed using an excess of unlabeled β_3CRE3 or β_3CRE2 (self), or the consensus CRE as competitors in the gel-retardation assay. As shown in Fig. 4A, a 100-fold molar excess of unlabeled β_3CRE3 (self) successfully competed with the DNA–protein complex (arrow) formed between β_3CRE3 and nuclear factors from human brown adipose tissue, whereas the same molar excess of consensus CRE had a weaker inhibitory effect. The same observation was made with β_3CRE2 and the human white adipose tissue nuclear extracts (Fig. 4B). Asterisk represents a possible nonspecific binding. These data suggest that the interaction between β_3CRE2 or β_3CRE3 and nuclear factors is really specific, and that the nuclear factor(s) involved does not belong to the CREB/ATF family.

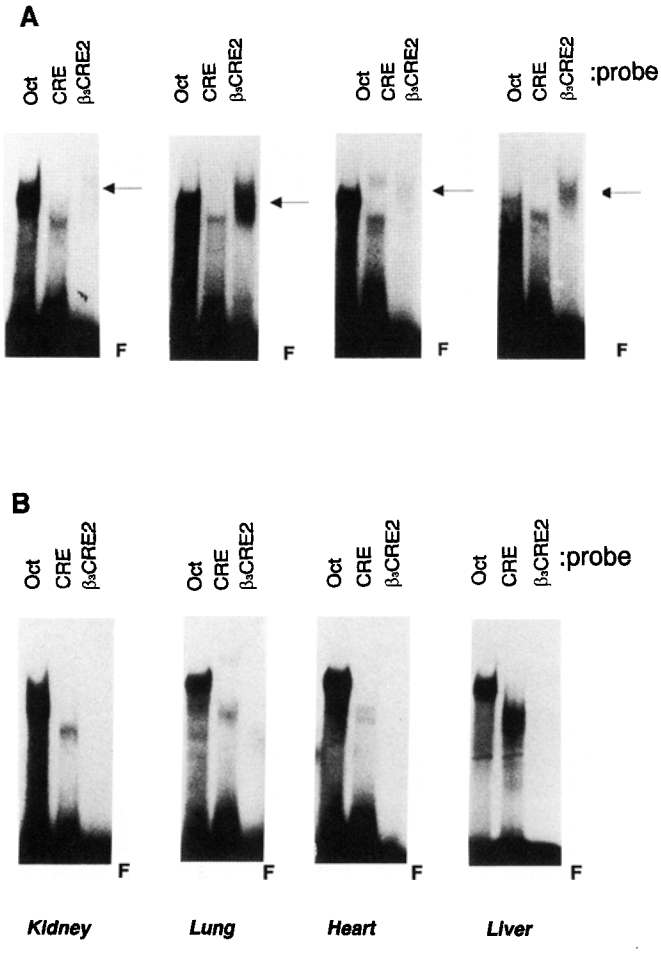

Fig. 5. Tissue-specific expression of the nuclear factor(s) complexed with β_3CRE2. Radiolabeled H2B octamer, canonical CRE, or β_3CRE2 was incubated with approx 5 μg of nuclear extract proteins from various tissues **(A)** HBAT = human brown adipose tissue; HWAT = human white adipose tissue; RBAT = rat brown adipose tissue; RWAT = rat white adipose tissue, **(B)** and rat kidney, lung, heart, and liver. The DNA binding was analyzed by the gel-retardation assay. The β_3CRE2-dependent DNA–protein complexes are indicated with arrows.

