DNA Elements and Protein Factors Involved in the Transcription of the β_2-Adrenergic Receptor Gene in Rat Liver. The Negative Regulatory Role of C/EBPα[†]

Liu Jiang, Bin Gao, and George Kunos*

Department of Pharmacology & Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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ABSTRACT: Primer extension and RNase protection analyses of the rat β_2-adrenergic receptor (β_2AR) gene identify two transcription start points at −64 and −220 nt, respectively. Transient transfections of putative promoter/*pCAT* constructs into DDT_1 MF-2 cells indicate that fragments −36 to −100 (P1) and −186 to −312 (P2) are sufficient to promote transcription, whereas −911 to −1122 contains a negative regulatory element(s). RNase protection analysis of the 3′ untranslated region (3′-UTR) indicates the presence of two transcripts with 3′-UTR of 111 and 604 nt exclusive of the poly(A^+) tails. Northern blots of β_2AR mRNA using full-length and partial cDNA probes indicate that a major 2.2 kb and a minor 1.6 kb species arise from the use of alternative promoters as well as different polyadenylation signals. DNase I footprinting and DNA mobility shift assays (DMSA) using rat liver nuclear extracts identify a number of transcription factors binding to sequence elements within or upstream from P1 and P2, including Sp1, CRE, CP1, AP-2, NF-1, NF-κB, and C/EBP. Supershift assays using antibodies against C/EBPα and C/EBPβ and mutational analyses indicate that the protein binding to the C/EBP consensus recognition site at −925 to −933 is C/EBPα. The activity of promoter/*CAT* constructs containing the C/EBP recognition site is significantly decreased by cotransfection of C/EBPα but not C/EBPβ into either DDT_1 MF-2 cells or primary rat hepatocytes. Partial hepatectomy causes a transient decrease in C/EBPα, as measured by DMSA, and an increase in β_2AR mRNA levels and rate of transcription in the remnant liver. Thus, derepression via C/EBPα is likely involved in the up-regulation of β_2AR in the regenerating rat liver.

The β_2-adrenergic receptor (β_2AR)[1] is a member of the family of G-protein coupled receptors, and it plays an important role in mediating the sympathoadrenal response to stress. β_2AR are thought to be located outside of the sympathetic neuroeffector junction where they preferentially respond to and mediate the diverse effects of circulating epinephrine, including bronchial, uterine, and vascular smooth muscle relaxation, cardioacceleration, and hepatic glucose mobilization. β_2AR are also involved in the control of cell growth and differentiation (Hen *et al.*, 1989), and are subject to complex, tissue-specific regulation by hormonal and developmental factors. Good examples of the multiple factors that regulate β_2AR gene expression are provided by studies in the rat liver (Kunos & Ishac, 1987). The expression of hepatic β_2AR is dependent on age (Blair *et al.*, 1979) and sex (Studer & Borle, 1982), and it is regulated by thyroid (Malbon, 1980; Lazar-Wesley *et al*., 1991) and glucocorticoid hormones (Wolfe *et al.*, 1976) as well as by stimuli that trigger hepatocyte proliferation, such as partial hepatectomy (Huerta-Bahena *et al.*, 1983), cholestasis (Aggerbeck *et al.*, 1983), liver damage induced by hepatotoxins (Garcia-Sáinz & Nájera-Alvarado, 1986), and acute enzymatic dissociation of hepatocytes (Refsnes *et al.*, 1983; Nakamura *et al.*, 1983).

Gene expression is a multistep process regulated at different levels, involving multiple regulatory elements. There is evidence that the expression of the β_2AR gene can be regulated at the level of its transcription (Collins *et al.*, 1990), posttranscriptionally at the level of mRNA stability (Hadcock *et al.*, 1989), or at the level of translation via the peptide product of a short open reading frame located in the 5′ flanking region of the β_2AR gene (Parola & Kobilka, 1994). The structure of the human and hamster β_2AR genes has been characterized (Kobilka *et al*., 1987), and some of the *cis*-regulatory elements in their regulatory domains have been identified and implicated in transcriptional regulation by glucocorticoids (Nakada *et al.*, 1989) or stimuli acting through cyclic AMP (Collins *et al*., 1989). However, the *cis*-regulatory elements involved in the transcription of the rat β_2AR gene, such as the promoter(s) and the start and end points of transcription, have not yet been determined. Recently, we have cloned the rat β_2AR gene (Jiang & Kunos, 1995), which had a 5′ flanking sequence different from that determined in an earlier study (Buckland *et al.*, 1990). The correct sequence has now allowed a detailed analysis of the mechanism of the transcription of the β_2AR gene. The results demonstrate the existence of two separate promoters

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* Correspondence should be addressed to this author at the Department of Pharmacology & Toxicology, Medical College of Virginia, Virginia Commonwealth University, Box 980613, Richmond, VA 23298. Telephone: 804-828-2073. Fax: 804-828-2117.

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[1] Abbreviations: AR, adrenergic receptor; *tsp*, transcription start point; UTR, untranslated region; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; CRE, cAMP response element; GRE, glucocorticoid response element; CPE, cytoplasmic polyadenylation element; NF-κB, nuclear factor kappa B; C/EBP, CCAAT enhancer binding protein; DMSA, DNA mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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as well as two functional polyadenylation sites, and indicate their alternative use in the generation of two β_2AR mRNA species in the liver. DNase I footprinting and DNA mobility shift assays (DMSA)[1] indicate the interaction of a number of transcription factors with the regulatory domain of the β_2AR gene. Of these, a negative regulatory role for C/EBPα is indicated by the results of cotransfection experiments with putative promoter/pCAT constructs. Since C/EBPα is shown to be down-regulated in the post-hepatectomy liver, derepression from C/EBPα may be involved in the up-regulation of the β_2AR gene in the regenerating liver.

MATERIALS AND METHODS

RNA Preparation and Northern Blotting Analysis. Total or poly(A^+) RNA was isolated from various tissues of juvenile, male Sprague-Dawley rats (75−100 g), and analyzed by Northern blotting as detailed previously (Gao & Kunos, 1994). Blots were hybridized with a full-length, 2.1 kb rat β_2AR cDNA probe (kindly provided by Dr. Claire Fraser) or by a probe corresponding to the 5′-UTR −186 to −2251, generated by restriction digestion of a 5.5 kb rat β_2-AR genomic clone with *Pst*I and *Hin*dIII (Jiang & Kunos, 1995). Loading control was provided by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The probes were ^{32}P-labeled by random priming.

Nuclear Run-On Transcription Assay. Liver nuclei were prepared according to Blobel and Potter (1966) except that the nuclear storage buffer was changed to 40% glycerol, 5 mM $MgCl_2$, 50 mM Tris-HCl, and 0.1 mM EDTA. Nuclear run-on transcription assays were carried out according to Greenberg and Ziff (1984) and Szentendrei *et al.* (1992), with some modifications. Liver nuclei (1×10^7) in 100 μL of nuclear storage buffer were mixed with 100 μL of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, CTP, and GTP, 100 units of RNAsin, and 200 μCi of [α-^{32}P]UTP) and incubated at 30 °C for 30 min with gentle shaking. The nuclei were digested with 10 μL of RNase-free DNase (10 mg/mL, Promega) and 10 μL of $CaCl_2$ (20 mM) and incubated at 37 °C for 30 min. They were then further treated with 2 μL of proteinase K (10 mg/mL) and 25 μL of 10 × SET buffer (5% SDS, 100 mM Tris, pH 8.0, and 50 mM EDTA) at 37 °C for 30 min. Nuclear ^{32}P-labeled RNA was extracted by adding 550 μL of RNAzol B and 500 μL of phenol/chloroform (1:1), precipitated with ethanol, and dissolved in Northern hybridization buffer. For binding on the nitrocellulose membrane, 5 μg of linearized plasmid DNA was denatured by boiling in 0.l mM NaOH for 5 min and neutralized with 10 μL of 20 × SSC, and then spotted onto the membrane using the slot-blot apparatus, washed with 6 × SSC, and UV-cross-linked. The membranes containing either GAPDH cDNA (as control) or β_2AR cDNA were prehybridized for 6 h, hybridized for 72 h at 42 °C, and washed the same way as for Northern analysis.

