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Expression of the Human β_3-Adrenergic Receptor Gene in SK-N-MC Cells Is Under the Control of a Distal Enhancer

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ABSTRACT

Mechanisms of transcriptional regulation of the human β_3-adrenergic receptor were studied using SK-N-MC cells, a human neuroblastoma cell line that expresses β_3- and β_1-adrenergic receptors endogenously. Deletions spanning different portions of a 7-kb 5′-flanking region of the human β_3-adrenergic receptor gene were linked to a luciferase reporter and transfected in SK-N-MC, CV-1, and HeLa cells. Maximal luciferase activity was observed when a 200-bp region located between −6.5 and −6.3 kb from the translation start site was present. This region functioned only in SK-N-MC cells. Electrophoretic mobility shift assays of nuclear extracts from SK-N-MC, CV-1, and HeLa cells using double stranded oligonucleotides spanning different portions of the 200-bp region as probes and transient transfection studies revealed the existence of three *cis*-acting regulatory elements: A) −6.468 kb-AGGTGGACT-−6.458 kb, B) −6.448 kb-GCCTCTCTGGGGAGCAGCTTCTCC-6.428 kb, and C) −6.405 kb-20 repeats of CCTT-6.385 kb. These elements act together to achieve full transcriptional activity. Mutational analysis, antibody supershift, and electrophoretic mobility shift assay competition experiments indicated that element A binds the transcription factor Sp1, element B binds protein(s) present only in nuclear extracts from SK-N-MC cells and brown adipose tissue, and element C binds protein(s) present in both SK-N-MC and HeLa cells. In addition, element C exhibits characteristics of an S1 nuclease-hypersensitive site. These data indicate that cell-specific positive *cis*-regulatory elements located 6.5 kb upstream from the translation start site may play an important role in transcriptional regulation of the human β_3-adrenergic receptor. These data also suggest that brown adipose tissue-specific transcription factor(s) may be involved in the tissue-specific expression of the β_3-adrenergic receptor gene. (*Endocrinology* **142:** 1935–1949, 2001)

THE β_3-ADRENERGIC receptor (β_3AR) is an important regulator of metabolic activity in brown and white adipose tissues (BAT and WAT, respectively), two major sites for regulation of energy balance. The β_3AR belongs to a family of G protein-coupled receptors. Binding of endogenous ligand or specific synthetic agonists to this receptor activates adenylate cyclase and increases cAMP levels, leading to increased protein kinase A activity. These effects are thought to be responsible for increased thermogenic activity and heat production in BAT and increased lipolysis in WAT. As β_3AR stimulation causes an increase in thermogenic activity and less efficient utilization of metabolic fuels, its sustained activation may be important for the treatment of obesity and improvement of glycemic control in type II diabetes. Indeed, numerous reports have shown that stimulation of β_3ARs causes weight loss and improved glycemic control in rodent models of these diseases (1–8).

Despite the well characterized role of β_3ARs in rodents, its role in the regulation of energy balance in man is not clear. A positive correlation between an Arg to Trp mutation at position 64 within the human β_3AR gene and the early onset of noninsulin-dependent diabetes mellitus (9–13), insulin resistance (9), increased weight gain (10, 11, 14–16), and abdominal obesity (9) has been reported (9–16). However, other investigators have failed to observe this correlation (17–20).

The successful treatment of obesity and diabetes in rodent models with selective β_3AR agonists (1–8) supports a role for this receptor as a therapeutic target in man and has prompted a great effort toward the development of compounds with a high affinity and selectivity for the human β_3AR. However, some agonists with high affinity and selectivity for β_3AR in transfected CHO cells that express high levels of human β_3ARs showed little or no activity *in vivo* when tested in nonhuman primates and clinical trials (21, 22). These conflicting observations could be due to a variety of factors, including pharmacokinetic and metabolic issues, the involvement of cell- and species-specific factors in shaping the β_3AR response, and variability of β_3AR expression in target tissues in different pathophysiological contexts. The efficacy and potency of different human β_3AR agonists can also depend on the expression level of the β_3AR (23).

Previous studies suggested that in rodents β_3AR mRNA is expressed abundantly in both BAT and WAT (24, 25),

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whereas in man it is expressed in BAT, but appears to have little or no expression in WAT (26–28). In contrast to these findings, treatment with a selective agonist for the human β_3AR (CGP-12177) increased lipolysis and glycerol formation *in vivo* and *in vitro*, suggesting the presence of functionally active β_3ARs in human WAT (29, 30). However, the significance of these studies remains to be clarified, as CGP-12177 might interact with another adrenergic receptor(s) (31).

Although the genes for mouse (32), rat (33), and human (h) β_3ARs (33–35) have been cloned, little additional data (36, 37) are available regarding the structure of regulatory regions and possible transcription factors involved in β_3AR transcriptional regulation. Recently, Ito *et al.* (38), using transgenic mice, showed the importance of the 500-bp sequence in the promoter that may be responsible for BAT-specific expression of human β_3ARs. However, both the level of β_3AR expression as well as tissue distribution differ in mice and man. These differences indicate the possible existence of different mechanisms and elements that direct its expression in human cells and tissues compared with rodents.

The data presented here used SK-N-MC cells, a human neuroblastoma cell line that expresses β_3AR endogenously (33, 34). In this paper we show that SK-N-MC cell-specific expression of the human β_3AR gene is dependent on interactions among three regulatory elements, A, B, and C, located between the −6.5 to −6.3 kb region upstream of the translation start site of the gene. Characterization of transcription factors that bind to these elements showed that element A binds the ubiquitous factor Sp1, whereas element B binds a factor(s) present only in SK-N-MC cells and mouse BAT, suggesting its involvement in directing adipose tissue-specific expression of the β_3AR gene. Element C binds a factor(s) present in both SK-N-MC and HeLa cells.

Materials and Methods

Cloning of human β_3AR genomic DNA

To isolate a 5′-flanking region of the hβ_3AR gene, a human fibroblast genomic library (Stratagene, La Jolla, CA) was screened using human β_3AR cDNA. cDNA was constructed by ligating four PCR products using the following primers: an ATG-*Nar*I fragment [sense primer, 5′-CTTTCCCTACCGCCCCACGCGCGAC-3′ (nucleotides 606–630); antisense primer, 5′-GTGGCGCCCAACGGCCAGTGGCCAGTC-3′ (nucleotides 934–961)], a *Nar*I-*Acc*I fragment [sense primer, 5′-TTGGCG-CTGACTGGCCACTGGCCGTTG-3′ (nucleotides 926–953); antisense primer, 5′-GCGCGTAGACGAAGAGCATCACGAG-3′ (nucleotides 1288–1313)], an *Acc*I-*Sty*I fragment [sense primer, 5′-CTCGTGATGCTC TTCGTCTACGCGC-3′ (nucleotides 1288–1313); antisense primer, 5′-GT-GAAGGTGCCCATGATGAGACCCAAGG-3′ (nucleotides 1515–1542)], and a *Sty*I-TAG fragment [sense primer, 5′-CCCTGTGCACCTTGGGTCT-CATCATGG-3′ (nucleotides 1506–1533); antisense primer, 5′-CCTCTGC-CCCGGTTACCTACCC-3′ (nucleotides 1842–1850)]. The corresponding primer sequences were taken according to the sequence with GenBank Accession No. X72861. The four fragments were ligated into a pUC18 plasmid (Life Technologies, Inc., Gaithersburg, MD) and sequenced. Using cDNA as a probe, two genomic clones were isolated. In addition, a PCR product representing a 1.3-kb hβ_3AR promoter that was previously reported (36) was used to identify 7 kb of the hβ_3AR gene 5′-flanking region. This 7-kb promoter region was subcloned into pSP72 (Promega Corp., Madison, WI) between *Pst*I and *Hin*dIII restriction enzyme sites and mapped extensively. The full length of the promoter was sequenced in both directions using automated sequencing.

Rapid amplification of 5′-cDNA ends (5′RACE)

To identify the transcription start site of the hβ_3AR gene, 5′RACE was performed on an SK-N-MC polyadenylated [poly(A)] RNA that was isolated using a Micropo (A) pure kit (Ambion, Inc., Austin, TX). The 5′RACE was performed according to the protocol provided with the Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA). The primers used included the sense adapter primer AP2 (CLONTECH Laboratories, Inc.; 5′-ACTCACTATAGGGCTCGAG-CGGC-3′) and the antisense primer (5′-GGCAGCCCACTGGTGTT GGCGGTAT-3′) that corresponded to the hβ_3AR gene sequence at positions 729–703 (GenBank Accession No. X72861). The PCR products were then cloned into a pCRII vector using the TA cloning kit (Invitrogen, Carlsbad, CA). The clones were sequenced using the ABI 373 automated sequencer (PE Applied Biosystems, Foster City, CA).

Human β_3AR gene promoter deletion constructs

Serial deletions of the hβ_3AR gene 5′-flanking region were designed to identify the region(s) responsible for transcriptional regulation. The 7-kb hβ_3AR promoter was cloned into the *Kpn*I/*Hin*dIII sites of a pGL3 basic vector (Promega Corp.) to obtain the full-length promoter that drives the expression of the reporter gene (luciferase). This full-length promoter is labeled −7 hβ_3/Luc. The pGL3 basic vector contains luciferase cDNA as a reporter gene and an upstream synthetic poly(A) signal to reduce background. The deletion constructs labeled −5.5hβ3/Luc, −3hβ3/Luc, and −0.5hβ3/Luc were made by digestion with *Kpn*I and *Avr*II, *Eco*RV, and *Bst*EII, respectively, blunt-ended, and religated. The −1.3hβ3/Luc construct was made by ligating a PCR product obtained using HeLa cell genomic DNA and 5′-ggtaccTCTAGGTGGAAAGGT-GCATG-3′ as sense primer and 5′-aagcttAGTCCCCTCCCTGTCGT-3′ as antisense primer (GenBank Accession No. M62473).

Constructs with deletions within the promoter region (dEVhβ_3AR/Luc, dEIhβ_3AR/Luc, and dBhβ_3AR/Luc) were made by digesting parental vector −7hβ_3AR/Luc with *Avr*II (−5.6 kb) and *Eco*RV (−3.1 kb), *Eco*RI (−2.3 kb), and *Bst*EII (−0.5 kb), respectively, blunt-ended, and religated.