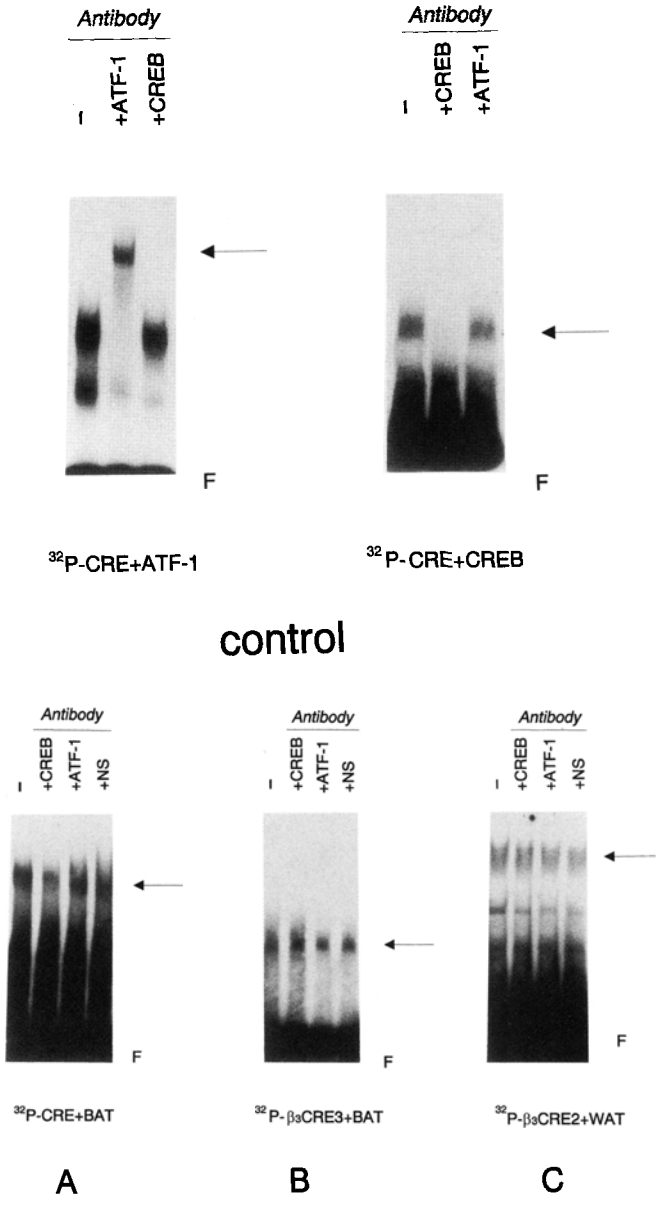

Fig. 6. Immunological properties of the adipose tissue nuclear factor(s) complexed with the β_3CRE2 and β_3CRE3. The radiolabeled consensus CRE was incubated with 50 ng of ATF-1 or CREB-1 protein that were pretreated with 1 μg of indicated antibodies on ice for 1 h. The DNA binding was analyzed by the gel-retardation assay. Supershift (ATF-1, left panel) or inhibition (CREB, right panel) is indicated with an arrow. **(A)** Same as control panel, except that approx 5 μg of nuclear extract proteins from human brown adipose tissue were used and nonimmune IgG was included. DNA–protein complex formed is indicated with an arrow. **(B)** Same as A, except that radiolabeled β_3CRE3 was used. **(C)** Same as A, except that radiolabeled β_3CRE2 and nuclear extract proteins from human white adipose tissue were used.

The immunological properties of these factors were then characterized using monoclonal IgG antibodies raised against ATF-1 or CREB-1. First, the specificity of these antibodies was assessed. Anti-ATF-1 antibody supershifted the ATF-1-dependent DNA–protein complex, whereas anti-CREB-1 antibody had no effect (Fig. 6, control panel). Anti-CREB-1 antibody inhibited the CREB-1-dependent DNA–protein complex, whereas anti-ATF-1 antibody had no effect (Fig. 6, control panel). These results confirm that anti-ATF-1 and anti-CREB-1 antibodies are specific and do not crossreact.

The interaction between CRE and nuclear factors from human brown adipose tissue (Fig. 6A, arrow) was partially inhibited by the CREB-1 antibody, and not influenced by anti-ATF-1 antibody or nonspecific preimmune IgG. These results suggest that in nuclear extracts from human brown adipose tissue, it is CREB-1, but not ATF-1, that is complexed with the consensus CRE sequence. As shown in Fig. 6B and C, none of the antibodies tested had an effect on the interaction between β_3CRE3 and nuclear factors from human brown adipose tissue or between β_3CRE2 and nuclear factors from human white adipose tissue. These results, together with the competition assays, strongly suggest that these nuclear factors differ from CREB/ATF family.

Tissue-Specific Expression/DNA Binding of the Nuclear Factor(s) Interacting with β_3CRE2

As an initial step toward characterizing the nuclear factors complexed with β_3CRE3 and β_3CRE2, we investigated their tissue-specific expression or DNA binding patterns. Nuclear extracts were prepared from human and rat brown and white adipose tissues and from rat nonadipose tissues, such as kidney, lung, heart, or liver. Gel-retardation assays were performed using the radiolabeled H2B octamer, consensus CRE, or β_3CRE2 oligonucleotides as probes.