Primer Extension Analyses. To determine the *tsp* of the β_2AR gene, an oligonucleotide primer complementary to the coding region sequence +1 to +28 or to the 5′ upstream sequence −98 to −122 was annealed to 10−15 μg of poly-(A^+) RNA from rat liver. Primer extension was achieved using Superscript reverse transcriptase at 41 °C for 60 min, using a primer extension kit (Promega). In order to define the position of the 5′ end of the extended products, they were run through a 6% polyacrylamide/7 M urea sequencing gel along with the corresponding genomic sequence, determined by using the same primer.

RNase Protection Mapping of the 5′ and 3′ Termini of Rat Liver β_2AR mRNA. The antisense RNA probes used to map the 5′ and 3′ ends of the β_2AR mRNA were obtained by in vitro transcription of PCR-generated DNA fragments designed to have a T7 RNA polymerase promoter at their 5′ ends. By using primers which flank the phage promoter in a transcription vector, an insert DNA fragment can be amplified and used directly as template in in vitro transcription (Mullis & Faloona, 1987). For generating the template for the antisense RNA probe used to map the 5′ end of β_2-AR mRNA, the downstream primer was 5′-**TAATACGACTCACTATAGGG**CTCCATGGCCGGGCAGGT-3′. The 20 nucleotides in boldface type correspond to the T7 promoter sequence, whereas the 3′ end of the probe, including the last 2 nucleotides in boldface, is complementary to the sequence +8 to −12 of the rat β_2AR gene. The upstream primers used in the same reaction correspond to the 5′-UTR sequences −338 to −320 (probe 1).

For generating the template for the 294 nt long antisense RNA probe used to map the 3′ end of the β_2AR mRNA, the PCR downstream primer was 5′-**TAATACGACTCACTATAGGGA**AAACATTAAGCACTGAA-3′, again containing the T7 promoter (boldface), and the 18 3′-most nucleotides being complementary to the 3′-UTR sequence +1496 to +1479; the upstream primer corresponded to the coding sequence between +1223 and +1240. (The G in the translation stop codon is at +1257.) The single-stranded, ^{35}S-labeled cRNA probe was generated by in vitro transcription from the cDNA templates desribed above, using T7 RNA polymerase (Promega). The DNA template was then degraded by RQ1 RNase-free DNase I; the RNA probe was purified on a 5% polyacrylamide/8 M urea gel and eluted, and its specific activity was determined [$(3-5) \times 10^4$ cpm/ng of RNA]. The RNase protection assays were performed using the RPA II kit (Ambion); 5−20 μg of total tissue RNA was hybridized with the radiolabeled RNA probe (10^6 cpm) overnight at 45 °C. Unprotected single-stranded probe and RNA were digested by 2.5 units/mL RNase A plus 100 units/mL RNase T1 for 30 min at 37 °C. The protected fragments were separated on a 6% polyacrylamide/8 M urea gel along the corresponding genomic sequence, and the bands were visualized by phosphorimaging, using ImageQuant software (Molecular Dynamics).

Polymerase Chain Reaction (PCR). DNA fragments used in *pCAT* constructs or employed as templates in in vitro transcriptions, to generate RNA probes, were obtained by PCR. The PCR reaction mixture contained Taq enzyme and enzyme buffer, 100 μM $MgCl_2$, 0.2 μM of each dNTP, 50 pmol of the appropriate sense and antisense primers, and 1 ng of DNA template. PCR products were generated by 30 amplification cycles.

Plasmid Construction. The *pCAT* SV40-enhancer reporter vector (Promega) was used for the construction of plasmids harboring putative promoter fragments of the β_2AR gene. Fragments generated by PCR amplification of DNA using suitable primers were inserted between the *Hin*dIII and *Pst*I or the *Pst*I and *Xba*I restriction sites upstream of the *CAT* gene in the *pCAT* vector. All subcloning and transformation techniques were performed as described (Sambrook *et al.*,

1989). Plasmid DNA was purified by a plasmid purification kit (Qiagen), and the correct insertion of fragments was verified by restriction analysis and dideoxy sequencing.

Cell Culture and Transient Transfections. The DDT_1 MF-2 hamster smooth muscle cell line used in transfection experiments was obtained from the American Type Culture Collection and cultured as specified by the supplier. Primary rat hepatocytes used in transfection experiments were obtained from male Sprague-Dawley rats (100−120 g), using a collagenase perfusion protocol described elsewhere (Preiksaitis *et al*., 1982). The isolated cells were washed with hepatocyte wash medium (GIBCO) and plated onto polylysine-coated culture dishes in attachment medium (GIBCO). After 3 h, the medium was changed to DMEM containing 5% fetal calf serum, 1×10^{-8} M dexamethasone, 10 ng/mL EGF, 5 μg/mL insulin, 2.5 μg/mL fungizone, 50 μg/mL gentamycin, 67 μg/mL penicillin, and 100 μg/mL streptomycin. Transient transfections were performed using the Lipofectin reagent (GIBCO/BRL), as recommended by the manufacturer. Briefly, cells were cultured in their ususal, serum-containing medium until they reached 40−60% confluency. The cells were then washed twice with Opti-MEN I reduced-serum medium (GIBCO/BRL), and transfected with 2 μg of plasmid DNA/2 mL culture dish using 10 μL of Lipofectin. In some experiments, plasmids containing β_2-AR promoter/CAT constructs were cotransfected with expression plasmids harboring the C/EBPα (pMEX-C/EBP 3′UT) or C/EBPβ (pMEX-CRP2) coding regions. The expression plasmids had been prepared and kindly provided by Dr. Peter F. Johnson. In each experiment, 1 μg of a pSV-β-galactosidase vector (Promega) was cotransfected to allow assessment of transfection efficiency. After transfection, the cells were incubated in reduced-serum medium for 8 h, and then changed to normal growth medium and harvested at 48−72 h. Cells were lysed by freeze−thawing and assayed for CAT and β-galactosidase activities, as described (Gao & Kunos, 1994). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. Values of CAT activity were expressed as cpm of [^{14}C]butyrylchloramphenicol. In each experiment, parallel plates of cells were transfected with *pCAT* control and *pCAT* enhancer plasmids (Promega) that served as positive and negative controls, respectively. All transfections were repeated at least 3 times with similar results.

Preparation of Nuclear Extracts. Nuclear extracts used in DNase I footprinting were prepared from the liver of male rats weighing 75−100 g, as described by Gao and Kunos (1995). Nuclear extracts used in DNA mobility shift assays were prepared as described by Roy *et al*. (1991).

DNase I Footprinting. The double-stranded DNA fragments used in footprinting represent different segments of the 5′ flanking domain of the rat β_2AR gene, as indicated in Figure 5. These probes were obtained by PCR, using genomic DNA as template and appropriate oligonucleotide primers, with one of the primers being end-labeled using [γ-^{32}P]ATP. The labeled probes were purified on a 2.5% agarose gel. Binding reactions were carried out in a volume of 20 μL, containing 5000 cpm of the labeled probe, 20−60 μg of nuclear extract, and 1.5 μg of poly(dI-dC) as nonspecific competitor, under conditions described previously (Gao & Kunos, 1995).