To further analyze and more precisely identify the sequence located between −7 and −5.6 kb of the hβ_3AR promoter that contains *cis*-regulatory elements, expression plasmids were generated containing

TABLE 1. PCR primers for analysis of the hβ_3-AR promoter

Primer no.	Primer sequence	Amplified genome region
	Sense Primers	
1S	5′-CTGCAGGGGTTGAGAAC3′	(−7.12 kb–7.105 kb)
3S	5′gctagcGCAAGTGCAATCTATAACACAGGGG3′	(−6.96 kg–6.94 kb)
	Antisense Primers	
4AS	5′gtcgacGCTGGGATTACAGGTCCGTGC3′	(−6.71 kg–6.69 kb)
6AS	5′gtcgacATGCTTAGGCTTCCTTCCAGG3′	(−6.60 kb–6.48 kb)
8AS	5′gtcgacCTTTTGCAAGTGGCACGAAGG3′	(−6.32 kb–6.30 kb)
14AS	5′gtcgacACCTGCCAGTCTGCCTTCTC3′	(−5.90 kb–5.88 kb)
12AS	5′gtcgacCCTAGGTGGCAGAGCGAGACTCT3′	(−5.64 kb–5.62 kb)

Sequences of primers used in PCR in order to make vectors that contain regions necessary for transcription of hβ_3-AR. Positions of primers are determined from the translation start site at +1. *Smaller letters* represent *Kpn*I and *Nhe*I restriction enzyme sites introduced within sense and antisense primers, respectively.

PCR products made using a series of primers shown in Table 1. *Nhe*I and *Bam*HI restriction enzyme sites were introduced for cloning purposes. Amplified fragments were cloned into a pCRII vector. The PCR fragments were further ligated either in front of the minimal promoter herpes simplex virus thymidine kinase (TK) obtained from pTKb plasmid (CLONTECH Laboratories, Inc.) or 0.5 kb of the hβ_3AR gene promoter. PCR products were first ligated into a pSP72 plasmid containing the TK minimal promoter. After digestion with *Nhe*I/*Bgl*II, the fragments containing PCR products/TK minimal promoter were gel-purified and cloned back into the pGL3 basic vector. As a measure of basal transcriptional activity, the construct containing only the TK minimal promoter was used. Data from experiments were expressed as the fold increase over TK/pGL3 activity. PCR products between primers 3Sx12AS, 3Sx14AS, and 3Sx8AS were also ligated in the reverse orientation in front of a TK minimal promoter. The accuracy of all clones was confirmed by sequencing.

When 0.5 kb of the promoter served as a minimal promoter, the primers shown in Table 1 but containing a *Kpn*I site in the sense orientation and a *Bst*EII site in the antisense orientation were used. PCR products were ligated in front of −0.5hβ_3AR/Luc constructs.

To test for the contribution of specific elements (*i.e.* elements A, B, and C) to the transcriptional activity of the 3x8 region, a series of DNA constructs was made that contained mutations in elements A and/or B, keeping element C intact in the context of the 3x8−0.5hβ_3AR/luc. Elements A, B, and C are described in *Results*. The importance of element C was examined by mutating elements A and B (vector 3x8 AmBmC/0.5β_3/Luc), or examining the transcriptional activity when element C alone was present, *i.e.* construct C-0.5β3/Luc. To introduce mutations within these constructs, we designed PCR primers that contained mutations within elements A and/or B. In addition, a unique *Bss*SI site that is used for cloning purposes was conveniently located at position −6.46 kb within element A. Briefly, to generate 3x8, A mutated (m), B, C, primer 3 as sense (see Table 1), and primer 5′-GTTG**TTC**CTGGGACTCGTGA-3′ (introduced mutation is in *bold* and BssSI site is *underlined*) as antisense were used. The PCR products were digested with *Kpn*I and *Bss*SI and ligated into 3x8 0.5hβ3/luc previously digested with the same enzymes. To generate 3x8 A, Bm, C, and 3x8 Am, Bm, C, sense primer 5′-TGGGACTCGTGACCTCTCCC**AGC**CAGACGGGAGC-3′, and primer 8 (see Table 1) as an antisense primer were used. The PCR products were digested with *Bss*SI/*Bst*EII and ligated into previously *Bss*SI/*Bst*EII-digested plasmids 3x8 0.5β3/luc and 3x8 Am, B, and C, respectively. Constructs that carried only element A with or without mutations (3xAwt, 3xAc,mut) and only element B with or without mutations (3xBwt, 3xBa,mut) in the absence of element C were also prepared and tested in transient transfection assays.

Cell culture

Three cell lines were used in these experiments: SK-N-MC cells, (American Type Culture Collection, Manassas, VA), CV-1 kidney cells from the green monkey, and HeLa cells. SK-N-MC cells were grown in monolayer in MEM supplemented with 10% FBS and nonessential amino acids (Life Technologies, Inc.). The CV-1 cells were grown in DMEM (10% FBS), and HeLa cells were maintained in Ham's F-12 (Life Technologies, Inc.) supplemented with 10% FBS. All media also contained penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown at 37 C with 5% CO_2.

Transient transfection experiments

DNA constructs were transiently transfected into cells using either $CaPO_4$ precipitation (Life Technologies, Inc.) or Lipofectamine Plus (Life Technologies, Inc.) according to the manufacturer's recommendations. Cells were transfected in a condition of subconfluence. Three hours before transfection, medium was changed. We used 10 μg construct containing luciferase as a reporter gene and 1 μg pRSVβ-gal (RSV, Rous sarcoma virus; β-gal, β-galactosidase; CLONTECH Laboratories, Inc.) as a control for transfection efficiency when $CaPO_4$ precipitation was used. When cells were transfected using Lipofectamine Plus, 1.5 μg DNA constructs and 0.17 μg pSVβ-gal were used. After incubation for 16 h, the medium was replaced and incubated for an additional 24 h. The next day cells were assayed for luciferase and β-galactosidase activity. In each experiment all constructs were tested in triplicate. Each experiment was repeated four or five times with two or three different DNA preparations. Two different passages of SK-N-MC cells were tested. Luciferase activity was determined as previously described (39). The activity of β-galactosidase was measured using a Tropix kit (Cambridge, MA).

RNA analysis and RT-PCR

Total RNA was isolated from perirenal adipocytes differentiated in culture (Zen-Bio, Inc., Research Triangle Park, NC), using RNAsol (Biotecx Laboratories, Inc., Houston, TX). RT-PCR was performed as suggested by the TaKaRa RT-PCR kit (TaKaRa Biomedicals). For RT reactions 500 ng total RNA were used. cDNA was synthesized for 50 min at 42 C, and the PCR reaction was performed using conditions previously described (40). Briefly, cDNA was amplified by 30 cycles under the following conditions: 95 C for 30 sec, 76 C for 10 sec, 58 C for 30 sec, 62 C for 10 sec, 68 C for 10 sec, 72 C for 30 sec, and 1-min extension at 72 C. Primers for human uncoupling protein 1 (UCP1) (40), peroxisome proliferation-activating receptor-γ (PPARγ), and human actin were used. For UCP1, sense primer 5′-TAGGTATAAAGGTGTCCTGG-3′ and antisense primer 5′-CACTTTTGTACTGTCCTGGTGG-3′ were used. Sequences for sense and antisense primers were, respectively, 5′-TGGCCGCAGGAAATGACCATGGTTGA-3′ and 5′-CGGAGAACAATCAGATTGAAGC-3′ for PPARγ and 5′-CGACGAGGCCCAGAGCAAGC-3′ and 5′-CCAGGGCGACGTAGCACAGC-3′ for actin (40). PCR reactions were run on 1.2% agarose gels.

Nuclear extract and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were isolated from SK-N-MC and CV-1 cells as well as from mouse BAT, WAT, liver, and muscle tissue. Nuclear extract from HeLa cells was purchased from Promega Corp. For isolation we used a method described previously (41). Cells that were 80–90% confluent were washed three times with PBS buffer and incubated with buffer A composed of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, and a cocktail of several protease inhibitors (Roche, Indianapolis, IN). After 10-min incubation on ice, cells were centrifuged for 10 min at 250 × *g*. Pellets were resuspended in 3 vol ice-cold buffer A with 0.05% Nonidet P-40, and homogenized with 20 strokes in a Dounce homogenizer (Kontes Co., Vineland, NJ). After centrifugation at 250 × *g*, pellets were resuspended in buffer B containing 5 mM HEPES (pH 7.9), 26% glycerol (vol/vol), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and a cocktail of protease inhibitors. NaCl was added to a concentration of 300 mM, and the suspension was incubated for 30 min on ice. After centrifugation at 24,000 × *g* for 50 min, supernatants were aliquoted and stored at −70 C. Protein concentrations were measured using the Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

Nuclear extracts were isolated from tissue using the method of Varshavsky (42). Tissues of interest (BAT, WAT periovarian depot, liver, and muscle) were isolated from mice and placed in ice-cold PBS. All procedures were performed at 4 C. Tissues were weighed, minced, and homogenized in the presence of Nonidet P-40 in buffer A1 [15 mM HEPES (pH 7.6), 60 mM KCl, 15 mM NaCl, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM spermine, and protease inhibitor cocktail]. Tissue homogenates were centrifuged at 1200 × *g* for 10 min; pellets were resuspended in 0.3 M sucrose/buffer A1 and layered in an equal volume on a cushion of 1.7 M sucrose/buffer A1. After centrifugation at 24,000 × *g* for 45 min, pellets were resuspended in buffer B1 [10 mM HEPES (pH 7.6), 350 mM NaCl, 5% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EGTA, and protease inhibitor cocktail] and incubated at 4 C for 30 min. After centrifugation at 100,000 × *g* for 60 min, supernatants were aliquoted and stored at −70 C, and protein concentrations were determined.

To increase the presence of proteins that bind for regions A and B, we further purified crude nuclear extracts using a heparin column. The purification was performed as described previously (43). Crude extracts were diluted in appropriate buffer to make a final concentration of 20 mM HEPES (pH 7.9), 20% glycerol, 150 mM KCl/NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, and protease inhibitor cocktail. The heparin-Sepharose CL-6B column (Pharmacia Biotech, Uppsala, Sweden) was made from a 50% slurry. The columns were equilibrated with 10 ml of the equilibration buffer described above. Crude nuclear extracts were loaded onto columns, flow-through was collected, and after washing, proteins were

eluted with elution buffer (same as the dilution buffer described above only containing 0.8 M KCl). Aliquots were stored at −70 C.

EMSAs were performed using double stranded T4 polynucleotide kinase (Life Technologies, Inc.) labeled oligonucleotides (oligos). Binding reactions contained 10 μg crude or 2 μg heparin-purified nuclear extract protein, 1.5 μg poly(dI-dC) (Pharmacia Biotech), 70,000–100,000 cpm radiolabeled probes in a binding buffer consisting of 0.24 mM $ZnSO_4$, 20% glycerol, 100 mM KCl (this final concentration was obtained by addition of KCl to the binding reaction after first calculating the concentration of KCl resulting from the addition of the nuclear extract), 0.05% Nonidet-P-40, 0.1 mM EDTA, and 20 mM HEPES, pH 8.4. Nuclear extract and poly(dI-dC) were added to the binding buffer and incubated at room temperature for 30 min with radiolabeled probes. In some experiments cold competitor or antibodies for Sp1 and AP2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) proteins were used. After incubation to allow binding, samples were run on 6% acrylamide, 0.5 × Tris-borate buffer gel. Oligonucleotides used in EMSA are described in the text and figure legends. Oligonucleotides containing Sp-1 or AP2 consensus binding sites were bought from Santa Cruz Biotechnology, Inc. All EMSA experiments were repeated at least four times.