In all tissues examined, the H2B octamer or the consensus CRE oligonucleotides used as controls formed DNA–protein complexes (Fig. 5A,B). In contrast to the level of the constitutively expressed octamer binding protein (OTF-1), the expression level or DNA binding pattern of CRE binding protein(s) varied from tissue to tissue. In both human and rat, somewhat higher CRE binding activities were observed in brown than in white adipose tissue. In rat brown adipose tissue, kidney, and heart, two signals were observed.

The nuclear factor(s) interacting with the β_3CRE2 is present in each adipose tissue tested and is expressed at a higher level in white than in brown adipose tissue of both rat and human (Fig. 5A). Interestingly, nuclear factor(s) from nonadipose tissues (kidney, lung, heart, or liver) failed to form complexes with the β_3CRE2 (Fig. 5B). Therefore the factor interacting with β_3CRE2 appears to have an adipose tissue-specific DNA binding activity or expression pattern. Experiments using β_3CRE3 as a probe showed an interaction with a nuclear factor(s) in all tissues tested, suggesting that this factor is ubiquitously expressed (results not shown).

A computer search has revealed that β_3CRE2 (TGAGG TCT) is very similar to the half-binding site for some members of the steroid/thyroid/retinoic acid receptor superfamily (Evans, 1988). Response elements for members of this family usually contain two or more repeats of the TGA GGTCA motif (Monia et al., 1995). We were then interested in testing whether the nuclear factor interacting with β_3CRE2 shows a DNA binding pattern similar to that of the nuclear hormone receptor superfamily. To answer this question, an excess of unlabeled TGAGGTCA motif containing only a few flanking sequences was incubated in the binding reaction. As shown in Fig. 7, this unlabeled oligonucleotides successfully eliminated the binding of the adipocyte-specific nuclear factor to β_3CRE2, whereas octamer elements have no effect. This result has established that this adipocyte-specific nuclear factor binds to the same or similar cognate DNA sequences as the nuclear hormone receptor superfamily.

Reporter Gene Assay

To gain insight into the effect of the nuclear factors interacting with β_3CRE2 and β_3CRE3 on transcription, these elements were subcloned upstream of the tymidine kinase (tk) promoter of the pBLCAT2. These constructs were transfected into differentiated rat white adipocytes, and the CAT activities were measured. As illustrated in Fig. 8, both β_3CRE2 and β_3CRE3 slightly repressed the basal level of the tk-dependent promoter activity. On cAMP treatment, the CAT activity driven by β_3CRE2 increased, whereas there was no significant difference observed in the CAT activity driven by β_3CRE3.

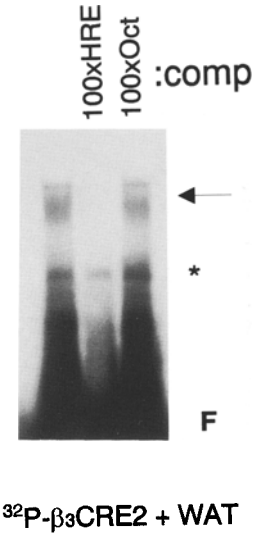

Fig. 7. Competition between nuclear hormone receptor response elements and β_3CRE2. Radiolabeled β_3CRE2 was incubated with approx 5 μg of nuclear extract proteins from human white adipose tissue in the absence (–) or presence of a 100-fold molar excess of unlabeled nuclear hormone receptor response elements (HRE) or histone H2B octamer element (Oct). The β_3CRE2-dependent DNA–protein complex is indicated with an arrow, and * indicates a possible nonspecific binding.

Discussion

In this study, we have shown that ATF-1 or CREB-1 interacts with the putative CREs present in the human β_3-AR gene (β_3CRE1–4) with different affinities (Fig. 1). The specificity of the interaction was confirmed by the competition assays using these CREs as competitors (Fig. 2).

It has been previously shown that the DNA binding affinity of ATF-1 to some CREs is lower than that of CREB owing to binding instability (Hurst et al., 1990). In analogy to this observation, our DNA binding (Fig. 1) and competition experiments (Fig. 2) have shown that the affinity of ATF-1 is weaker than that of CREB-1 for the β_3CREs with the exception of β_3CRE3.