DNA Mobility Shift Assays (DMSA).[1] Oligonucleotide probes were synthesized by an automated DNA synthesizer (Milligen). One of the strands was end-labeled with [γ-^{32}P]-ATP, and the complementary strands were denatured at 90 °C and annealed by slow-cooling. The binding reaction was carried out in a total volume of 20 μL, containing 5000 cpm of labeled probe, 10 μg of nuclear extract with and without a specific consensus oligonucleotide competitor, and 1.5 μg of poly(dI-dC), under conditions described previously (Gao & Kunos, 1995). The following consensus oligonucleotides were used: NF-1, 5′-TAT TTT GGA TTG AAG CCA ATA TGA TAA TGA-3′; TFIID, 5′-GCA GAG CAT ATA AGG TGA GGT AGG A-3′; GRE, 5′-GAT CAG AAC ACA GTG TTC TCT A-3′; CRE, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; AP-2, 5′-GAT CGA ACT GAC CGC CCG CGG CCC GT-3′; AP-3, 5′-CAT GTG GGA CTT TCC ACA GAT C-3′; Sp1, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; C/EBP, TGC AGA TTG CGC AAT CTG CA-3′; NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; CP1, 5′-GCC ACA AAC CAG CCA ATG AGT AAC TGC TCC AAG-3′. For supershift assays, 1−2 μL of antiserum was added to the binding reaction at the indicated dilutions, and the incubation was continued for another 30 min at 4 °C. Shifted bands were size-fractionated by polyacrylamide gel electrophoresis and visualized by phosphorimaging. The antisera against C/EBPα and C/EBPβ were gifts of Dr. Peter F. Johnson, and had been raised against synthetic peptides corresponding to the N terminus of C/EBPβ or an internal sequence of C/EBPα. The antiserum against CTF/NF-1 was a gift of Dr. Naoko Tanese, and contains a rabbit polyclonal antibody against the CTF factor, which was shown to recognize the NF-1L protein in rat liver (Adams et al., 1995).

Partial Hepatectomy. Male rats weighing 75−100 g were anesthetized with 40 mg/kg pentobarbital, and subjected to 2/3 hepatectomy. Weight-matched control rats (sham) were anesthetized and subjected to laparatomy and handling of the liver. The animals were sacrificed by decapitation 2−6 h after surgery, and the residual liver tissue was removed for preparation of poly(A^+) RNA or nuclear extract (see above).

Statistical Analyses. For comparing values obtained in three or more groups (as in Figures 5 and 10), one-factor ANOVA was used followed by Tukey's *post hoc* test.

RESULTS

Northern Analysis of β_2AR mRNA. Previous Northern blot analyses of β_2AR transcripts indicated a major hybridizing band ranging in size from 2.0 kb in rat liver (Baeyens & Cornett, 1993) and DDT_1 MF-2 cells (Collins *et al*., 1988) to 2.2 kb in mouse lymphoma cells (Bahouth *et al*., 1988), or 2.1 kb in rat lung and prostate (Frielle *et al*., 1987). In some studies, a minor band of 1.6−1.8 kb was also detected (Collins *et al*., 1988). As illustrated in lane 1 of Figure 1, Northern blot analysis of poly(A^+) RNA from rat liver yielded a major band of 2.2 kb and a minor band of 1.6 kb.

Mapping of 5′ Termini of β_2AR Gene Transcripts. Transcription start points (*tsp*) were identified by primer extension and RNase protection analyses (Figure 2). Using rat liver poly(A^+) RNA as template and an oligo complementary to the coding region sequence +1 to +28, primer extension yielded a single product at 64 bp upstream from the translation start codon, indicating a transcription start point at this locus (*tsp*1, Figure 2A, bottom). Possible additional products upstream from this site may have been missed due

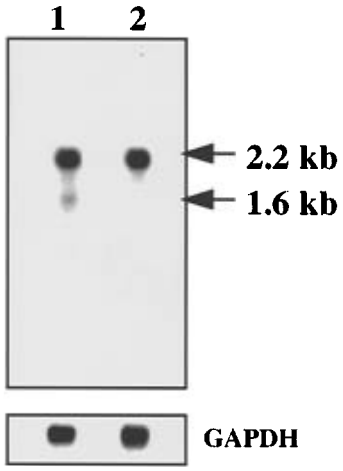

FIGURE 1: Northern blot analysis of β_2AR mRNA. Five microgram aliquots of poly(A^+) RNA from rat liver were hybridized with the 2.l kb cDNA probe (lane 1) or with a DNA probe corresponding to the 5′-UTR −186 to −2251 (lane 2). Note that the latter probe only hybridized with the major 2.2 kb species.

to the high G+C content of the first 200 nt in the 5′ untranslated region (5′-UTR), which is known to interfere with the generation of longer extended products. Therefore, primer extension was repeated using an oligo primer complementary to the upstream sequence −98 to −122 in the 5′-UTR. Using this primer, another prominent extended product terminating at −220 bp was detected (*tsp*2, Figure 2A, top).

The functional role of *tsp*1 and *tsp*2 was confirmed by RNase protection analysis (Figure 2B). To generate the RNA probe, we have used a recently introduced technique of incorporating the T7 RNA polymerase promoter into the 5′ end of the PCR-generated cDNA primer, from which the ^{35}S-labeled RNA probe is derived by in vitro transcription (Browning, 1989). Using a probe complementary to the sequence +8 to −338, a major protected fragment was identified with a 5′ terminus corresponding to the A at −220 nt upstream from the translation start codon (Figure 2B, top). By running the sequencing gel for a shorter time, a major fragment and a minor protected fragment with 5′ termini at −64 and 50 bp, respectively, were also detected (Figure 2B, bottom). The 5′ ends of these products were in very good agreement with the 5′ ends of the primer extension products described above, which confirms the role of the adenines at −64 and −220 as putative major *tsp* in the rat β_2AR gene.

The size of the 1.6 kb mRNA band suggests that its transcription originates at *tsp*1, which has not been previously identified. In order to test this more directly, Northern blots of rat liver poly(A^+) RNA were hybridized with the full-length cDNA probe as well as a probe complementary to the 5′-UTR upstream from *tsp*1 but including *tsp*2 (−186 to −2251). As illustrated in Figure 1, the full-length probe hybridized with both the major 2.2 kb band and the minor 1.6 kb band (lane 1), whereas the 5′-UTR probe only hybridized with the 2.2 kb band (lane 2). This supports the notion that the 5′ terminus of the 1.6 kb mRNA corresponds to *tsp*1 rather than *tsp*2.

Mapping of the 3′ Termini of β_2AR Transcripts. The previously cloned rat β_2AR cDNA contains a 604 nt long 3′-UTR exclusive of the poly(A^+) tail (Gocayne *et al*., 1987), which defines the distal polyadenylation site. Since the coding region is 1254 nt long, the 1.6 kb mRNA species

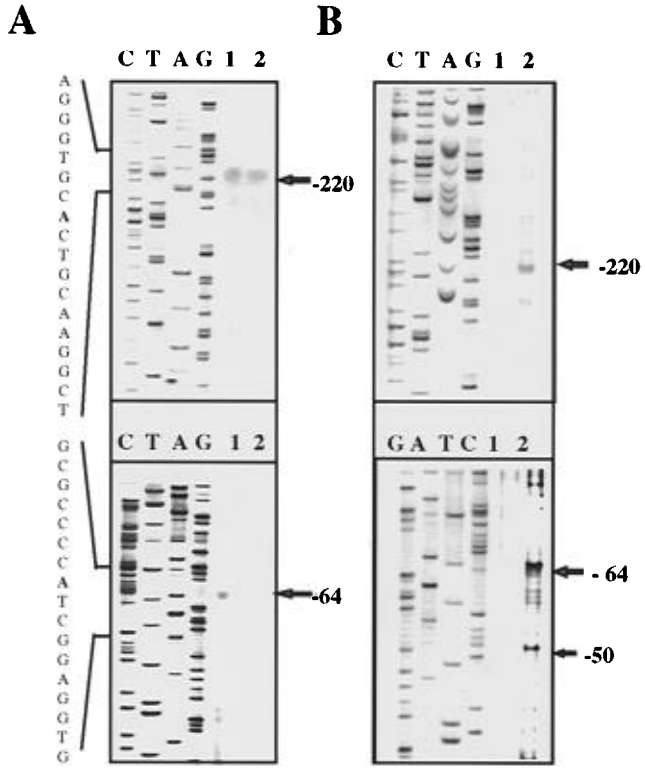

FIGURE 2: Identification of two *tsp* in the rat β_2AR gene by primer extension (A) and RNase protection analysis (B). (A) ^{32}P-Labeled oligonucleotides complementary to the upstream sequence −98 to −122 (top) or to the coding region sequence +1 to +28 (bottom) were hybridized with 15 μg (lanes 1) or 10 μg (lanes 2) of rat liver poly(A^+) RNA. The extended products were size-fractionated on a polyacrylamide−urea sequencing gel next to the sequencing reaction using β_2AR genomic DNA as template and the corresponding oligos as sequencing primers (lanes C, T, A, G). Arrows and numbers indicate the position and size of the extended products. (B) Rat liver poly(A^+) RNA was hybridized to a ^{35}S-labeled antisense RNA probe complementary to the 5′ flanking region sequence +8 to −338 of the β_2AR gene, and the protected fragments were separated on a 6% polyacrylamide/8 M urea gel run for 6 h (top) or 2 h (bottom). Lanes C, T, A, and G represent the corresponding sequence of β_2AR genomic DNA. Lanes 1 contain yeast tRNA. Lanes 2 contain protected products of probe + 10 μg of poly(A^+) RNA after digestion with RNase T_1 + RNase A. Numbers and arrows represent the position and size of protected products.