Examination of S1 nuclease hypersensitive sites

The 80-bp CCTT region of the hβ_3AR promoter was amplified by PCR using primer 6 at a position −6.508 kb to −6.488 kb of the hβ_3AR promoter (5′-CCTGGAAGGAAGCCTAAGCAT-3′) as sense and primer 16 at position −6.20 kb to −6.18 kb (5′-GGCACTGCTAGGAACACACTC-3′) as antisense. The PCR product was subcloned in pGEM7 at *Eco*RI and *Bgl*II sites. The supercoiled form of plasmid was digested with *Sca*I after 30-min treatment with S1 nuclease. S1 hypersensitivity was tested in the presence of increasing salt concentrations as well as in different pHs. One microgram of DNA was treated for 30 min with 5 U S1 nuclease in buffer containing 50 mM sodium acetate (pH 4.5), 0.1 M $ZnCl_2$, and increasing concentrations of NaCl (0, 0.05, 0.1, 0.3, and 0.5 M). In addition, experiments were performed in buffer at pH 6 or 7 in the presence of 0.3 M NaCl.

Results

hβ_3AR genomic clones

To obtain the 5′-flanking region of the hβ_3AR gene, we screened a human fibroblast genomic library using partial cDNA as a probe. Two positive clones were identified. The entire 7.0 kb of the 5′-flanking region was sequenced from both directions (Accession No. AF359565).

Identification of the transcription initiation site

After cloning the 7-kb 5′-flanking region of the human β_3AR, we undertook experiments to identify the transcription start site of the human β_3AR. Previous work (35, 44) suggested the presence of several transcription start sites between −200 and −130 bp upstream of the translation start site. The strongest start site appeared to be located at position −180 bp. We confirmed these findings by 5′RACE using poly(A) RNA from SK-N-MC cells. Four different populations of PCR products were isolated using a sequence 81 bp downstream from the translation start site as a primer. Of the resulting 20 clones, 6 stopped at position 130, 4 clones stopped at position 150, 6 clones stopped at position 200, and 4 clones stopped at position 100 from the translation start site respectively (Fig. 1). These results confirm the previously published data (35, 44) and suggest a functional role for the two TATA-like sequences located 25 bp upstream of the transcription start site.

Functional analysis of −7 kb hβ_3AR promoter region

A 7-kb 5′-flanking region of the human β_3AR gene was isolated from a human fibroblast genomic library. To identify the sequences involved in the regulation of hβ_3AR gene expression, vectors containing serial deletions of segments of the hβ_3AR promoter ligated to a luciferase reporter gene (Fig. 2A) were transiently transfected into SK-N-MC cells. Luciferase activity was measured, corrected for transfection efficiency, and expressed as the fold increase over the activity of the control reporter (pGL3 basic). The data in Fig. 2B show that a region of 0.5 kb of the hβ_3AR promoter was sufficient to drive a low level of expression in all cell types tested, suggesting that this DNA region could serve as a minimal promoter. Constructs containing 1.3, 3, and 5.6 kb of the hβ_3AR gene promoter also caused low level expression, as measured by luciferase activity (Fig. 2B). However, additional elements located upstream of −5.6 kb appear to be required for maximal expression of the hβ_3AR, because a construct containing the full 7 kb (−7hβ_3/Luc) increased luciferase activity approximately 50-fold over basal levels (Fig. 2B). These data suggest that the region between −7 and −5.6 kb contains a strong positive regulatory element(s) that, at least in SK-N-MC cells, may control expression of the hβ_3AR.

The cell lines used in our study showed different transfection efficiencies when the same transfection methods used. To achieve comparable transfection efficiency we used two different methods of transfection for different cell types. In addition to normalization of transfection data by RSV/β-gal cotransfection, we also compared luciferase activity in all three cell types transfected with RSV/Luc vector, which was used as a positive control. As shown in the small panel in Fig. 2B, RSV/Luc induced luciferase activity in all three cell types in a range of 250-, 450-, and 380-fold over basal levels in SK-N-MC, CV-1, and HeLa cells, respectively.

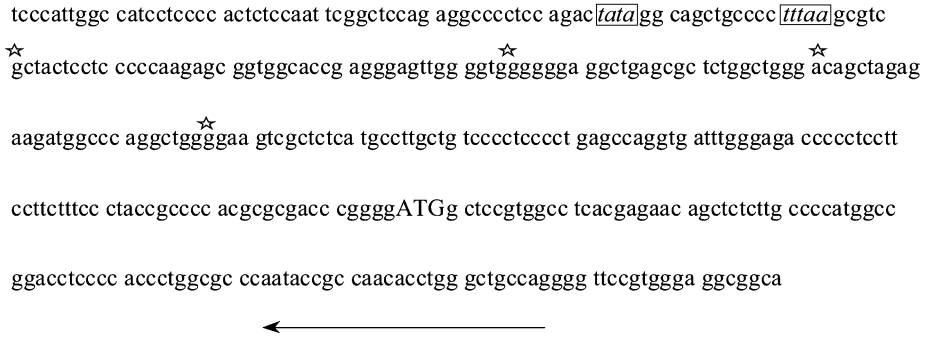


FIG. 1. Determination of transcription start site of hβ_3AR gene using 5′RACE. 5′RACE was performed on poly(A) RNA isolated from SK-N-MC cells using the Marathon cDNA amplification kit (CLONTECH Laboratories, Inc.). Twenty subcloned RACE-PCR products were sequenced. The capitalized ATG sequence represents the translation start sites. The *underlined* sequence represents the primer used in 5′RACE; determined transcription start sites are indicated with asterisks. TATA-box like sequence shown in *italics*.

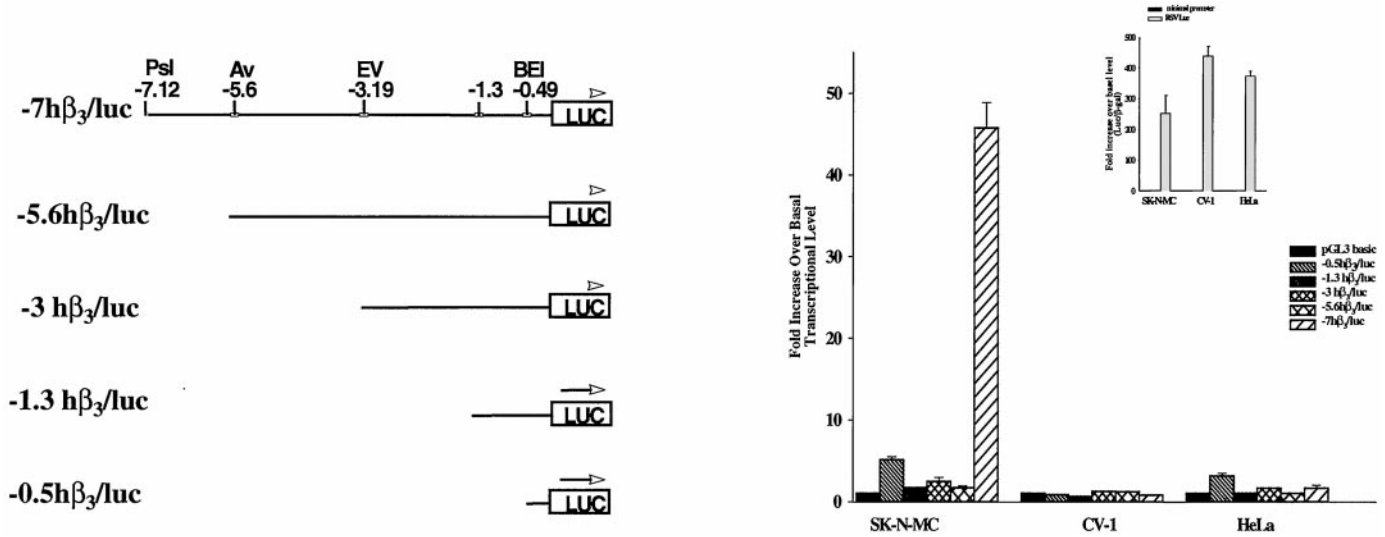


FIG. 2. Transient transfection experiment using hβ_3AR promoter constructs in SK-N-MC, CV-1, and HeLa cells. Transient transfection experiments were performed using calcium phosphate precipitation and Lipofectamine Plus methodology. The amounts of DNA constructs used in these experiments along with experimental methodology are described in *Materials and Methods*. A, Partial restriction enzymes map of constructs used in the experiments. All promoter regions are cloned in a pGL3 basic vector. The positions of the restriction enzyme sites are labeled, with the translation start site being +1. B, Results from transient transfection experiments in SK-N-MC, CV-1, and HeLa cells. Data are presented as fold increases over transcriptional activity achieved with the pGL3 basic vector and are corrected for transfection efficiency measured by the level of cotransfected β-gal activity. The value for each construct was obtained from at least five experiments; each experiment was performed in triplicate. During the course of these experiments two or three different preparations of DNA were made. The *small panel* in B represents the fold increase in RSV/Luc over pGL3 basic vector used as a positive control for transfection efficiency and cell viability. *Error bars* represent the SEM.

Cell-specific expression

Constructs carrying the deletions described above (see Fig. 2A) were also introduced into CV-1 and HeLa cells. These cell types do not endogenously express β_3ARs. Unlike the transfected SK-N-MC cells, when the -7hβ3/Luc was introduced into CV-1 or HeLa cells, no luciferase activity was observed. In addition, none of the other constructs with the exception of -0.5hβ_3/Luc showed any transcriptional activity. -0.5hβ_3/Luc showed a low level of luciferase activity in HeLa cells.

Regulatory elements present in −7 to −5.6 kb are strong activators of hβ_3AR gene expression in SK-N-MC cells

To allow for a detailed analysis of the 5′-flanking region of the hβ_3AR gene, we made a series of internal deletion constructs that contained the region between -7 to -5.6 kb, but lacked the regions between *Avr*II and *Eco*RV (dEV), *Eco*RI (dEI), and *Bst*EII (dB; see Fig. 3A). All constructs were transfected into SK-N-MC and CV-1 cells. In SK-N-MC cells constructs, dEV, dEI, and dB showed the same activity as the parental construct -7hβ_3/Luc (Fig. 3B), suggesting that regulatory elements located within the -7 to -5.6 kb distal region are responsible for maximal transcriptional activity of the hβ_3AR gene promoter in these cells. No activity of any of the constructs was observed in transfected CV-1 cells.