Using nuclear extracts from human brown adipose tissue and monoclonal antibodies (MAbs), we have shown that it is CREB-1 or an immunologically related protein, but not ATF-1 that is bound to the consensus CRE (Fig. 6A), despite the fact that ATF-1 is a ubiquitously expressed protein (Meyer and Habener, 1993; Orten et al., 1994). This might be owing to a lower affinity of ATF-1 compared to

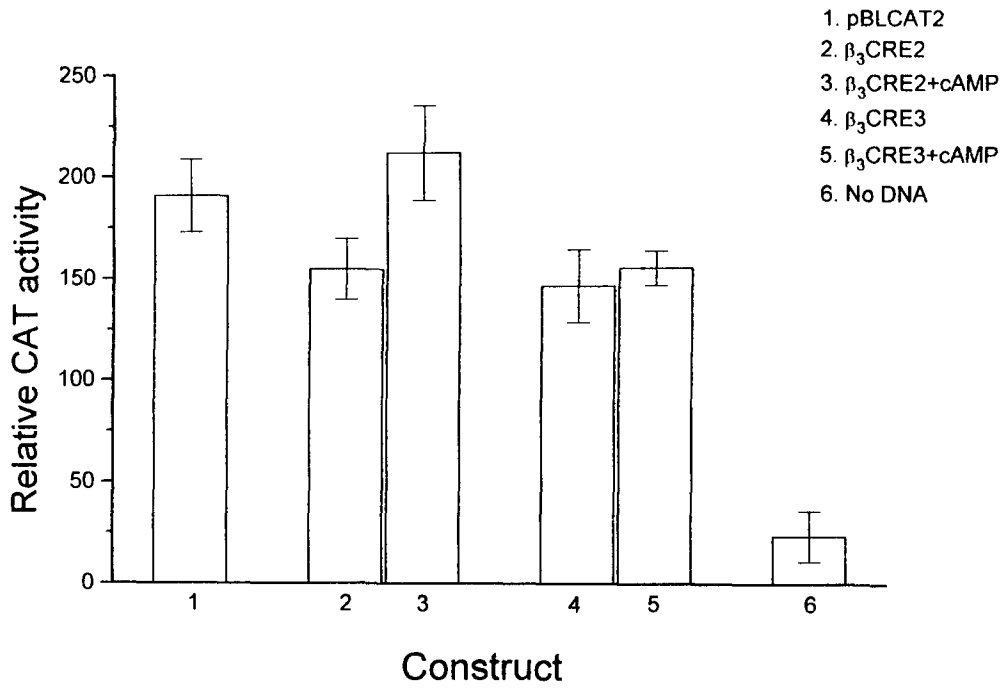

Fig. 8. Reporter gene assay. β_3CRE2 or β_3CRE3 was inserted in front of the tk promoter of the pBLCAT2; 5 μg of each CAT and 0.5 μg of tk-GH plasmids were transfected into the rat white adipose cells. 8-Bromo-cAMP (500 μ*M*) was added to the cells 6 h before harvesting. After 48 h from the transfection, cells were lysed, and CAT activities were measured as described in Materials and Methods. The transfection efficiencies were normalized by the secreted GH in the medium. The results are the mean of two independent measurements with the two values shown by the error bars.

CREB-1 for the CRE site. In addition, differential interaction of CREB/ATF-1 with the CREs may be owing to the differentiation stage of the cells. It has been shown that in the undifferentiated teratocarcinoma cells (F9), the transcriptional activity of CRE is low, despite the presence of CREB and ATF-1. However, when F9 cells are induced to differentiate by treatment with retinoic acid, the cAMP-induced activity is restored (Masson et al., 1992). The low activity of the CRE in undifferentiated cells is correlated with low levels of the regulatory subunit of PKA and high levels of CBP 100, an inhibitor of CREB action (Masson et al., 1992).

