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Transcriptional Regulation of α_{1b} Adrenergic Receptors (α_{1b}AR) by Nuclear Factor 1 (NF1): a Decline in the Concentration of NF1 Correlates with the Downregulation of α_{1b}AR Gene Expression in Regenerating Liver

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The 5′ upstream region from −490 to −540 (footprint II) within the dominant P2 promoter of the rat α_{1b} adrenergic receptor (α_{1b}AR) gene is recognized by a sequence-specific DNA-binding protein (B. Gao, M. S. Spector, and G. Kunos, J. Biol. Chem. 270:5614–5619, 1995). This protein, detectable in Southwestern (DNA-protein) blots of crude nuclear extracts as 32- and 34-kDa bands, has been purified 6,000-fold from rat livers by DEAE-Sepharose, heparin-Sepharose, and DNA affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and UV cross-linking of the purified protein indicated the same molecular mass as that in crude extracts. Methylation interference analysis revealed strong contact with a TTGGCT hexamer and weak contact with a TGGCGT hexamer in the 3′ and 5′ portions of footprint II, respectively. Nucleotide substitutions within these hexamers significantly reduced protein binding to footprint II and the promoter activity of P2 in Hep3B cells. The purified protein also bound to the nuclear factor 1 (NF1)/CTF consensus sequence, albeit with lower affinity. Gel mobility supershift and Western blotting (immunoblotting) analyses using an antibody against the NF1/CTF protein identified the purified 32- and 34-kDa polypeptides as NF1 or a related protein. Cotransfection into Hep3B cells or primary rat hepatocytes of cDNAs of the NF1-like proteins NF1/L, NF1/X, and NF1/Red1 resulted in a three- to fivefold increase in transcription directed by wild-type P2 but not by the mutated P2. Partial hepatectomy markedly decreased the levels of NF1 in the remnant liver and its binding to P2, which paralleled declines in the rate of transcription of the α_{1b}AR gene and in the steady-state levels of its mRNA. These observations indicate that NF1 activates transcription of the rat α_{1b}AR gene via interacting with its P2 promoter and that a decline in the expression of NF1 is one of the mechanisms responsible for the reduced expression of the α_{1b}AR gene during liver regeneration.

The α_{1b} adrenergic receptor (α_{1b}AR) is a G-protein-coupled receptor that mediates the acute metabolic effects of catecholamines in the liver and is also involved as a comitogen in the regenerative response after the loss or injury of liver tissue (13, 14, 35, 36). Expression of the α_{1b}AR gene in the rat liver is controlled by hormonal and developmental factors as well as by conditions associated with hepatocyte dedifferentiation (8, 33, 35, 36, 49). Such regulation has been shown to occur at the transcriptional level under many of these conditions, such as after partial hepatectomy (36), during primary culturing of rat hepatocytes (28), and in response to glucocorticoids (53), cycloheximide (25), phorbol esters (26), and thyrotropin and cyclic AMP (31). In order to understand the molecular mechanisms involved in the transcription of the α_{1b}AR gene under a variety of physiological and pathological conditions, we have cloned the rat α_{1b}AR gene and identified its multiple promoters and the *cis*-acting elements in its regulatory domain (18, 19). Subsequent experiments have identified the sequence-specific factors that interact with the dominant P2 promoter, including an unidentified ubiquitous transcription factor, tentatively referred to as αAR transcription factor (αARTF), which bound to footprint II within the P2 promoter and was found to be indispensable for the transcription of the α_{1b}AR gene and to be widely distributed among various rat tissues (20). The CCAAT-binding factor CP1 (7, 10) was also found to bind to the footprint II sequence (20), as demonstrated by competition with a CP1 binding sequence from the 5′-flanking region of the mouse α-globin gene (7, 10). Furthermore, the binding of CP1 and αARTF to the footprint II sequence was mutually exclusive (20).