To precisely identify the location of potential regulatory elements in this region, a series of PCR products that spanned the regions between the designated primers (primers are labeled with numbers, see Fig. 4A) were made and ligated to herpes simplex virus TK/pGL3 vector. These constructs were transiently transfected into SK-N-MC and CV-1 cells, and data are shown in Fig. 4B. The data suggest the following: 1) the region between primers 6 and 8 (200 bp) contains regulatory elements necessary for transcriptional activity of the distal promoter region; 2) the region between primers 14 and 8 may contain elements that behave as repressors; and 3) the activities of the regions between primers 3 and 12, 3 and 14, and 3 and 8 are cell specific. To exclude the possibility that nonspecific interactions between the TK promoter and positive regulatory elements in the hβ_3AR promoter cause increased transcriptional activity, we tested whether the region between primers 3 and 8 (a region that is transcriptionally active in SK-N-MC cells) can increase transcriptional activity of the -0.5 kb endogenous hβ_3AR promoter (45). As shown in Fig. 4C, an even greater positive response was observed when endogenous minimal promoter was connected to the 3x8 region. These data raise the possibility of functional synergy between 5′-upstream regulatory elements and the basal promoter. Finally, as shown in Fig. 4D, fragments 3x12 and 3x8 are active in both forward and reverse orientations.

Three sites within the hβ_3AR 5′-flanking region (−6.5 to −6.3) bind nuclear proteins in SK-N-MC cells

The ability of the region between primers 3 and 8 to cause maximal stimulation of luciferase activity suggests that DNA elements within this region may bind transcription factors in SK-N-MC cells. As the region between primers 3 and 6 was only weakly active in driving transcription compared with the region between primers 3 and 8, we focused our attention on the region between primers 6 and 8. We constructed nine 40-bp double stranded oligos that covered the entire region between primers 6 and 8 (see Fig. 5A). These oligos were used

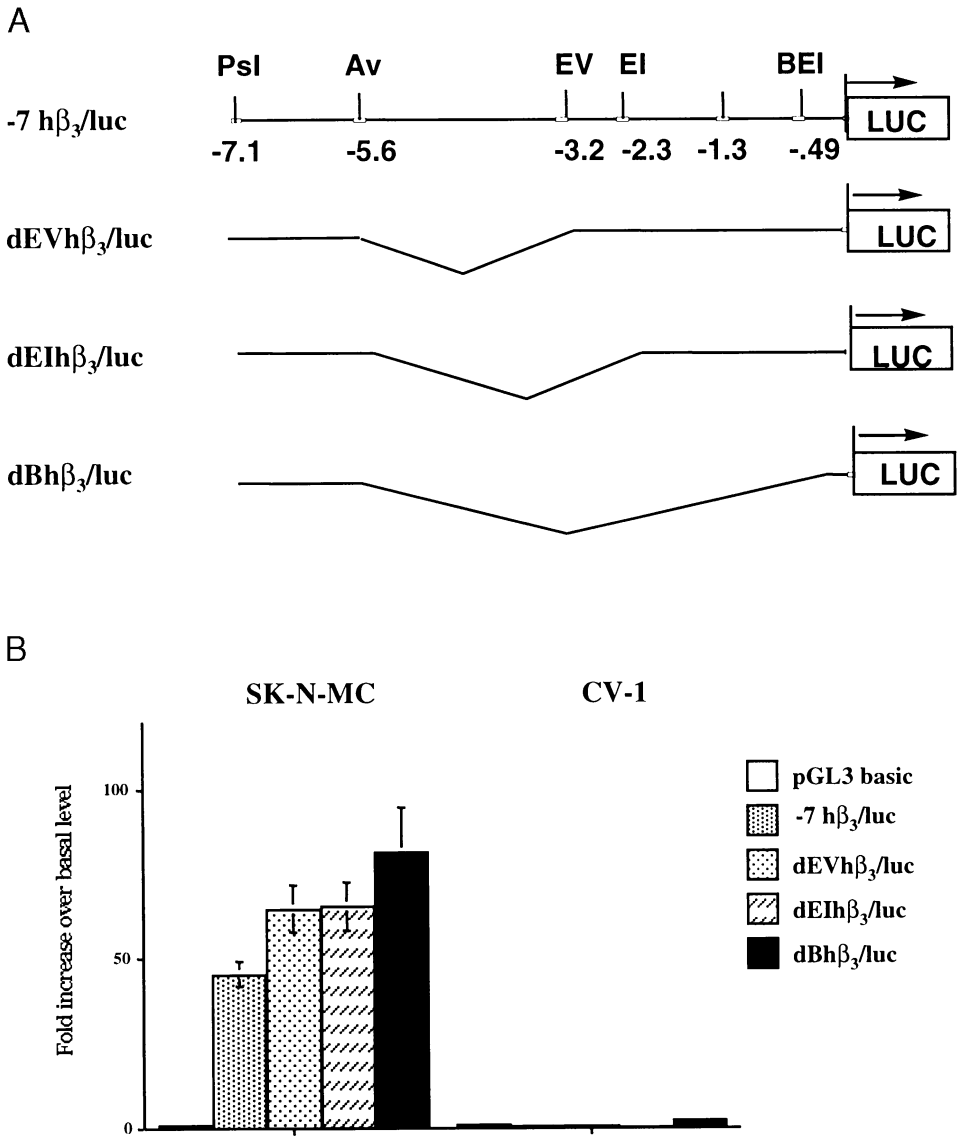


FIG. 3. Effect of the far upstream region on transcription activity. A, Transfection constructs were made by keeping the region between −7 to −5.6 and deleting regions between *Avr*II and *Eco*RV, *Eco*RI, and *Bst*EII to make dEVhβ_3AR/luc, dEIhβ_3AR/luc, and dBhβ_3AR/luc, respectively. B, A series of deletion constructs was introduced into SK-N-MC and CV-1 cells. All experiments were performed as described previously. Data are presented as the fold increase over basal transcription activity after correction for transfection efficiency (β-gal activity). The values are the results of at least five experiments, each performed in triplicate. *Error bars* represent SEM.

for EMSA with nuclear extracts from SK-N-MC cells. Oligos 2, 2A, 3A, 4A, and 1B all bound proteins (Fig. 5, B and C). The specificity of binding was confirmed by competition EMSA experiments in the presence of excess homologous unlabeled oligos (Fig. 5D). Competition EMSA with heterologous oligo combinations revealed that oligo 2 competes with oligo 3A (Fig. 5D), and oligo 1B competes with oligo 4A (Fig. 5C), suggesting common binding proteins for these pairs of oligos. Further, although oligo 2A overlaps with oligos 1 and 2, oligos 1 and 2 individually did not compete off nuclear protein binding of oligo 2A (Fig. 5D), suggesting the presence of an additional binding site in the overlapping region.

Similar experiments were performed using nuclear extracts from HeLa and CV-1 cells. Oligo 2A formed a complex with protein extracts from both CV-1 and HeLa cells, and oligos 1B and 4A formed complexes with proteins from SK-N-MC and HeLa cell nuclear extracts (Fig. 5, B and C). As no transcriptional activation of the hβ_3AR promoter was observed in HeLa cells in transient transfection experiments, it is likely that the protein(s) that bind to oligos 2A and 1B is either unable to function appropriately in our model system or is not sufficient in the absence of cell-specific factors (see below) for activation of the hβ_3AR promoter. Importantly, oligo 2 binds nuclear proteins from SK-N-MC cells, but not from CV-1 or HeLa cells (Fig. 5B). All EMSA experiments were performed at least four times, and data were highly reproducible.

Mutational analysis of sequences corresponding to regions A and B

In the experiments described below we have designated the regions represented by oligos 2A, 2(3A), and 4A(1B) as elements A, B, and C, respectively, for further clarity and ease of discussion.

Mutated elements A (overlap between 1 and 2, see Fig. 5A) and B (overlap between oligos 2 and 3A, see Fig. 5A) were used as probes for EMSA with nuclear extracts from SK-