The four putative CREs of the human β_3-AR gene were linked to the reporter CAT gene and transfected into the differentiated VERO cells. A clear stimulatory effect of cAMP on the CAT expression was observed with β_3CRE1, β_3CRE2, and β_3CRE4 but not with β_3CRE3 constructs (Thomas et al., 1992). In analogy to this observation, we have shown in this study that β_3CRE2, but not β_3CRE3 mediates cAMP responsiveness (Fig. 7). However, in our gel retardation experiments, β_3CRE3 was found to bind most efficiently to CREB-1 or ATF-1 (Figs. 1 and 2). There are several explanations for this paradox. First, binding affinities do not always correlate with transcriptional activity. For example, three clustered Sp1 sites near the cap site of the insulin-like growth factor binding protein-2 gene have been shown to bind Sp1 protein with affinities that did not correlate with transcriptional activity (Kutoh et al., 1993). The site displaying the weakest binding affinity for Sp1 protein had almost the same transcriptional activity as the sites with the strongest affinity (Kutoh et al., 1993). Second, the human adipose tissue nuclear factor that binds with β_3CRE3 (Fig. 3A,B) might be a repressor and block the cAMP responsiveness. Indeed, we have shown that the nuclear factor from human brown adipose tissue, which binds to β_3CRE3, is distinct from CREB/ATF family (Figs. 4 and 6), and this factor slightly represses transcription and does not confer cAMP responsiveness (Fig. 7). In fact, c-*jun* is known to repress transcription of the human chorionic gonadotropin gene through CRE (Pestell et al., 1994). However, we have eliminated the possibility that this factor, which binds to β_3CRE3, is c-*jun* by using antibodies raised against c-*jun* (result not shown).

We have also shown that the half-binding site for some members of the steroid/thyroid/retinoic acid receptor superfamily could bind specifically to the adipocyte-specific nuclear factor that binds to β_3CRE2 (Fig. 5B), suggesting that this factor displays a similar DNA binding pattern to that of the nuclear hormone receptor superfamily. Response elements for members of this family usually contain two or more repeats of the TGAGGTCA motif (Monia et al., 1995, references therein). Some members of this family, however, can act through a single motif. One of them is retinoid X receptor β (RXRβ), which binds to the single TGAGGTCA motif within region II of the major histocompatibility complex class I transcriptional regula-

tory elements (Hamada et al., 1989; Nagata et al., 1992). We have examined the possibility that the factor complexed with β_3CRE2 in human white adipose tissue is RXRβ by using an antibody raised against RXRβ. The binding pattern in the gel-retardation assay was not altered by the antibody, suggesting that this factor differs from RXRβ (results not shown). Several orphan members of the nuclear receptor superfamily are expressed in a tissue-specific manner (Law et al., 1992, Luo et al., 1994; Chawla and Lazar, 1993). Thus, it is tempting to speculate that the adipose tissue-specific nuclear factor that binds to β_3CRE2 is an orphan member of this family. To this end, it could be mentioned that our experiments in this study were performed by an in vitro approach using cis-element oligonucleotides and purified or extracted trans-factors. The observed interactions, however, may not necessarily reflect the molecular events occurring in vivo. To confirm this point, in vivo footprinting experiments could be considered.

β_3-AR (protein or mRNA) is highly expressed in rodent, but significantly less in human brown and white adipose tissues. There are four possible CREs present in the human gene (Liggett and Schwinn, 1991), but only one in the mouse (Van Spronsen et al., 1993), and none in the rat gene (Brown and Machida, 1994). Therefore, it might be that CREs present in the human β_3-AR gene mediate an inhibitory effect on transcription. Though CREB-1 or ATF-1, which is usually a transcription activator, is able to bind to β_3CREs (Figs. 1 and 2), adipose tissue nuclear factors that are distinct from CREB/ATF family also bind to β_3CRE2 and β_3CRE3. (Figs. 3, 4, and 6). These factors may therefore function as repressors. To support this idea, they have a slight inhibitory effect on transcription (Fig. 7), and it is noteworthy that there is an inverse correlation in human brown and white adipose tissue between the level of the β_3CRE2-dependent DNA–protein complex (Fig. 5) and the degree of the β_3-AR expression.

In analogy to our observation, recent reports have provided evidence that the nonconsensus CREs of the human calcitonin and rabbit surfactant protein A genes can bind to an unidentified nuclear factor that is distinct from CREB/ATF family, and it can mediate cAMP responsiveness (Monia et al., 1995; Michael et al., 1996). However, owing to their tissue-specific (thyroid and lung) expression pattern, these factors are unlikely to be the adipose tissue-specific factor described in this present work. It is known that multiple factors can interact with a single cis-element, thereby producing a greater variety of transcriptional responses (Rauscher et al., 1990; Kutoh and Schwander, 1993) than would be possible with a single transcription factor.