Here we report the extensive purification and further characterization of the αARTF protein and analysis of its binding to the P2 promoter. The results of methylation interference analyses, DNA mobility shift assays (DMSA), supershift assays, Western blotting (immunoblotting), and mutational analyses indicated that the purified protein is identical or closely related to the nuclear factor 1 (NF1)/CTF protein. Cotransfection experiments using cDNAs for three different molecular forms of NF1 confirmed the role of this transcription factor as a positive transcriptional regulator of the α_{1b}AR gene in normal hepatocytes as well as in malignantly transformed hepatocytes. Finally, differential DMSA and Western blotting using nuclear proteins from normal and posthepatectomy remnant livers demonstrated that NF1 expression and binding to DNA are downregulated in the regenerating liver, which parallels the similar downregulation of the transcription and expression of the α_{1b}AR gene. Thus, NF1 is one of a group of transcription factors whose expression is turned off in the regenerating liver, which, in turn, is responsible for or at least contributes to the decreased expression of similarly regulated target genes.

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MATERIALS AND METHODS

Southwestern (DNA-protein) blot analysis. Southwestern blotting was performed as previously described (60). Nuclear proteins (30 to 150 μg) from rat livers or brains were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) in transfer buffer (50 mM Tris-HCl, 40 mM glycine, 20% methanol) with a Bio-Rad semidry apparatus and subsequently renatured by treatment with and then withdrawal of 6 M guanidinium chloride in Z′ buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.6], 12.5 mM $MgCl_2$, 10 μM $ZnSO_4$, 20% glycerol, 0.01% Nonidet P-40). After renaturation, the membranes were incubated with blocking buffer [Z′ buffer containing 3% nonfat dried milk and 3 μg of poly(dI-dC) per ml] for 30 min at 25°C, washed twice for 10 min with binding buffer (Z′ buffer containing 0.25% nonfat dried milk), and hybridized with ^{32}P-labeled concatemers of annealed synthetic oligonucleotides II, IIa, and IIb (20) for 30 min at 25°C. The membranes were washed three times with Z′ buffer for a total of 15 min and analyzed with a PhosphorImager (Molecular Dynamics).

Preparation of rat liver nuclear extracts. Nuclear extracts were prepared from the livers of 120-day-old male Sprague-Dawley rats by the method of Latchman (37), with modifications. All steps were carried out at 0 to 4°C; all buffers contained 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg (each) of pepstatin, aprotinin, and leupeptin per ml, 0.1 mM benzamidine, and 10 mM β-glycerophosphate. Minced tissue (1,000 g of liver) was brought to 3,000 ml with buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA) containing 0.25 M sucrose and homogenized with a motor-driven Potter homogenizer (10 to 15 strokes, 1,400 rpm) until 90% cell lysis occurred. The homogenate was filtered through cheesecloth and centrifuged at 5,000 rpm for 15 min in a GSA rotor (Sorvall). The combined nuclear pellets were resuspended in 2,000 ml of buffer A containing 0.25 M sucrose and 0.5% Triton X-100, homogenized very gently, and spun as described above. The pelleted nuclei were resuspended in 5 volumes of buffer A containing 2.4 M sucrose and spun at 24,000 rpm for 1 h in an SW28 rotor (Beckman). The packed nuclei were resuspended in buffer A containing 0.3 M NaCl, stirred gently for 45 min, and spun at 10,000 rpm for 15 min in an SS34 rotor (Sorvall). After the addition of Nonidet P-40 to 0.01%, the clear supernatant was dialyzed twice for 2 h against 20 volumes of buffer E (20 mM HEPES [pH 7.9], 300 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.01% Nonidet P-40). The dialysate was centrifuged at 10,000 rpm for 15 min in a GSA rotor to eliminate the precipitate formed during dialysis. The protein concentration of the supernatant (crude nuclear extract) was determined by Bio-Rad protein assay, with bovine serum albumin as standard. The usual yield was 1 mg of nuclear protein per g of liver.