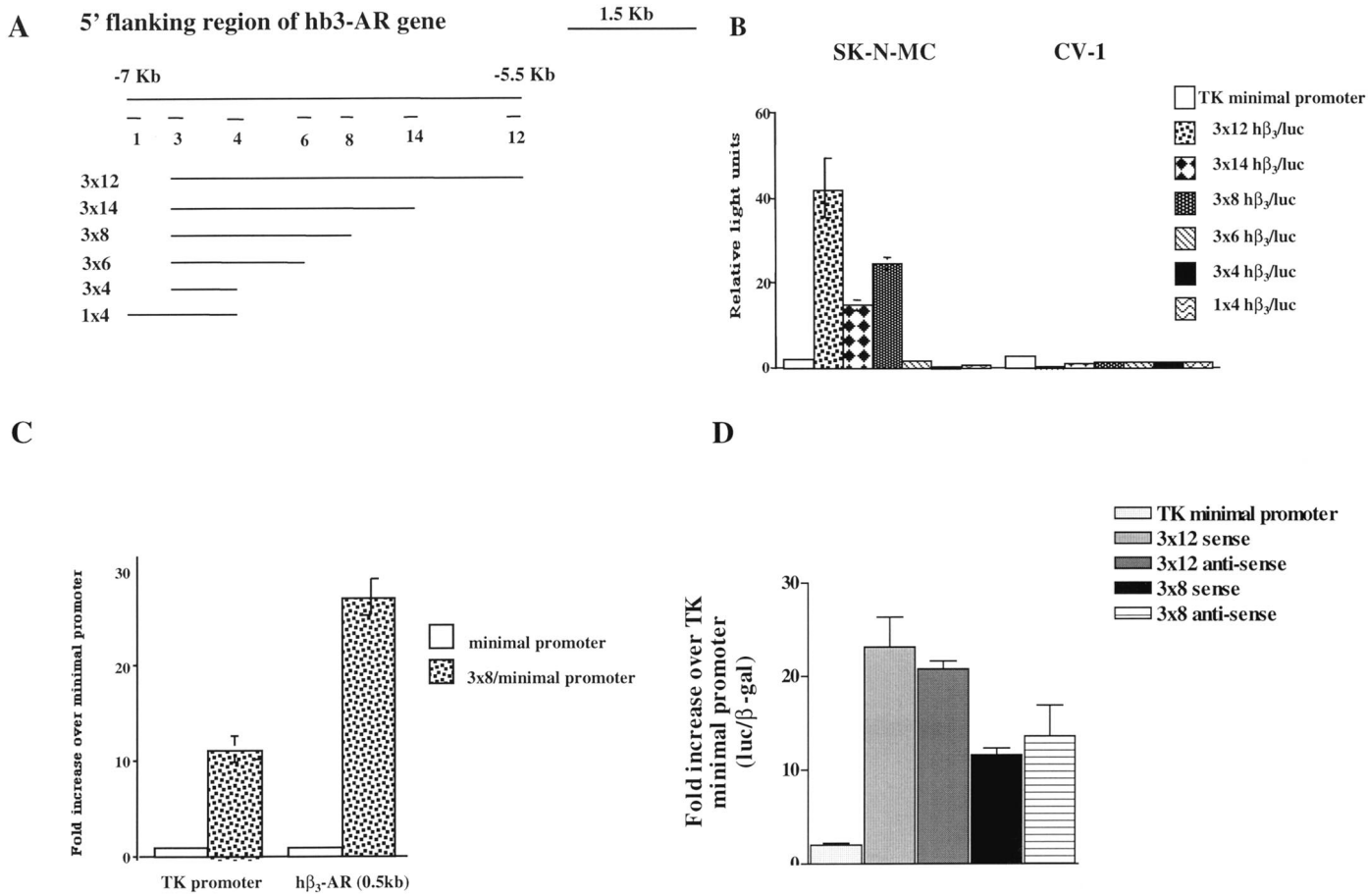


FIG. 4. Further analysis of 1.5 kb of distal promoter. A, Series of PCR products were made and ligated to a TK minimal promoter within the pGL3 basic. Vector sequences of primers used are described in *Materials and Methods*. B, Transient transfections were performed in SK-N-MC and CV-1 cells using calcium phosphate precipitation as described previously. Data are presented as relative light units corrected for transfection efficiency. *Error bars* represent the SEM. Each vector was transfected at least five times, and each time the experiment was performed in triplicate. The DNA used in these experiments was a product of two or three different preparations. C, Luciferase activity obtained after transfection of the 3x8 region ligated to a TK (heterologous promoter) or 0.5 kb (endogenous promoter) as a minimal promoter. Data are presented as fold increases over transcription activity of the minimal promoter itself. All data were corrected for transfection efficiency. D, The regions between primers 3x12, 3x14, and 3x8 were ligated in sense (3x12, 3x14, and 3×shβ_3/luc) and antisense (3x12, 3x14, and 3×ashβ_3/luc) orientation to TK in pGL3 and transfected into SK-N-MC cells. Luciferase activity was measured and presented as the fold increase over the TK minimal promoter level after correction for transfection efficiency.

N-MC cells. The mutations were introduced within these elements as a block of three nucleotides covering the entire sequence (Fig. 6A). Based on the results shown in Fig. 6B, we conclude that the core sequence in element A that is necessary to bind nuclear protein is -AGGTGGACT-, a sequence that resembles the binding site for the transcription factor Sp1. Similar mutational analysis of element B indicated that the -GCCTCTGGGGAG- sequence is necessary for protein binding in this element (Fig. 6C).

Element C (CCTT-rich region) is recognized by S1 nuclease

Element C is represented by 20 repeats of a CCTT motif. EMSA experiments with oligos 4A and 1B that cover the 80-bp element C (Fig. 5C) showed binding of nuclear proteins from SK-N-MC and HeLa cells. Previous work from Cantor and Efstratiadis (46) indicates that homopurine-homopyrimidine-rich regions similar to element C are sites that are hypersensitive to S1 nuclease. It has been suggested that such sites play a role in transcriptional regulation (47, 48). To determine whether element C is hypersensitive to S1 activity, which would indicate its potential role in transcriptional regulation of hβ_3AR, we performed S1 nuclease experiments as described by Evans and Efstratiadis (48). As shown in Fig. 7, S1 nuclease recognized sequence(s) in the region between primers 6 and 8 (Fig. 5A). S1 nuclease cleavage occurred in all salt concentrations, although the efficiency was lower with increased salt, thus confirming the observation by Htun *et al.* (49). Interestingly, DNA that was first digested with the nuclease *Sca*I was not sensitive to S1 nuclease (Fig. 7B), suggesting that structural features in addition to primary sequence are important. Primer extension experiments using primers 6 and 16 indicated that the S1 nicking occurred within the TTCC-rich region (data not shown).

Interactions among elements A, B, and C

To further characterize the specific roles of the individual elements, A, B, and C, in transcriptional regulation of the hβ_3AR gene, element A and/or element B were mutated in

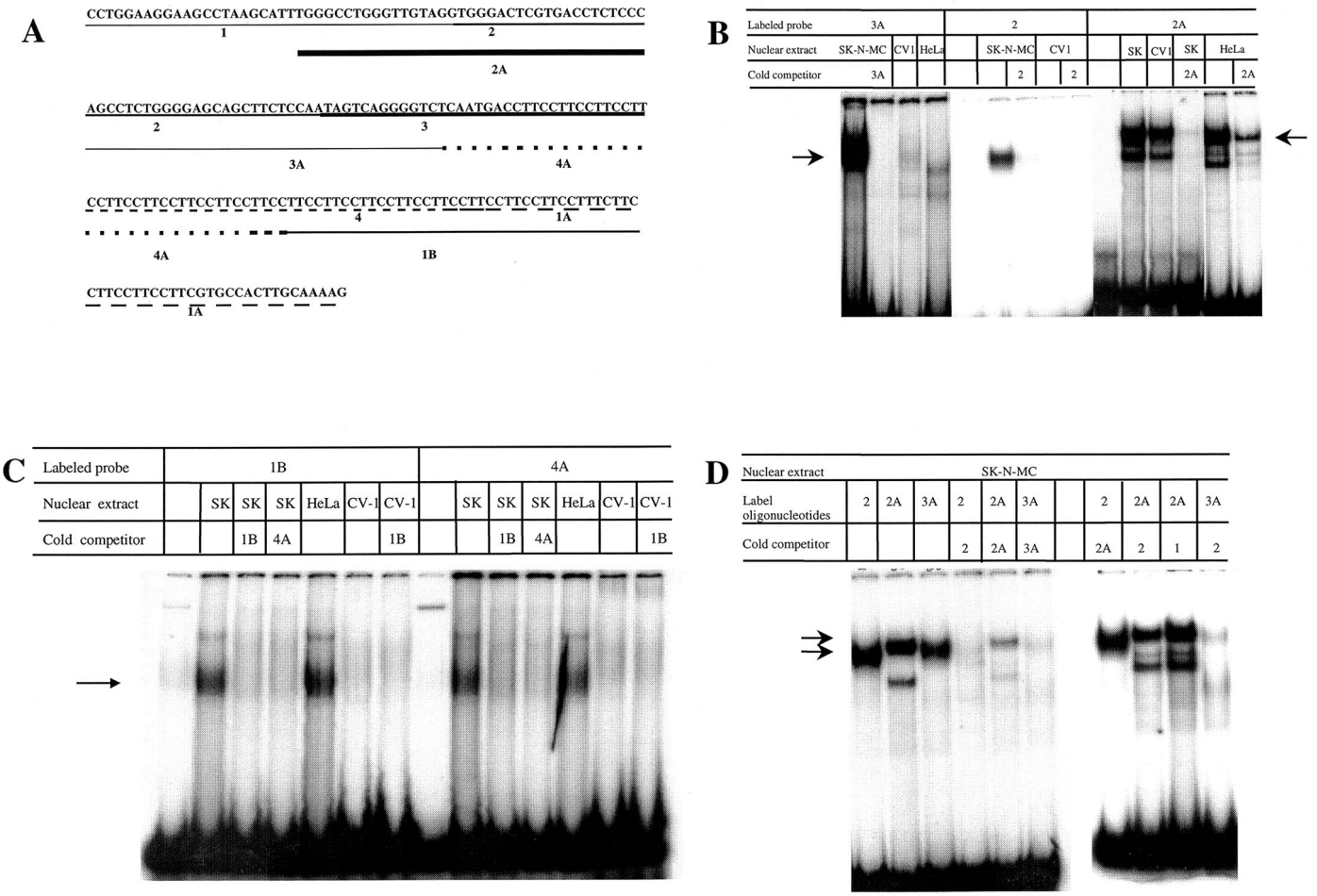


FIG. 5. EMSA experiments with oligos generated from the sequence between −6.50 and −6.30 kb from the 5′-flanking region of the hβ_3AR gene. A, Sequence of 200 bp between primers 6 (−6.508 kb) and 8 (−6.308 kb). EMSAs were performed using *underlined* sequences as oligos marked as 1, 2, 3, 4, and 1A to cover the 200 bp and oligos 2A, 3A, 4A, and 1B, representing overlaps between oligos 1 and 2, 3 and 3, 3 and 4, and 1A and 4, respectively. Experiments were performed with double stranded, labeled oligos under the conditions described in *Materials and Methods*. B and C, Nuclear extracts from SK-N-MC, CV-1, and HeLa cells were incubated with the radiolabeled oligos described above. The figure shows only oligos that demonstrate binding. B, Oligonucleotides 2, 2A, and 3A. C, Oligonucleotides 1B and 4A. D, EMSAs were performed in the presence of an excess of the indicated cold oligos. The amount of cold oligos was a 50-fold molar excess of the amount of labeled oligos unless otherwise indicated. *Arrows* indicate the position of the major DNA-protein complex.

the context of the 3x8 vector containing the 0.5-kb endogenous minimal promoter and luciferase reporter gene. In addition, element C (80 bp CCTT repeats) was deleted from constructs, leaving sequences corresponding to elements A and B intact. Constructs that carried only element A with or without mutations (3xAwt, 3xAc,mut) and only element B with or without mutations (3xBwt, 3xBa,mut) in the absence of element C were also tested in transient transfection assays. The results of these experiments are shown in Fig. 8. Constructs with a mutation in either element A or B showed 50% and 60% decreases in transcriptional activity, respectively. Constructs containing mutations in both elements A and B showed 70% lower transcriptional activity compared with the intact, fully active 3x8 hβ_3-/Luc. Element C maintained 30% of maximal luciferase activity when part of 3x8 hβ_3-/Luc. However, element C alone, when connected to the 0.5-kb endogenous minimal promoter has no transcriptional activity. Similarly, elements A and B themselves did not increase luciferase activity in the absence of element C. These data suggest that interaction among elements A, B, and C is necessary to achieve the full activaty of the hβ_3AR gene enhancer.