The nuclear factor complexed with β_3CRE2 showed an adipose tissue-specific expression pattern. It might be that, in addition to CREB/ATF-1, this nulcear factor is involved in the regulation of adipose tissue-specific gene expression, including the β_3-AR and the recently discovered obese gene (Zhang et al., 1994). Further biochemical and functional analyses of these factors, such as an in vitro transcription using purified components, are required for a better understanding of the gene regulation of the β_3-AR.

Materials and Methods

Materials

All materials used were of analytic or molecular-biology grade, and were purchased from Merck (Darmstadt, Germany), Sigma (St. Louis, MO), Fluka (Buchs, Switzerland), Gibco-BRL (New York, NY), Pharmacia (Uppsala, Sweden), or Boehringer Mannheim (Mannheim, Germany). *Escherichia coli* expressed recombinant human CREB-1 (bZIP domain) and ATF-1 (full-length) proteins and ATF-1, CREB-1, c-*jun*, and RXR β monoclonal IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Human Tissue

Pieces of human omental adipose tissue weighing between 10 and 40 g were obtained during intra-abdominal surgery and immediately frozen in liquid nitrogen. The patients were men below the age of 65, and were operated on for cancer, inflammatory bowel disease, or cholecystectomy. Their body mass index was 23 ± 1 kg/m^2, i.e., in the normal range. No patient had any identified metabolic or endocrinological disorder. Pieces of perirenal brown adipose tissue weighing about 1.5 g were obtained during renal surgery from male or female patients (mean age: 3 mo) and immediately frozen in liquid nitrogen. Subjects had been fasted for at least 12 h; general anesthesia was obtained with isoflurane or enflurane. The project had been approved by the Ethics Commission of the Department of Surgery of the University of Geneva, Faculty of Medicine.

Animals

Sprague Dawley male rats (9 wk old) were kept at room temperature (about 21°C) with 12 h of illumination/d and fed *ad libitum* with Provimi Lacta chow (Cossonay, Switzerland). They were killed by decapitation. Interscapular brown adipose tissue, epididymal white adipose tissue, kidney, lung, heart, and liver were rapidly excised and frozen in liquid nitrogen until use. For transfection experiments, precursor cells from epididymal adipose tissue were isolated by incubation for 30 min at 37°C in the presence of collagenase and cultured according to Champigny et al. (1992).

Preparation of Nuclear Extracts

A modification of the method of Schreiber et al. (1989), initially developed for the preparation of cultured cell nuclear extracts, was used. Approximately 0.5 g of tissue were homogenized in 10 mL of an ice-cold solution of 10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 5 m*M* DTT, and 1.25 m*M* PMSF with a Potter Elvejhem homogenizer. NP-40 was added to a final concentration of 0.5% and, after 15 min at 4°C, the homogenate

was centrifuged at 20,000*g* for 10 min at 4°C. The pellet was resuspended by gentle homogenization in 5 mL of the above buffer and recentrifuged. The resulting pellet was resuspended in 200 µL of an ice-cold solution of 20 m*M* HEPES (pH 7.9), 25% glycerol, 0.4*M* NaCl, 1 m*M* EDTA, and 1.25 m*M* PMSF, incubated for 15 min at 4°C with gentle shaking, and centrifuged at 15,000*g* for 5 min at 4°C. Supernatants were collected, and their protein concentrations were measured by the Bio-Rad system (Hercules, CA). Nuclear extracts (about 5 mg/mL) were frozen in aliquots at –80°C until use.

Oligonucleotides

The following complementary synthetic oligonucleotides, including the original flanking sequences, were synthesized by Schmidheini (Balgach, Switzerland), annealed and used for the gel-retardation assay. Mismatches from the consensus sequences (underlined) are shown with small letters; 1 pmol of each double-stranded oligonucleotides was labeled with ^{32}P-dCTP, purified by Nick Column (Pharmacia, Uppsala, Sweden), and used for the gel-retardation assay. Sequences of the sense strands of the annealed oligonucleotides are shown below. Consensus sequences are underlined. A fill-in reaction was performed by adding the *Hin*dIII (5'-end) and *Bam*HI (3'-end) restriction sites.