Protein purification. Crude liver nuclear extract (1.1 g obtained from 1,000 g of liver tissue) was applied to a 200-ml DEAE-Sepharose column preequilibrated with buffer E containing 300 mM KCl. The flowthrough containing the active fractions was diluted to 100 mM KCl with buffer E and loaded onto a 90-ml heparin-Sepharose CL-6B column equilibrated with buffer E containing 100 mM KCl. The column was eluted with buffer E containing 100 to 700 mM KCl increased in 50 mM steps, and fractions were analyzed by DMSA using ^{32}P-labeled oligonucleotide II as the probe. The DNA binding activity inhibited by a CP1 consensus oligonucleotide which was eluted at 250 to 350 mM KCl (20) was not analyzed further. The DNA binding activity eluted at 550 to 650 mM KCl and designated αARTF (20) was pooled, dialyzed against buffer Z′ containing 100 mM KCl, and loaded onto an oligonucleotide II affinity column, which had been prepared by coupling concatemers of annealed synthetic oligonucleotide II to cyanogen bromide-activated Sepharose CL-4B beads (30). The column was washed extensively with buffer Z′ containing 100 mM KCl and eluted stepwise with buffer Z′ containing 100 to 600 mM KCl. The active fractions recovered at 250 to 350 mM KCl were then loaded onto an oligonucleotide IIm (see Fig. 4C) affinity column, prepared with concatenated mutated oligonucleotide II. The flowthrough containing the αARTF activity was subjected to repeated chromatography over a second oligonucleotide II affinity column. The concentration of nonspecific competitor DNA [poly(dI-dC)] in the buffer was 1 μg/ml during the first pass and 3 μg/ml during the second pass through the oligonucleotide II affinity column.

SDS-PAGE and silver staining. SDS-PAGE was carried out by the method of Sambrook et al. (54). The gels were calibrated with prestained molecular weight standards (GIBCO). Electrophoresis was performed at 30 mA. Silver staining was performed with silver nitrate by using a Bio-Rad silver staining kit.

DNase I footprinting and DMSA. DNase I footprinting was performed as described previously (20). DMSA and preparation of nuclear extracts for DMSA were carried out as described previously (20). In competition experiments, radioactive probe and competitor oligonucleotides were mixed prior to the addition of nuclear extract. Of the synthetic oligonucleotides used, oligonucleotides II, IIa, and IIb correspond to −484 to −543, −484 to −518, and −515 to −543 bp in the 5′-flanking domain of the rat α_{1b}AR gene (20), respectively, and were synthesized in our laboratory with a Cyclone Plus DNA synthesizer (Milligen). The consensus oligonucleotide for NF1 (5′-TATTTTGGATTGAAGCCAATA TGATAATGA-3′) was purchased from Promega, and the other consensus oligonucleotides used were detailed previously (20). For antibody supershift experiments, the nuclear extract or purified protein and poly(dI-dC) were incubated with 1 μl of either preimmune serum or anti-NF1 serum (kindly provided by Naoko Tanese, New York University Medical Center) (1) for 1 h at room temperature. Labeled oligonucleotide II or NF1 consensus oligonucleotide was then added, and the mixture was incubated for another 20 min. The resulting complexes were analyzed as described for DMSA.

UV cross-linking. The procedure was carried out as previously described (11), with slight modifications. The probe used was prepared by annealing an oligonucleotide corresponding to the first 15 nucleotides of the sense strand of oligonucleotide II with its full-length antisense strand and then filling in the remaining 45 bases of the sense strand with 1 U of Klenow DNA polymerase I at 16°C for 3 h in the presence of 37.5 μM (each) dATP and 5-bromo-2′-dUTP and 6.7 μM (each) [α-^{32}P]dCTP and [α-^{32}P]dGTP. The specific activity of the probe was 2×10^8 dpm/μg. The purified protein was incubated with 2 ng of the ^{32}P-labeled DNA probe and 10 μg of poly(dI-dC) in 20 μl at room temperature for 20 min. The mixture was irradiated under a 312-nm UV source at a distance of 1 cm, and the DNA was then digested in the presence of 10 mM $CaCl_2$ for 30 min at 37°C with 1 U of DNase I (Promega) and 3 U of micrococcal nuclease (Sigma). The products were resolved by SDS-PAGE and analyzed with a PhosphorImager.

Methylation interference analysis. The oligonucleotide II coding and noncoding strands were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, respectively, and annealed with unlabeled complementary oligonucleotide strands. Labeled probes were partially methylated with dimethyl sulfate for 5 min at 25°C (41). DMSA were performed as described above, except that binding reaction mixtures were scaled up fivefold in a total volume of 40 μl. The free and complexed probes were visualized with a PhosphorImager and eluted from the polyacrylamide gel overnight in a solution containing 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 1% SDS at 37°C overnight. The DNA in the eluate was extracted with phenol-chloroform, ethanol precipitated, and cleaved with 1 M piperidine at 90°C for 30 min. Equal counts per sample were loaded in all lanes and resolved by 8 M urea–15% polyacrylamide gel electrophoresis.