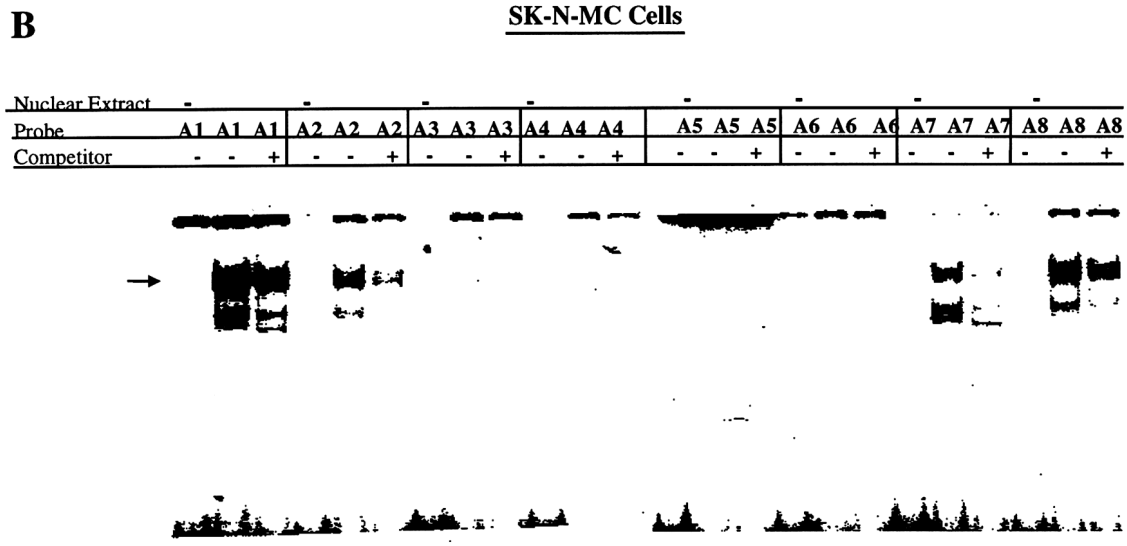
Proteins that bind to element A are Sp1- or Sp1-like

A computer search (using the Baylor College of Medicine database) of known consensus binding sites suggested the presence of binding sites for two known transcription factors (Sp1 and AP2) within the −6.9 to −6.3 kb region of the hβ_3AR promoter. Sequences found in oligos 2A (element A) and oligos 2 and 3A (element B) are homologous with Sp1 and AP2 binding sequences, respectively. EMSA using radiolabeled oligo 2A or 2 (3A) with SK-N-MC nuclear extracts and unlabeled competitor oligos identical to consensus binding sites for Sp1 and AP2 were performed to test for the presence of Sp1 and AP 2 binding sites. The data showed that the Sp1 cold oligo, at a 100-fold molar excess, displaced radiolabeled oligo 2A from its complex (Fig. 9A). In addition, supershift

A

Region A (overlap between oligo 1 and 2	**Region B** (overlap between oligo 2 and 3A)
A1gatccGGTTGTAGGTGGGACTCGTGAa	B1gatccGCCTCTGGGGAGCAGCTTCTCCa
A2gatccCTATGTAGGTGGGACTCGTGAa	B2gatccCGGTCTGGGGAGCAGCTTCTCCa
A3gatccGGTACAAGGTGGGACTCGTGAa	B3gatccGCCAGAGGGGAGCAGCTTCTCCa
A4gatccGGTTGTTCCTGGGACTCGTGAa	B4gatccGCCTCTCCCGAGCAGCTTCTCCa
A5gatccGGTTGTAGGACCGACTCGTGAa	B5gatccGCCTCTGGGCTCCAGCTTCTCCa
A6gatccGGTTGTAGGTGGCTGTCGTGAa	B6gatccGCCTCTGGGGAGGTCCTTCTCCa
A7gatccGGTTGTAGGTGGGACAGCTGAa	B7gatccGCCTCTGGGGAGCAGGAACTCCa
A8gatccGGTTGTAGGTGGGACTCGACTa	B8gatccGCCTCTGGGGAGCAGCTTGAGGa

B



C

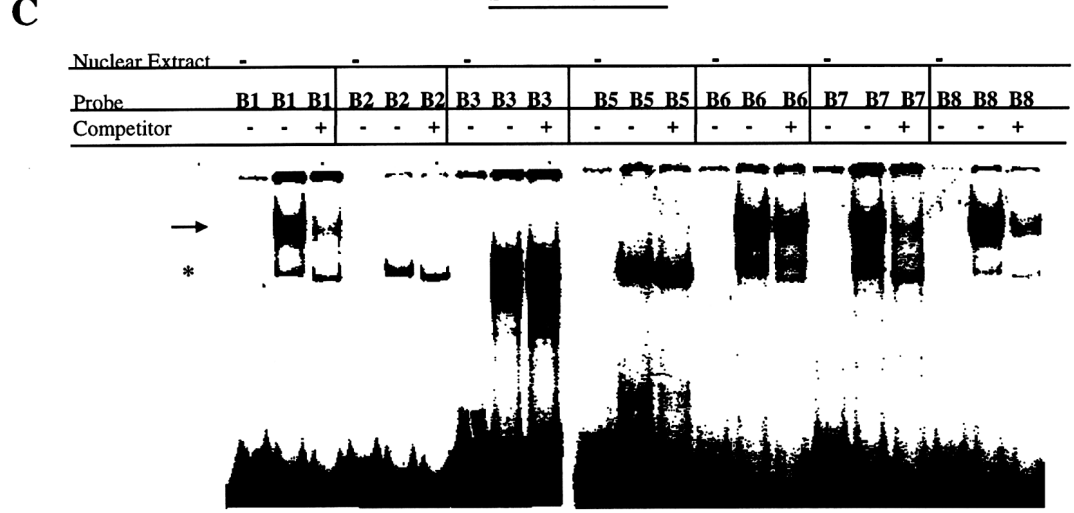


FIG. 6. Mutational analysis of elements A and B. A, As described in *Results*, element A represents overlap between oligos 1 and 2, element B is overlap between oligos 2 and 3A, and element C is presented with 20 repeats of a CCTT motif. Mutated oligos are shown, with mutated nucleotides *underlined*. Small letters correspond to sites for restriction enzymes used for easier cloning and purification of double stranded oligos. B and C, Results from EMSA experiments with mutated oligos and nuclear extracts from SK-N-MC cells. Each of the radiolabeled oligos was incubated in the absence (−) and presence (+) of the corresponding cold probe in a 100-fold molar excess. The *arrow* points to the bands representing specific binding, and the *star* indicates nonspecific binding.

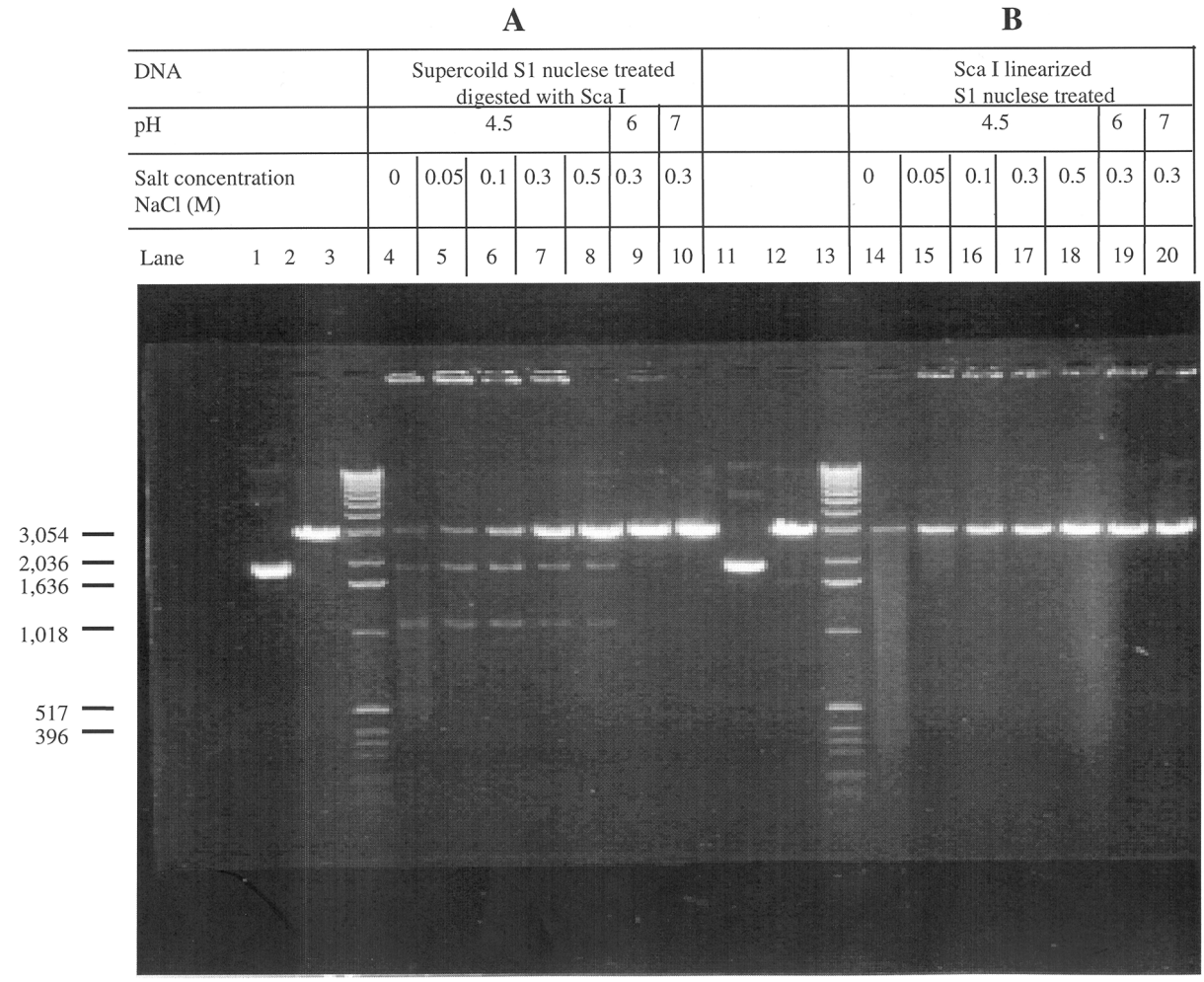


FIG. 7. Mapping of the S1 nuclease-sensitive site within −6.28 and −6.15 (element C). The insert between primers 6 and 16 was subcloned in pGEM7. A, Supercoiled plasmid (1 μg) was digested 30 min with 5 U S1 nuclease in S1 nuclease buffer at pH 4.5. After precipitation, the samples were digested with *Sca*I and run on 1% agarose gel. Each lane contains 1 μg S1 nuclease *Sca*I-treated supercoiled DNA in the presence of no salt (lane 4), 0.05 M NaCl (lane 5), 0.1 M NaCl (lane 6), 0.3 M NaCl (lane 7), or 0.5 M NaCl (lane 8). The band that runs at a level of 3 kb presents linearized DNA with *Sca*I, two bands that run at a level of approximately 2 and 1.1 kb and are products of S1 nuclease activity and *Sca*I digestion. Lanes 9 and 10 underwent the same treatment of supercoiled DNA, but in the presence of buffer of pH 6.0 and 7.0, respectively. Lanes 1 and 2, Supercoiled and *Sca*I-linearized plasmid, respectively. B, Linear DNA was first digested with *Sca*I and then incubated with S1 nuclease, as described above, in the presence of increasing salt concentrations. Mol wt markers are a 1-kb ladder DNA.