β_3CRE1: TTTGCTTGACtcCAGCGGGT
β_3CRE2: ACCAAGCTGAgGTCtTGGGA
β_3CRE3: CAGACATcACaTCATTGCAC
β_3CRE4: GGATCAcGAgGTAGGAGAT

Consensus CRE of the choriogonadotropin α-gene (Bohm et al., 1993):

CGAGAAATTGACGTCATGGTAAG

Histone H2B octamer (Gerster and Roeder, 1988):

CTTCACCTTATTTGCATAAGCG

Nuclear hormone receptor binding sites (Monia et al., 1995);

AGCTGAGGTCATGGG

Gel-Retardation Assays

The gel-retardation assay was performed as previously described (Alksnis et al. 1991). Briefly, binding reactions of 20 µL were carried out in buffer containing 10 m*M* HEPES (pH 7.9), 2.5 m*M* $MgCl_2$, 10% glycerol (v/v), 1 m*M* DTT, 1 µg poly (dI-dC), 50 m*M* KCl, 2.5 fmol of ^{32}P-labeled oligonucleotides, and 50 ng of human recombinant ATF-1/CREB-1 protein or 5 µg of nuclear extract proteins. Competitors (unlabeled probe) were added at the same time as the radiolabeled probes. MAbs were preincubated with the proteins at 4°C for 1 h before the start of the reaction. The incubation was carried out at room temperature for 15 min. Free and bound DNA were separated by electrophoresis on nondenaturing 4% polyacrylamide gels (acrylamide bis 29:1, crosslinked) with 0.5X TBE (1X TBE: 90 m*M* Tris-borate, 1 m*M* EDTA) as running buffer. The gels were dried and analyzed by autoradiography.

Reporter Plasmid Constructs

All enzyme reactions were performed according to standard procedures (Sambrook et al., 1989). Double-stranded oligonucleotides of β_3CRE2 and β_3CRE3 carrying the *Hin*dIII (5') and *Bam*HI (3') restriction sites at each end were subcloned into the *Hin*dIII/BamHI sites present upstream of the tk promoter of the pBLCAT2 plasmid (Luckow and Schütz, 1987). The plasmids were prepared by Qiagen columns (Diagen, Düsseldorf, Germany) for the transfection experiment. tk-GH plasmid (Selden et al., 1986) was used to normalize the transfection efficiency.

Cell Culture, Transfection, and CAT Assay

Cells were cultured in a mixture of Dulbecco's Modified Eagle's Medium (DMEM)/F-12 nutritive medium (1:1; v/v) supplemented with sodium bicarbonate 50 m*M*, penicillin 100 U/mL, streptomycin 50 µg/mL, dexamethasone 50 n*M*, isobutylmethylxanthine (IBMX) 0.2 m*M*, and carbacycline 1.4 n*M* in a 95% air/5% CO_2 atmosphere at 37°C. Insulin 20 n*M* and triiodothronine 2 n*M* were added to this complete medium, which was supplemented with 10% fetal calf serum (FCS) (Champigny et al., 1992). Medium was changed every 3 d, and a transfection experiment (cell number: approx 10^4/30-mm dish) was performed on day 6. Five micrograms of each CAT and 0.5 µg of tk-GH plasmids were transfected into the mouse white adipocytes differentiated in primary cultures using the calcium phosphate transfection method (Bohm et al., 1993). The efficiency of transfection was enhanced by using the CalPhos Maximizer (Clontech, Palo Alto, CA) at the final concentration of 20 µg/mL in the medium. A cell-permeable cAMP analog, 8-bromo-cAMP (500 µ*M*), was added to the cells 6 h before harvesting. The cells were harvested after 48 h of transfection, and CAT activity was measured using the CAT ELISA KIT (Boehringer Mannheim). Briefly, cells were lysed with 300 µl of the lysis buffer supplied with the kit. Equal amounts of proteins (150 µg per well) were loaded on each lane and CAT activity was measured according to the manufacture's protocol. Transfection efficiency was normalized by measuring the secreted GH in the medium using the GH ELISA KIT (Boehringer Mannheim).

Computer Analysis

To find homologies, each β_3CRE sequence was analyzed by the Vacs scan system (F. Hoffmann La-Roche, Basel, Switzerland).

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