must have a substantially shorter 3′-UTR. Analysis of the 3′-UTR of the β_2AR gene indicates the presence of two AATAAA polyadenylation signals, starting at 83 and 572 bp downstream of the translation stop codon, respectively (Figure 3C). Also, in the region between these two polyadenylation signals, there are two cytoplasmic polyadenylation elements (CPE, Figure 3C) that have been implicated in the stage-specific poly(A^+) elongation as well as translation of mRNAs of developmentally regulated genes (Simon *et al*., 1992). To verify whether the proximal polyadenylation signal defines an alternative 3′ terminus, an RNase protection assay was performed to identify the corresponding proximal poly(A^+) addition site in the 3′-UTR of β_2AR mRNA. The antisense RNA probe used in the assay corresponds to the sequence starting at 34 bp upstream and ending at 222 bp downstream of the G in the translation stop codon. As illustrated in Figure 3A, a fragment ending at 108 bp downstream from the stop codon (at +1365) was protected, which indicates a second poly(A^+) addition site 21 bp downstream from the proximal polyadenylation signal,

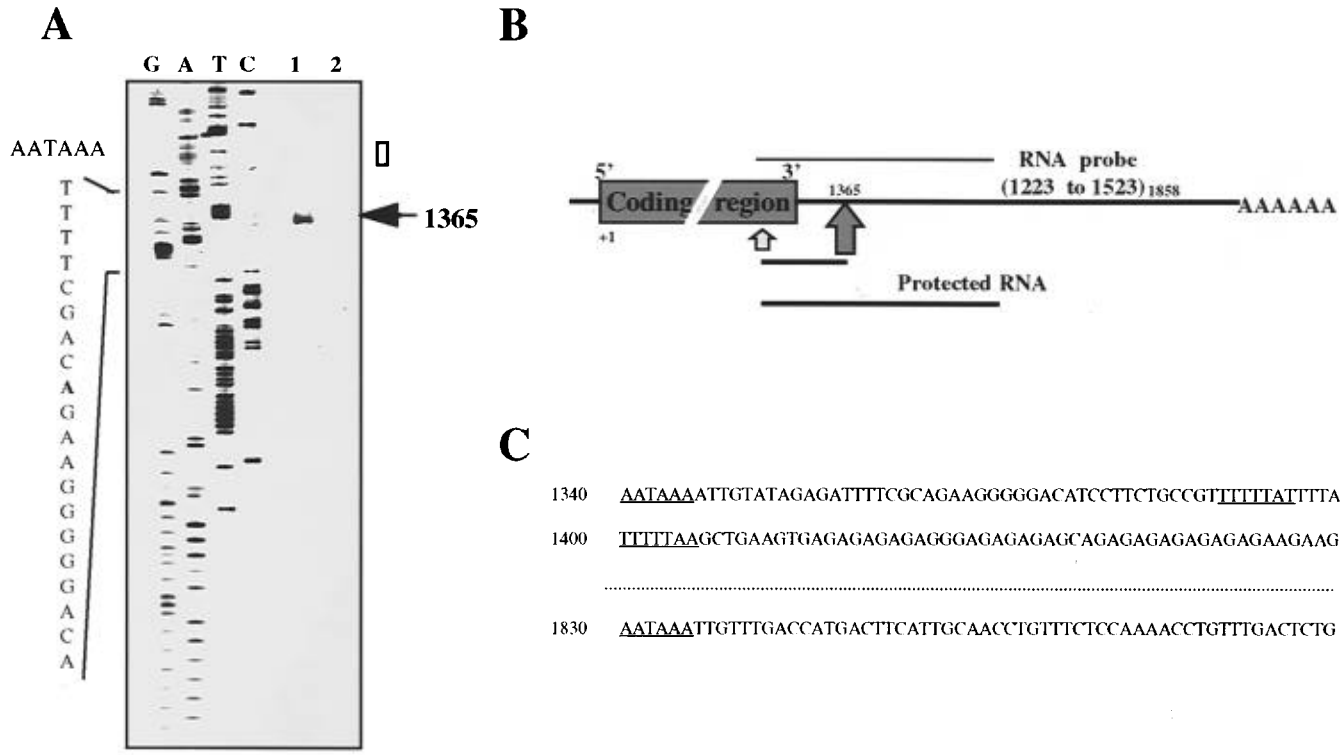

FIGURE 3: RNase protection mapping confirms the use of a second, more proximal polyadenylation signal in β_2AR transcripts. (A) Ten micrograms of poly(A^+) RNA from rat liver was hybridized with a ^{35}S-labeled RNA probe complementary to the sequence +1223 to +1496. In panel A, lane 1 shows a protected band at −1365; lane 2 is minus RNase control. Lanes G, A, T, and C represent the corresponding sequence of β_2AR genomic DNA. (B) Schematic illustration of the position of the protected fragments. (C) Sequence of the 3′-UTR of the rat β_2AR gene, with the two polyadenylation signals and CPE underlined. Arrows are at the two polyadenylation sites.

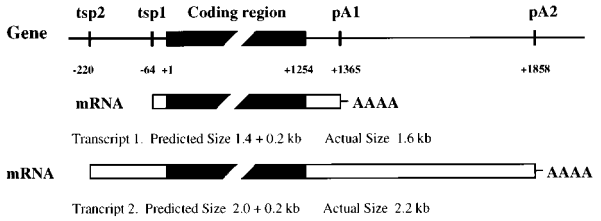

FIGURE 4: Schematic representation of the structure of the two β_2-AR mRNA transcripts. pA1 and pA2 stand for the proximal and distal polyadenylation sites, respectively. The shaded boxes represent the coding region.

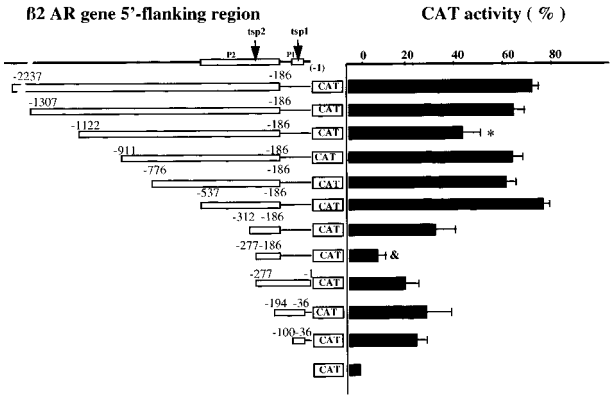

FIGURE 5: Characterization of two promoters of the rat β_2AR gene. The left side is the schematic representation of the pCAT constructs used in cell transfection experiments; the right side shows relative CAT activities in DDT_1 MF-2 cells, expressed as percent of the positive control (not shown). The construct on the bottom is negative control (minus SV40 promoter). CAT activities were corrected for transfection efficiencies, as described under Materials and Methods. Horizontal columns and bars represent means ± SE from at least three separate transfections. Values were analyzed by one-factor ANOVA followed by Tukey's test. Statistically significant ($P < 0.05$) differences from values in the immediately adjacent groups are indicated by * (compared to −1207, −186 and −911, −186) or & (compared to −312, −186 and −277, −1). The positions of the two promoters and two major *tsp* are marked on the left abscissa.

indicated by the open box above the arrow in Figure 3A. The expected longer fragment corresponding to the full-length probe is off the limit of this sequencing gel. Based on the localization of the two *tsp* and the two poly(A^+) addition sites, and the results of Northern hybridization with different cDNA probes, it is likely that the 1.6 and 2.2 kb mRNAs are generated by using the internal vs external *tsp*/polyadenylation sites, respectively, as illustrated in Figure 4.