EMSA experiments using Sp1 antibodies showed that after incubation of HeLa and SK-N-MC nuclear extracts with radiolabeled Sp1 and 2A oligos (Fig. 9B) the complex formed was supershifted in a manner similar to that of the protein/Sp1 oligo complex (Fig. 9B). These data strongly suggest that the protein that binds oligo 2A is on Sp1- or Sp1-like protein. In contrast, unlabeled AP2 oligo even in a 200-fold molar excess did not displace radiolabeled oligo 3A from its complex (Fig. 9A). In addition, radiolabeled AP2 oligo forms a complex with protein from SK-N-MC cells that cannot be displaced by the presence of 100-fold molar excess of 2(3A) unlabeled oligos (data not shown). Also, although the AP2 antibody supershifted the AP2 complex with protein from either SK-N-MC or HeLa cells, it did not affect the 2(3A) complex with SK-N-MC cell nuclear extracts (Fig. 9C). These results strongly suggest that transcription factors in SK-N-MC cells that bind to the sequence within oligo 2 (element B) represent proteins that cannot be identified as an AP2 or Sp1 transcription factor. Given the sequence similarity between the AP2 consensus binding site and element B, it is conceivable that this protein(s) represents an AP2-like protein that is expressed in SK-N-MC, but not in CV-1 or HeLa, cells.

BAT contains factors that bind to elements A and B

β_3AR is expressed in BAT and WAT in rodents and in BAT in man. To test for the presence of the binding proteins described earlier in these tissues, nuclear extracts from mouse WAT (periovarian depot), BAT, and primary cultures of human white adipocytes (perirenal depot) were isolated and used for EMSA experiments as described above. Nuclear extracts from liver and muscle were also tested as controls. Nuclear extract from WAT showed binding to oligo 2A, but not 2. However, nuclear proteins from BAT bound all labeled oligos in a sequence-specific manner, as shown by EMSA

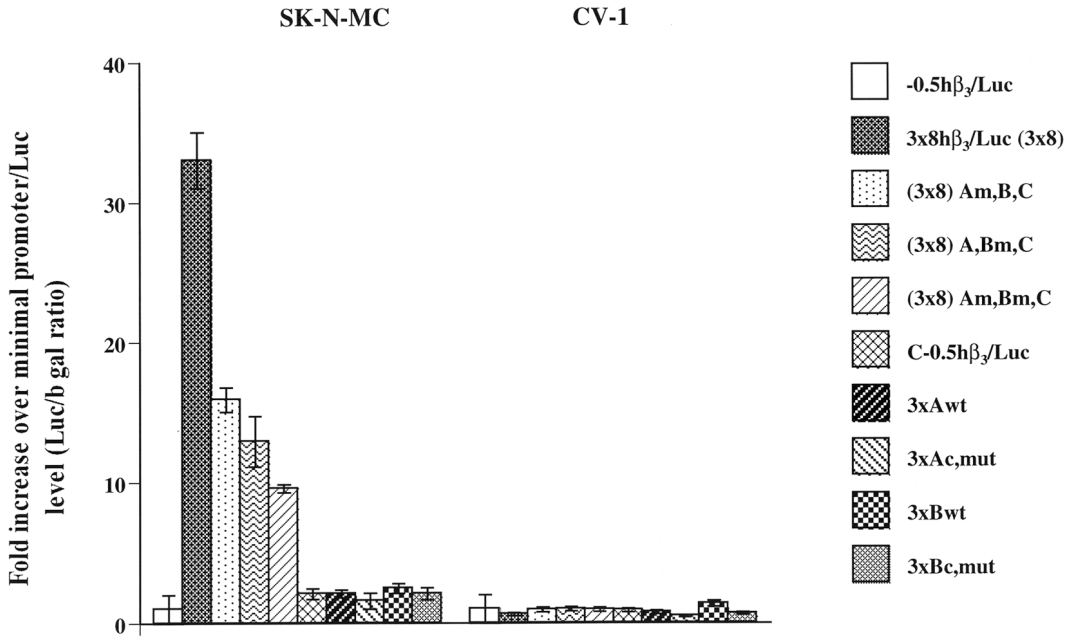


FIG. 8. Effects of mutation within element A and/or B and C on transcription activity of the 3x8 region. Constructs that contain mutations within A (3x8 Am, B, C) and/or B (3x8 A, Bm, C and 3x8 Am, Bm C) are made as described in *Materials and Methods* using primers that contain mutations previously shown to eliminate binding for SK-N-MC nuclear extracts (A3 and B2; Fig. 7A). The mutation in C is achieved by deleting 80 bp of CCTT regions (3xA wt or 3xB wt). All mutations are introduced within region 3x8 (−6.96 to −6.30 kb) ligated to the 0.5-kb hβ_3AR promoter and luciferase in a pGL3 basic vector. All constructs were transfected in SK-N-MC and CV-1 cells, and data are presented as a fold increase over 0.5 kb promoter activity after correction for transfection activity. *Error bars* represent ±SEM.

competition experiments (Fig. 10A). Nuclear extract isolated from a primary culture of human WAT (perirenal depot) showed a binding pattern similar to that obtained with SK-N-MC cell and BAT extracts (Fig. 10C). Primary adipocyte cultures developed from the human perirenal depot showed the morphological appearance and genetic characteristics (expression of UCP-1 and β_3AR) of BAT (40). Using RT-PCR analysis of RNA isolated from human perirenal adipocytes, we showed the presence of UCP1 mRNA (Fig. 10D). Thus, it is plausible that the protein(s) that forms complexes with the radiolabeled probes come from cells with a BAT phenotype rather than from white adipocytes. In contrast, liver and muscle nuclear extracts bound oligo 2A, (element A) but did not form complexes with oligo 3A (element B; Fig. 10B).

Discussion

In this report we used SK-N-MC cells, a human neuroblastoma cell line that expresses β_3AR endogenously, to identify *cis*-acting regulatory elements within the hβ_3AR promoter. The data show that in addition to the proximal promoter elements (36, 37), an enhancer element located further upstream is necessary for maximal expression of the hβ_3AR gene. In addition, the data suggest that interactions of several different hβ_3AR enhancer-binding proteins may play important roles in the cell- and tissue-specific expression of the gene. This enhancer was shown to be necessary and sufficient to activate both the hβ_3AR basal promoter and the heterologous TK promoter in a cell type-specific (*i.e.* SK-N-MC, but not CV-1 or HeLa, cells), but orientation-independent, fashion.

Deletion mapping analysis of the 5′-flanking region of the hβ_3AR gene identified a 1.4-kb region located between −7 and −5.6 kb from the translation start site that induced a 50- to 70-fold increase in luciferase activity in a cell-specific manner. This induction was achieved even when the region between −5.6 and −0.5 kb was deleted. The data suggest that no additional enhancer or repressor elements exist within the sequence between −5.6 and −0.5 kb. Alternatively, a repressor or weak enhancer may be present within the region between −5.6 and −0.5 kb, but its activity is not sufficient to have a measurable effect on the activity of the enhancer present within a sequence between −5.6 and −7 kb.

Further dissection of the sequence between −7 and −5.6 kb revealed a 200-bp enhancer located between positions −6.50 and −6.30 kb that contains three elements (A, B, and C), which all bind nuclear proteins. Element A binds a protein(s) present in the nuclear extract of all tested cell types. In addition, sequence analysis of element A indicated similarity to the Sp1-binding site. Indeed, both EMSA experiments with the consensus binding site for the Sp1 protein as a competitor and the supershift analysis using a Sp1 antibody showed that this protein is probably Sp1 or a Sp1-like protein. The proximal location of this Sp1-binding site is consistent with other reports showing Sp1-binding sites to be located in the proximal region of promoters of numerous genes (50). These elements are usually involved in the linking of a distal control element(s) (51).

Element B binds nuclear proteins expressed in SK-N-MC, but not CV-1 or HeLa, cells, suggesting that it may play an important role in the cell-specific expression of the receptor. Mutational analysis of element B identified the sequence −6.448 kb-GCCTCTGGGGAG-−6.428 kb as the core se-

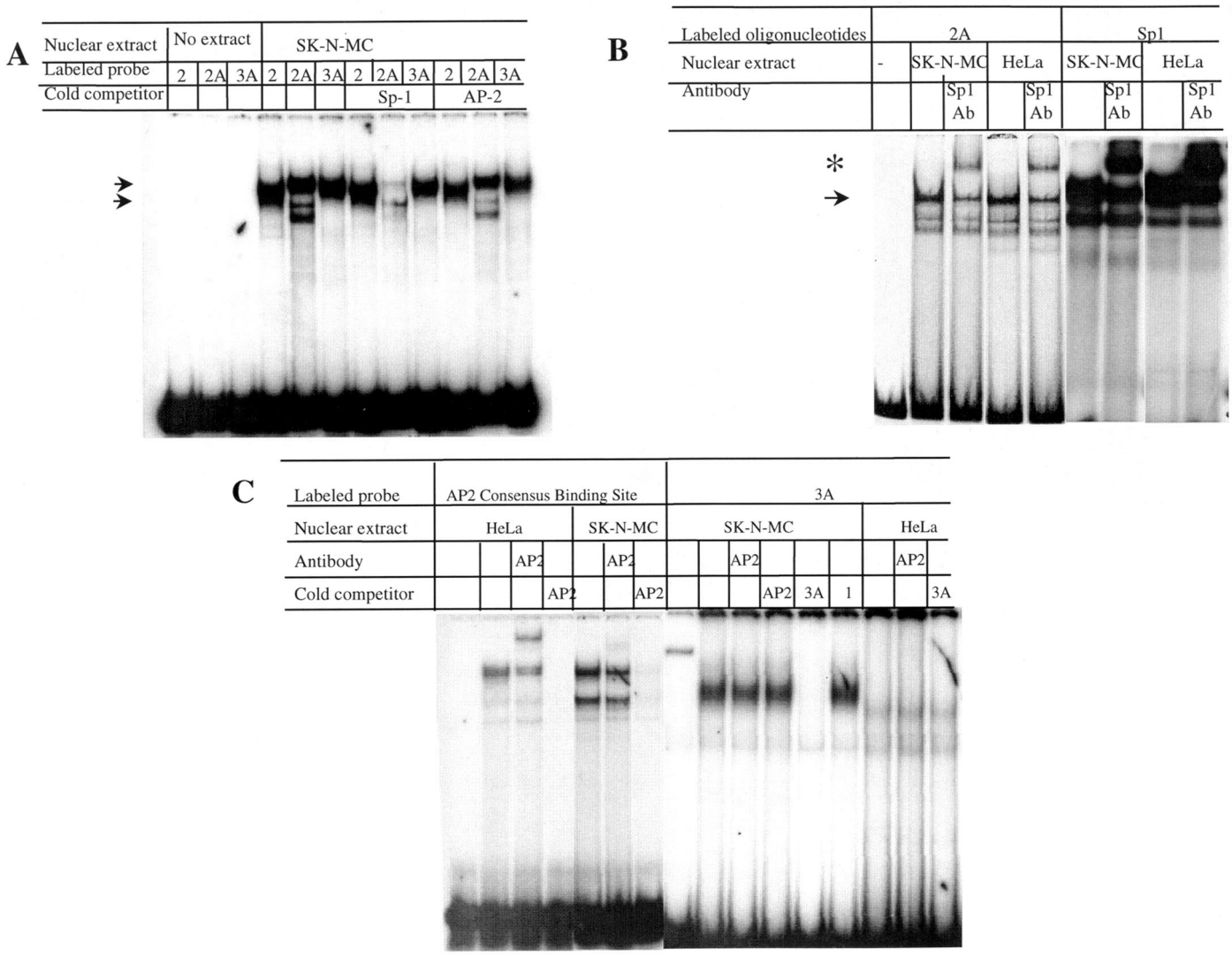


FIG. 9. The protein that competes with binding of 2A oligos is Sp1 or Sp1-like protein, whereas protein that binds for oligo 2 represents a novel transcription factor. A, Nuclear extract proteins from SK-N-MC cells bind for radiolabeled probes 2A and 2(3A). Oligo 2A was competed from its complex with the Sp1 consensus binding site, whereas oligo 2 (3A) is not competed out from its complex with AP2 oligos. EMSA experiments were performed in the presence of a 100-fold molar excess of Sp1 and AP2 consensus binding sites. B, Supershift experiments using antibody against Sp1 protein with radiolabeled oligos 2A and nuclear extracts from SK-N-MC and HeLa cells. C, EMSA experiments with labeled oligos 3A and nuclear extracts from SK-N-MC and HeLa cells in the presence of antibody against AP2 protein. An *arrow* indicates major complexes, and supershifted bands are shown with *asterisks*.