Functional Mapping of the Promoters of the Rat β_2AR Gene. As noted earlier in the case of the hamster β_2AR gene (Kobilka *et al*., 1987), the sequence around the two *tsp* of the rat β_2AR gene is G+C-rich and there is no TATA box in the expected positions upstream from the two *tsp* (Jiang & Kunos, 1995), which are features of housekeeping-like promoters (Melton *et al*., 1984). In order to localize the regions around the *tsp* which are necessary and sufficient for promoter activity, fusion constructs containing PCR-generated fragments of the 5′ flanking region and the bacterial *CAT* gene were transiently transfected into DDT_1 MF-2 cells, and CAT activity was measured. DDT_1 MF-2 cells were used because they express high levels of β_2AR and display excellent transfection efficiency. As shown in Figure 5, the 5′ flanking region between −186 and −2237 bp had promoter activity. Removal of 5′ sequences from −2237 to −1122 was associated with reduced CAT activity, which appears to recover upon further deletion to −911. This suggests the presence of a negative regulatory element

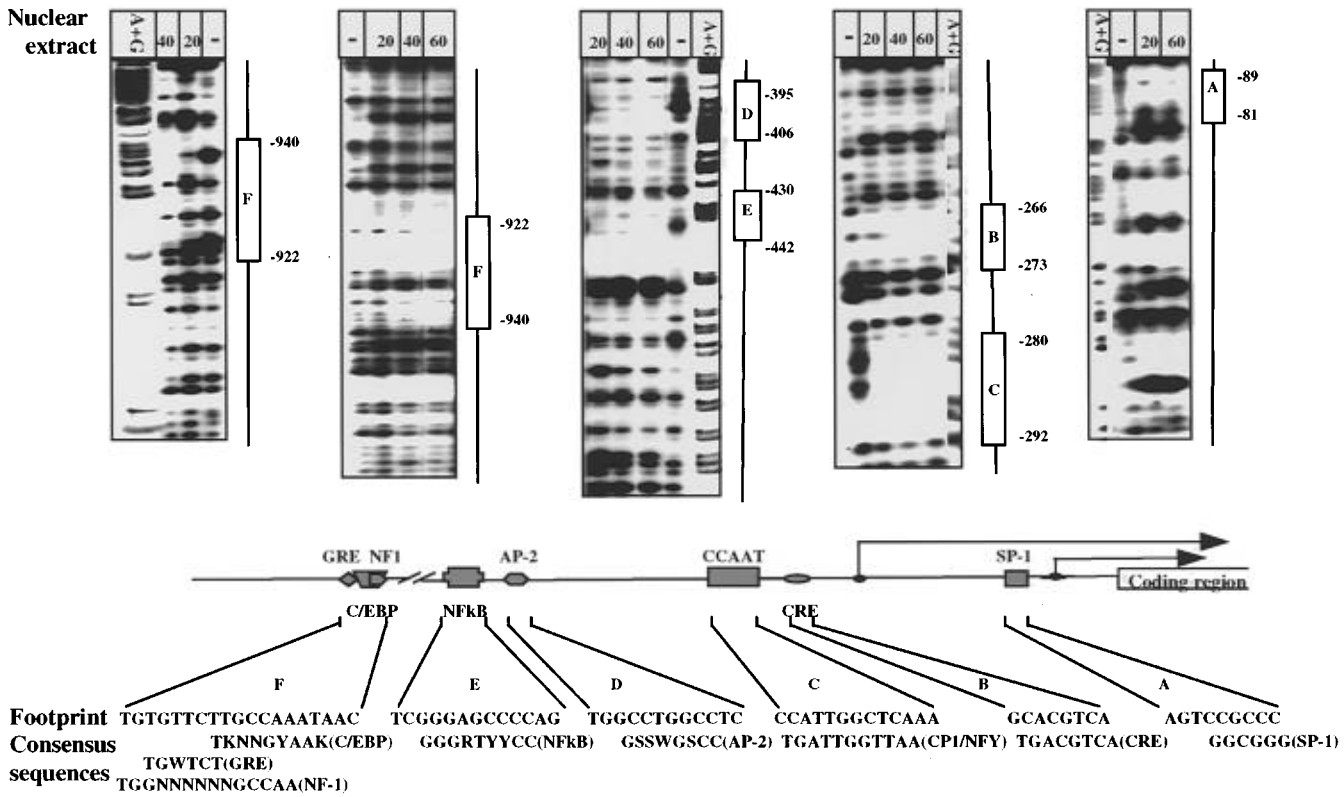

FIGURE 6: DNase I footprinting of the regulatory domain of the rat β_2AR gene. The five radiolabeled probes used, from left to right, correspond to the regions −853 to −1122 (antisense), −1122 to −853 (sense), −537 to −186 (sense), −312 to −186 (sense), and −1 to −312 (antisense), respectively. The amount of nuclear extract (in micrograms) is indicated on the top of the lanes. A+G: Maxam−Gilbert sequencing reaction. The position of footprints A−F is indicated by the open boxes and negative numbers. Consensus recognition sequences identified in and aligned with the corresponding footprint sequences are indicated in the lower part of the figure. Filled dots and arrows indicate the position of the two *tsp*.

between −911 and −1122 (see below). The shortest fragment still providing significant promoter activity is located between −312 and −186, whereas further shortening to −277 to −186 nearly abolishes activity. The fragment −36 to −194 also displays significant promoter activity, which is similar in magnitude to the activity of the fragment −36 to −100. This indicates the presence of a second promoter between −36 and −100. Thus, transcription of the β_2AR gene from the two *tsp* is controlled by two separate promoters: P2 located between −312 and −186 and P1 located between −36 and −100.

Analysis of Proteins Binding to the Regulatory Domain of the β_2AR Gene. In order to analyze the binding of transcription factors to the 5′ flanking region of the β_2AR gene, we employed DNase I footprinting and DMSA. Figure 6 illustrates the results of footprinting experiments using rat liver nuclear extracts and labeled probes corresponding to the indicated sequences. Six footprints were detected (A−F), and analysis of their sequence revealed consensus binding sites for various transcription factors (Figure 6). To analyze the proteins binding to these sites, we employed DMSA using radiolabeled, double-stranded oligonucleotides corresponding to the footprint sequences as probes, and rat liver nuclear extract with or without competing consensus oligos.

Footprint A in the P1 promoter contains a consensus binding site for transcription factor Sp1 (Figure 6). As illustrated in Figure 7A, DMSA using the footprint A probe yielded one minor and two major bands, which were competed by an excess of the unlabeled probe or the Sp1 consensus oligo, but not by consensus oligos corresponding to binding sites for other factors. Similar analysis of footprint C, which contains a consensus binding site for the ubiquitous transcription factor CP1, indicated that the shifted band was selectively competed by an excess of the CP1 consensus oligo (Figure 7B). The sequence element GGGAGCCCC in footprint E (−432 to −440) represents a close match to the NF-κB consensus sequence GGGRNNYYCC (Grilli *et al*., 1993). As illustrated in Figure 7C, excess NF-κB consensus oligo, but not other consensus oligos, effectively competed the shifted doublet. In addition to the experiments in Figure 7, we also did DMSA using the footprint B and D oligos and found that the shifted bands could be competed by an excess of a consensus CRE and AP-2 oligo, respectively (not shown). These experiments therefore suggest that Sp1, CREB, CP1, AP-2, and NF-κB bind to footprints A, B, C, D, and E, respectively.

Footprint F was of particular interest, as the experiments with putative promoter/*CAT* constructs suggest that it contains a negative regulatory element. This footprint contains overlapping consensus binding sites for the transcription factors GRE, NF-1, and C/EBP, as illustrated in Figure 6. DMSA analysis of this footprint yielded three bands (Figure 7D). The lowest band was completely blocked by a 200-fold excess of an NF-1 consensus oligo and partially competed by a similar excess of the GRE consensus oligo. Interestingly, the NF-1 oligo increased the intensity of the upper two bands. In contrast, a consensus oligo for C/EBP competed the upper two bands and increased the intensity of the low band. Furthermore, preincubation of the liver nuclear extract at 95 °C for 2 min abolished the lower band,

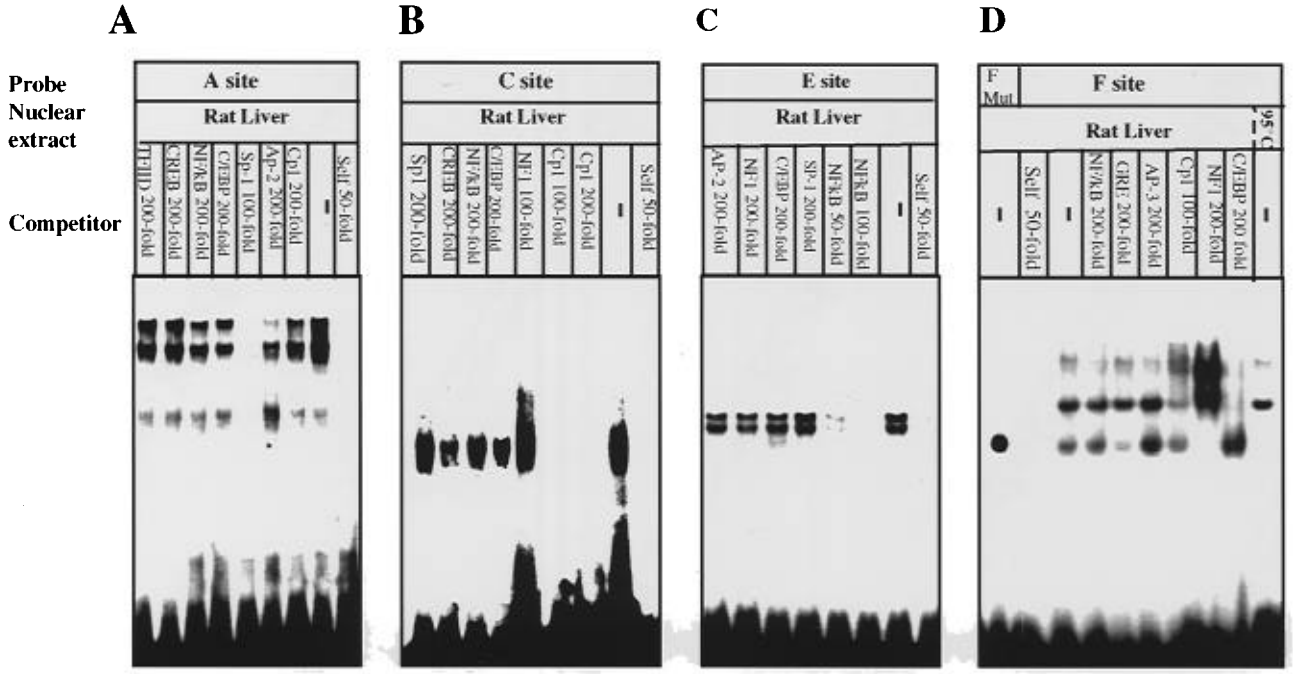

FIGURE 7: DNA mobility shift analysis of the proteins interacting with footprints A, C, E, and F. Rat liver nuclear extract (10 μg) was incubated with radiolabeled oligos corresponding to the indicated footprint, in the absence (−) or presence of the indicated competitor. The sequence of the competing consensus oligonucleotides is given under Materials and Methods, and the sequence of the labeled footprint oligos is shown in Figure 6. The sequence of the mutated F oligo was (mutations underlined): 5′-TCC TGT GTT CTA GCC AAC AAA CTG AAA-3′. The labeled complexes were separated on a polyacrylamide gel and visualized by phosphorimaging. For further explanation, see text.

but did not affect the upper two bands, which is consistent with the heat resistance of C/EBP proteins. Finally, using a mutated F site oligo in which three nucleotides in the C/EBP consensus sequence had been replaced (see legend of Figure 7) as the labeled probe, the upper two shifted bands were absent, whereas the lowest band was retained. These findings therefore indicate that the top two bands are generated by C/EBP-related proteins, while the low band contains primarily NF-1, but also liganded glucocorticoid receptors. They also suggest that in the absence of competing oligos, NF-1 and C/EBP interfere with the binding of one another, probably due to steric hindrance at their overlapping binding sites, and that removal of one can increase the binding of the other factor.

The C/EBP binding site can recognize the C/EBP family of proteins, the two major members of which are C/EBPα and C/EBPβ. Supershift assays with selective antibodies were performed to verify the contribution of these two proteins to the upper two shifted bands. As illustrated in Figure 8A, the presence of different dilutions of an antibody against C/EBPα caused the upper two shifted bands to disappear and a supershifted band to appear, whereas similar dilutions of the antibody against C/EBPβ were without such effect. The presence of NF-1 in footprint F was also confirmed by supershift assays (Figure 8B): an antibody against the CTF/NF-1 family of proteins caused a supershifted band to appear and the lowest of the three shifted bands to be reduced in intensity.

Effects of Partial Hepatectomy and C/EBPα on β_2AR Gene Expression. There is evidence that β_2AR are transiently up-regulated in the regenerating liver (Aggerbeck *et al.*, 1983; Huerta-Bahena *et al.*, 1983), with peak increases observed 2 days following partial hepatectomy (Sandnes *et al.*, 1986). As illustrated in Figure 9A, an increase in the steady-state level of β_2AR mRNA in the liver is evident as early as 2 h and persists at 6 h following partial hepatectomy. No significant change in β_2AR mRNA is evident in the liver from a sham-operated rat. In order to test whether or not increased transcription of the β_2AR gene is the underlying mechanism, nuclear run-on assays were performed using isolated nuclei from the same four livers (Figure 9B). The increased rate of transcription of the β_2AR gene parallels the change in steady-state mRNA levels. In 3 sham-operated and 3 age-matched, 2/3-hepatectomized male rats sacrificed 2 h following surgery, the amount of ^{32}P-labeled β_2AR mRNA generated by the nuclei from the post-hepatectomy livers was 181 ± 10% of the amount generated by control nuclei, indicating increased rate of transcription.

The expression of C/EBPα has been shown to be supressed in the regenerating liver, suggesting a role in maintaining the quiescent, differentiated state of hepatocytes (Mischoulon *et al.*, 1992). Therefore, we tested the effect of partial hepatectomy on the bands containing C/EBPα in DMSA using the labeled F oligo as probe. As illustrated in Figure 9C, partial hepatectomy resulted in a major but transient suppression of the upper two cluster of bands, which is evident as early as 30 min following hepatectomy and lasts up to 12 h when recovery of the bands is evident. The selectivity of this decrease is evident from the finding that the NF-κB-like binding activity of the same nuclear extracts, as detected by using the labeled footprint E oligo as probe, was increased between 10 min and 3 h following hepatectomy (not shown). This latter finding is in agreement with the reported transient up-regulation of NF-κB following partial hepatectomy (Cressman *et al.*, 1994).