quence responsible for binding the SK-N-MC nuclear proteins. This sequence shows some similarity to the AP2 consensus binding site. However, no evidence to support AP2 binding could be obtained from EMSA experiments using an AP2 oligo as a competitor or from supershift experiments using AP2 antibody. Recently, the binding site sequence for estrogen receptor factor-1 (ERF-1) has been shown to share a 70% similarity with element B (43). The role that ERF-1, a transcription factor belonging to a family of AP2 transcription factors, plays in regulation of the hβ_3AR will be the subject of future studies.

Element C is composed of 20 repeats of a CCTT motif. The EMSA data showed that this region serves as a binding site for nuclear proteins even though it seems to lack the sequence complexity of sequence-specific DNA-binding sites. This homopyrimidine-rich sequence resembles previously recognized motifs that are hypersensitive to S1 nuclease digestion (46). S1 nuclease digestion experiments showed that S1 recognized this region and caused nicking within it. Based on sequencing and primer extension studies, all of the nicking and cleavages occurred within the CCTT region of element C (data not shown). In naked DNA templates, these regions are thought to present single stranded regions, perhaps occurring by a strand-sliding mechanism (46). *In vivo*, the sites recognized by S1 nuclease are usually designated hypersensitive sites. In these sites the organization of chromatin facilitates binding of transcription factors (47, 48). It remains to be determined whether the factors present in SK-N-MC cell nuclear extracts that bind to element C recognize its primary sequence, its structural organization, or both.

The importance and relative contributions of elements A,

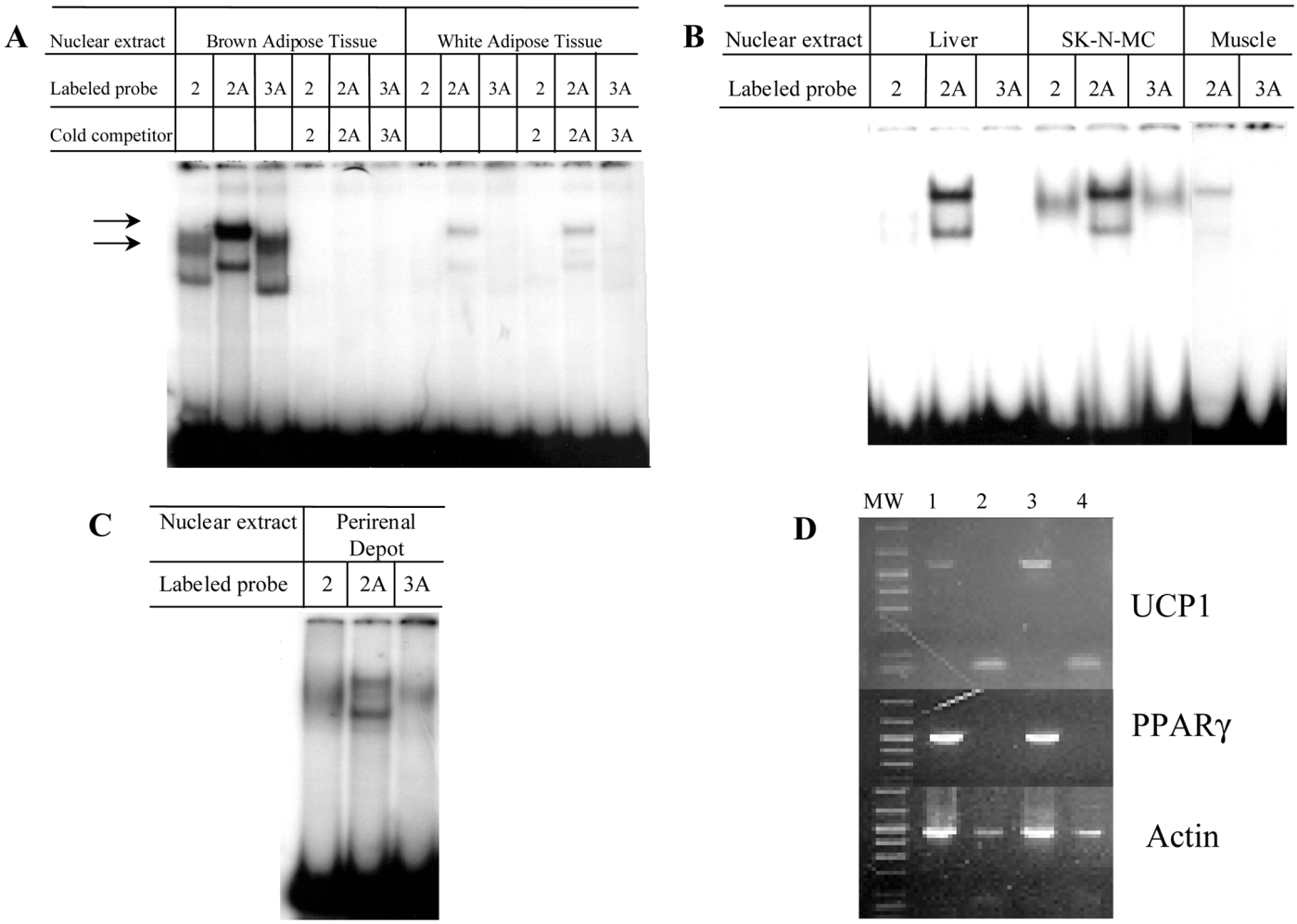


FIG. 10. Binding of nuclear extract proteins isolated from liver, muscle, BAT, WAT, and primary adipocyte cell cultures isolated from the perirenal adipose tissue depot. A, Nuclear extracts from WAT and BAT incubated with radiolabeled oligos 2A and 2(3A), with or without a 50-fold molar excess of cold probe. B, EMSA experiments with protein in nuclear extracts from liver and SK-N-MC cells and labeled 2A, 2, and 3A oligos. C, Binding of nuclear extract proteins from muscle tissue and the perirenal depot with the oligos 2A, 2, and 3A. D, RT-PCR on 500 ng total RNA from adipocytes isolated from the human perirenal WAT depot. MW, 100-bp ladder; lanes 1 and 3, RNA from cells differentiated 7 and 10 days, respectively; lanes 2 and 4, RT-PCR of RNA samples in the absence of RT.

B, and C to the maximal transcriptional activity of the hβ_3AR promoter was also examined. Maximal transcriptional activity is strongly dependent upon the presence of all three elements, A, B, and C, and indicates that these *trans*-acting factors act in concert to achieve full activity of the enhancer as has been reported for other systems (52, 53). The close localization of these *cis*-acting elements suggests that interactions between the binding factors may be necessary for full enhancer activity.

Although SK-N-MC cells provide a unique human-derived model system in which to study regulation of the β_3AR, it is important to understand the relevance of findings in this cell type to regulation in other human tissues. Although β_3AR is abundantly expressed in rodent BAT and WAT, data from several groups have shown only limited, if any, expression of β_3ARs in human WAT along with robust expression in BAT. To determine whether our findings in SK-N-MC cells can be extended to other tissues, we tested nuclear extract proteins from a variety of mouse and human tissues for the presence of enhancer-binding proteins. The pattern of binding in nuclear extracts from mouse BAT and human adipocytes cultured from perirenal adipose tissue, a location of predominantly WAT in man, was the same as that we observed with SK-N-MC nuclear extracts. Although the perirenal depot in man contains predominantly WAT, a number of laboratories (40, 54, 55) have also shown that this depot contains some cells that express UCP1, a characteristic of BAT. Therefore, it is possible that the presence of *trans*-acting factors in nuclear extracts from the human adipocytes that we obtained is due to the presence of contaminating brown adipocytes. Additional experiments using nuclear extract from a human WAT depot other than the perirenal tissue would be necessary to clarify the expression pattern of the B region binding factor. Nuclear extracts isolated from mouse epididymal fat depots did not show any binding to enhancer elements B or C and only weak binding to element A, indicating specificity for brown adipocytes. Nu-

clear extracts from liver and muscle showed binding to element A, but not elements B and/or C, a finding consistent with the tissue-specific expression of the β_3AR, as the receptor is not expressed in these tissues.

Recently Ito *et al.* studied the promoter of the human β_3AR gene using transgenic mice (38). The researchers concluded that 600 bp of proximal promoter are sufficient to provide BAT-specific expression of human β_3AR. However, these data do not exclude the existence of additional regulatory elements, such as the upstream enhancer that we have identified. Furthermore, our use of a heterologous reporter (*i.e.* luciferase) as a transcriptional read-out, instead of β_3AR mRNA used in the studies of Ito *et al.*, eliminates the possibility of posttranscriptional events confounding interpretation of the data. In addition, regulation of expression of the β_3AR by a human promoter inserted in murine tissues (as in Ito's studies) may be significantly different from regulation by its endogenous promoter. Consistent with this hypothesis, we recently obtained data showing that the human promoter with or without the distal enhancer causes a very low level of transcriptional activity when introduced into differentiated 3T3-L1 or HIB cells, two murine cell lines representative of WAT and BAT, respectively. Additionally, our data show that the 500-bp proximal promoter of the human β_3AR gene in SK-N-MC can direct only very low level transcription and that the enhancer located further upstream is essential for maximal transcription of the gene. To clearly understand the regulation of expression of the β_3AR in adipose tissues, experiments similar to those described in this manuscript need to be performed with human white and brown adipose cell lines.

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