The suppression of C/EBPα expression after partial hepatectomy could suggest that C/EBPα acts as a negative regulator of β_2AR gene transcription. To test this, expression

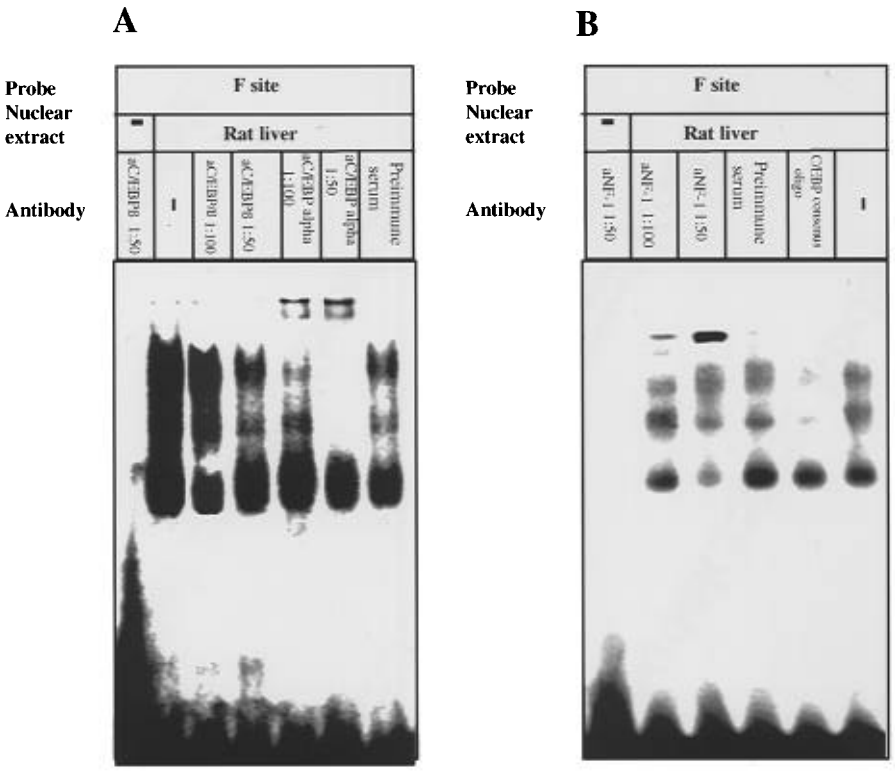

FIGURE 8: Supershift assays document the binding of C/EBPα (A) and NF-1 (B) to footprint F. DMSA assay mixtures contained radiolabeled footprint F oligo, 10 μg of liver nuclear extract, and the indicated antibodies. For further explanation, see text.

plasmids harboring the C/EBPα or C/EBPβ coding regions were cotransfected with promoter/*CAT* constructs containing different fragments of the 5′ flanking domain of the β_2AR gene into DDT_1 MF-2 cells as well as into primary cultured rat hepatocytes. As illustrated in Figure 10, transfection of C/EBPα but not C/EBPβ significantly suppressed CAT activity when the cotransfected promoter/CAT constructs contained the C/EBP binding site between −925 and −933, but not with a promoter fragment just short of this C/EBP site both in DDT_1 MF-2 cells (panel A) and in primary rat hepatocytes (panel B). In the absence of C/EBP, the CAT activity was lower with the −186 to −1122 construct than with the other two constructs, which is in agreement with the results shown earlier in Figure 5. Also, the relative CAT activities of the constructs compared to their respective positive controls were generally lower in primary hepatocytes than in DDT_1 MF-2 cells, which parallels the lower level of expression of the β_2AR gene in the former as compared to the latter type of cells.

DISCUSSION

The clarification of the primary structure of the 5′ flanking domain of the gene encoding the rat β_2AR (Jiang & Kunos, 1995) has made it possible to analyze the mechanism of its transcription. Northern blot analysis of poly(A^+) RNA from rat liver has documented two β_2AR transcripts: a major 2.2 kb and a minor 1.6 kb species. The β_2AR gene is intronless, and the identity of the genomic and cDNA sequences confirms the absence of introns in the coding as well as in the noncoding regions of the gene. Therefore, the most likely basis for the mRNA heterogeneity is variations in the 5′ and/or 3′ termini of the two transcripts, and here we present evidence for both.

Primer extension and RNase protection analyses using poly(A^+) RNA from rat liver indicate two major *tsp* at −64 (*tsp*1) and at −220 (*tsp*2), the latter of which is analogous to the single *tsp* identified at the same location in the human β_2AR gene (Emorine *et al.*, 1987; Kobilka *et al.*, 1987). The absence of *tsp*1 in the human β_2AR gene could be due to species- or tissue-specific expression of transcription factors that interact with the promoter which directs transcription from *tsp*1 (see below). In the human β_2AR gene, the promoter activity of two overlapping fragments of the 5′ flanking domain (−150 to −455 and −135 to −1575) has been documented (Collins *et al.*, 1989), but the core promoter region(s) of the rat β_2AR gene has (have) not yet been established. In the present experiments, transient transfections of putative promoter/*CAT* constructs have demonstrated promoter activity for two separate sequence domains of the rat β_2AR gene: one between −36 and −100 bp including *tsp*1 (P1), and a second one between −186 and −312 (P2) including *tsp*2. The possibility that P1 and P2 are responsible for generating the 1.6 and 2.2 kb mRNA species, respectively, is supported by the finding that a cDNA probe complementary to a 5′-UTR that includes *tsp*2 but not *tsp*1 detects only the 2.2 and not the 1.6 kb mRNA species (Figure 1B). Since the two *tsp* are only 156 bp apart, the 3′ termini of the 1.6 and 2.2 mRNA also must be different. Indeed, RNase protection assays of the 3′-UTR directly document the use of two distinct polyadenylation sites in the rat β_2AR gene (Figure 3).

The expression of the β_2AR gene is high in the fetal rat liver, but declines rapidly during ontogenic development (Baeyens & Cornett, 1993). Thus, this gene appears to belong to the group of developmentally regulated genes which are under the control of multiple promoters (Schibler & Sierra, 1987). At the same time, P1 and P2 have all the features of housekeeping gene promoters, including a high G+C content, lack of a TATA box, a high frequency of the CpG dinucleotide, and Sp1 elements upstream from their

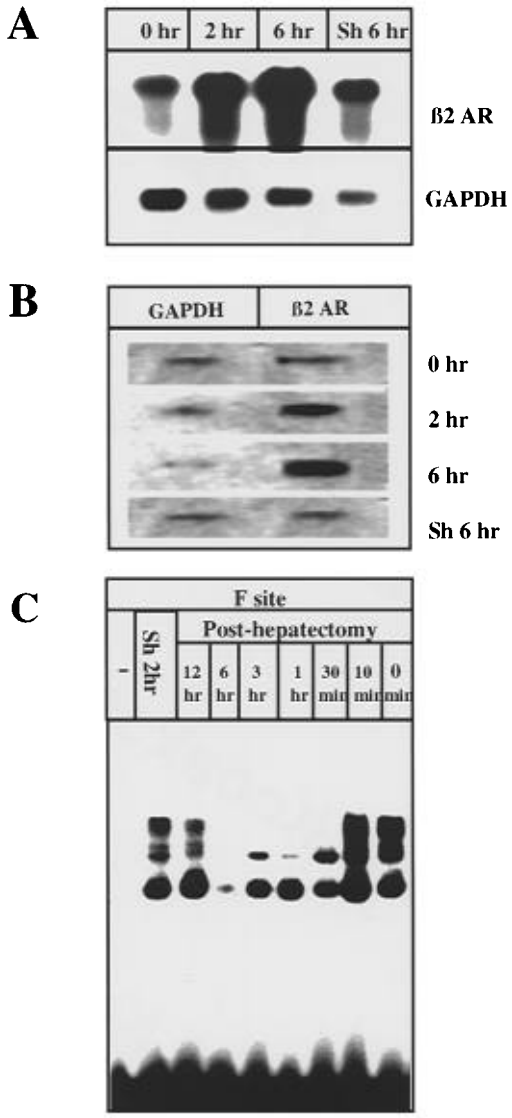

FIGURE 9: Effect of partial hepatectomy on the steady-state level of β_2AR mRNA (A), on the rate of transcription of the β_2AR gene (B), and on protein binding to footprint F (C). (A) Poly(A^+) RNA from the liver of control, partially hepatectomized (2 and 6 h) and a sham-operated rat (6 h) was analyzed by Northern blotting using a β_2AR and a GAPDH cDNA probe. This experiment was replicated 3 more times with similar results. (B) Nuclear run-on assay: ^{32}P-labeled RNA generated by liver nuclei from rats with the indicated pretreatment was hybridized to immobilized β_2AR and GAPDH cDNAs, as described under Materials and Methods. (C) DNA mobility shift analysis of proteins binding to footprint F in liver nuclear extracts from a sham-operated and from partially hepatectomized rats sacrificed at the indicated times after surgery. Note the marked, transient suppression between 30 min and 12 h of the upper two shifted bands, identified in Figures 7 and 8 as containing C/EBPα. Assay conditions were as described in Figure 7.

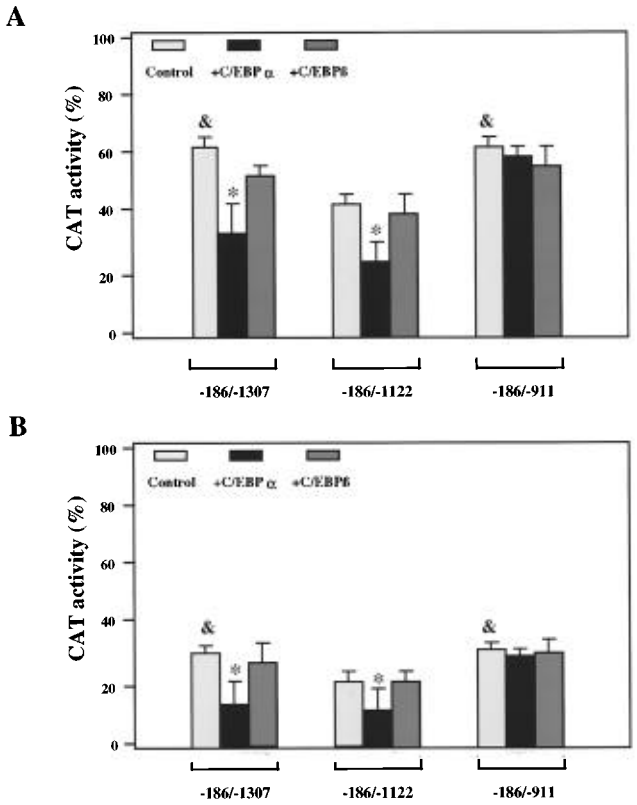

FIGURE 10: C/EBPα suppresses transcription by the β_2AR gene promoter. β_2AR promoter/*CAT* constructs containing different 5′ flanking region fragments as indicated below the abscissa were transfected alone or were cotransfected with expression plasmids harboring the C/EBPα or C/EBPβ coding region, into DDT_1 MF-2 cells (A) or into primary rat hepatocytes (B). Transfection efficiency was determined, and CAT activities were measured in both types of cells as described under Materials and Methods. Means from three separate experiments and their standard errors are shown. * indicates a significant difference from the corresponding control values, whereas & indicates a significant difference from the control value obained with the construct −186 to −1122 ($P < 0.05$).

respective *tsp* (Bird, 1986; Smale & Baltimore, 1989), as it is evident from analyzing the structure of the 5′-UTR of the β_2AR gene (Jiang & Kunos, 1995). A similar conclusion has been reached earlier in the case of the hamster and human β_2AR genes (Kobilka *et al*., 1987; Emorine *et al*., 1987). DNase I footprinting and DMSA also indicate the binding of NF-κB-like protein(s) to the consensus NF-κB binding site in footprint E. The NF-κB/Rel family of transcription factors is induced by a large variety of factors, including bacterial and viral pathogens and inflammatory cytokines. It is interesting to note that interleukin-1, which is a known inducer of NF-κB, was shown to induce the transcription of the β_2AR gene in A549 lung tumor cells (Szentendrei *et al.*, 1992).

Of the multiple footprints present in the 5′-UTR of the rat β_2AR gene, footprint F is probably the most interesting because of its possible involvement in negative transcriptional regulation (see above). This footprint, which could be documented using both sense and antisense probes (Figure 6), contains three overlapping consensus binding sites including GRE, NF-1, and C/EBP, and the binding of all three factors can be documented by DMSA (see Figure 7). We present several lines of evidence to suggest that C/EBPα, a member of the C/EBP family of basic leucine zipper transcription factors, may play the role of a transcriptional repressor of the β_2AR gene in rat liver. First, binding of a heat-stable factor displaceable by the C/EBP consensus oligo to footprint F is compatible with the presence of a C/EBP protein at this site (Figure 7), and supershift assays with selective antibodies (Figure 8A) as well as mutational analysis (Figure 7D) indicate that the major binding component is C/EBPα. The identification of two major bands as generated by C/EBP in the above assays is in agreement with the expression of two major C/EBPα translation products in the liver, $p30^{C/EBP\alpha}$ and $p42^{C/EBP\alpha}$ (Lin *et al.*, 1993). Second, the transient marked suppression of the shifted bands using nuclear extracts from post-hepatectomy

remnant livers (Figure 9C) is in agreement with the documented down-regulation of C/EBPα expression in the regenerating liver, whereas C/EBPβ and C/EBPδ are up-regulated under the same conditions (Mischoulon *et al.*, 1992; Flodby *et al.*, 1993). This not only confirms that the shifted bands represent C/EBPα but also is compatible with the postulated role of C/EBPα as a repressor of β_2AR gene transcription in the rat liver. β_2AR have been shown to be up-regulated in the remnant liver following hepatectomy (Aggerbeck *et al*., 1983; Sandnes *et al*., 1986), and this change is preceded by an increase both in the rate of transcription and in the steady-state level of the β_2AR mRNA [Figure 9A,B; also see Kunos *et al*. (1995)]. The third, and strongest, evidence is that coexpression of C/EBPα but not C/EBPβ with β_2AR promoter/CAT constructs containing the C/EBP binding site suppresses transcription not only in a hamster smooth muscle cell line but also in primary cultured rat hepatocytes (Figure 10). No suppression is observed when C/EBPα is cotransfected with a construct lacking the C/EBP binding site in either type of cell, which indicates the specificity of the observed effects. As expected, removal of the C/EBP binding site significantly increases CAT activity (compare −186 to −1122 vs −186 to −911 in Figure 10), probably as a result of abolishing the effect of C/EBP present in wild-type DDT_1 MF-2 cells or primary rat hepatocytes. CAT activity is also increased when a 5′ extended construct is used (−186 to −1307), which is probably due to as yet undefined DNA/protein interaction-(s) in the region between −1122 and −1307. Together, these findings support a repressor role of C/EBPα for β_2AR expression in the mature, differentiated liver.

The possible role of C/EBPα as a repressor of β_2AR gene expression in the rat liver is quite plausible. C/EBPα is preferentially expressed in liver and adipose tissue, is required for terminal differentiation of hepatocytes and adipocytes (Umek *et al.*, 1991), and is also involved in the control of energy metabolism, particularly as an activator of glycogen synthase (Wang *et al.*, 1995). The expression of C/EBPα is high in terminally differentiated hepatocytes, but low in dedifferentiated liver tissue, such as in fetal liver (Birkenmeier *et al.*, 1989), in liver tumor cells (Friedman *et al.*, 1989), in primary cultured hepatocytes (Mischoulon *et al.*, 1992), and in the regenerating liver (see above). In contrast, β_2AR activation inhibits glycogen synthase and leads to glycogen breakdown, and β_2AR expression is very low in the differentiated, adult rat liver, but high in fetal liver (Blair *et al.*, 1979; Baeyens & Cornett, 1993), in liver tumors (Bevilacqua *et al*., 1991), in primary cultured hepatocytes (Nakamura *et al.*, 1983; Refsnes *et al*., 1983), and in the regenerating liver (see above). This striking, inverse relationship makes it very likely that C/EBPα is a negative regulator of hepatic β_2AR expression in the rat in vivo.

The question arises whether C/EBPα is the only factor involved in the developmental regulation of hepatic β_2AR expression. In this regard, it is interesting to point out the mutually exclusive binding of C/EBPα and an NF-1-like factor to their partially overlapping sites in footprint F. Such an interaction, suggested by the mutual potentiation of the binding of these two factors upon removal of the other factor (Figure 7D), is not unprecedented. Overlapping binding sites for NF-1 and C/EBPα have been identified in the promoter of the liver-specific vitellogenin gene, and it has been suggested that such arrangements may allow fine-tuning of gene expression in response to various stimuli (Cardinaux *et al.*, 1994). In addition to the developmental factors listed above, hepatic β_2AR are also regulated by glucocorticoid (Wolfe *et al.*, 1976) and thyroid hormones (Malbon, 1980), and a GRE is also present in close juxtaposition to the C/EBP and NF-1 binding sites in footprint F. Further studies are required to understand the regulatory role of the mutually exclusive binding of different transcription factors to overlapping binding sites.

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