Western blot analysis. Nuclear extracts and purified protein were size fractionated on an SDS–8% polyacrylamide gel and transferred onto a nitrocellulose filter. The filter was blocked with TPBS buffer (0.05% [vol/vol] Tween 20 in phosphate-buffered saline [pH 7.4]) containing 3% nonfat dairy creamer for 2 h at 25°C and incubated with an antiserum against the NF1/CTF protein (1:500 dilution in TPBS buffer) overnight at 4°C. After three washes with TPBS, the filters were incubated with biotinylated secondary antibody (1:1,000 dilution in TPBS) for 30 min at 25°C. The immune complexes were detected by using streptavidin-alkaline phosphatase conjugate with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt as chromogens according to the manufacturer's (BRL) instructions.

Construction of plasmids. The mutated P2 promoter-pCAT construct (P2m) was prepared by sequential PCR as previously described (6). Briefly, partially overlapping sense (−536 to −511) (5′-CGTGGTGCCTTATTCGGGCGTGCG CG-3′) and antisense (−494 to −522) (5′-TACGGGTCCATAAGAGGCGCG CACGCCCG-3′) oligonucleotide primers containing the point mutations underlined were synthesized and used in the sequential PCR amplification steps. The primer pairs used in the sequential steps were sense primer + primer 1 (5′-TG ACTGCAGGGCGACATCAG-3′) containing a *Pst*I site and antisense primer + primer 2 (5′-GATGTGACTCAAGCTTCTGCCACTG-3′) containing a *Hin*dIII site. The template used in the sequential PCR was the P2 promoter-pCAT construct. PCR was carried out as described before (19). The two PCR products were combined and amplified by primers 1 and 2. The final PCR product was purified and subcloned into the pCAT enhancer vector. The mutations in P2m were verified by sequencing. The basic P2 promoter construct (P2b) and mutated basic P2 promoter construct (P2bm) were prepared by subcloning the wild-type or mutated −832 to −432 fragment of the 5′-flanking region of the α_{1b}AR gene into the pCAT basic vector (Promega). The constructs were verified by sequencing.

Transient transfections and CAT assays. Transient transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (19). The pNF1/X expression vector (pSV-pNF1/X) was constructed by subcloning the *Eco*RI-digested pNF1/X cDNA fragment (21) (obtained from the American Type Culture Collection) into the pcDNA3 expression vector (Invitrogen, San Diego, Calif.). The pNF1/Red1 and pNF1/L expression vectors (pSV-pNF1/Red1 and pSV-pNF1/L, respectively) were prepared by subcloning pNF1/Red1 and pNF1/L coding regions into pcDNA3 expression vectors. The pNF1/Red1 coding region was amplified by PCR from pNF1/Red1 cDNA in pGEM (21) (American Type Culture Collection). The 5′ PCR primer (5′-TCCAAGC TTCTCGAAGATTTTCTTGGGCAT-3′) containing a *Hin*dIII linker is located 30 bp upstream from the ATG start codon; the 3′ PCR primer (5′-TCTGGGC CCTCAGTTGCTTGTCTCTGC-3′) containing an *Apa*I linker is located around the stop codon. The PCR fragment was digested with *Hin*dIII and *Apa*I, and the purified fragment was ligated into pcDNA3 vector. The pNF1/L coding region was amplified from the psPUTK-NF1/L plasmid (kindly provided by Mary Ann Thompson, Vanderbilt University, Nashville, Tenn.). The 5′ primer (5′-GACC GAGAATACAAGCTTGCTT-3′) containing a *Xho*I linker is located 50 bp upstream from the ATG translation start codon; the 3′ primer (5′-TGGTCTG TCTAGAGGATGGTGGGA-3′) containing a *Xba*I linker is located 12 bp downstream from the stop codon. The PCR fragment was subcloned into pcDNA3. The inserts were verified by restriction mapping and sequencing.

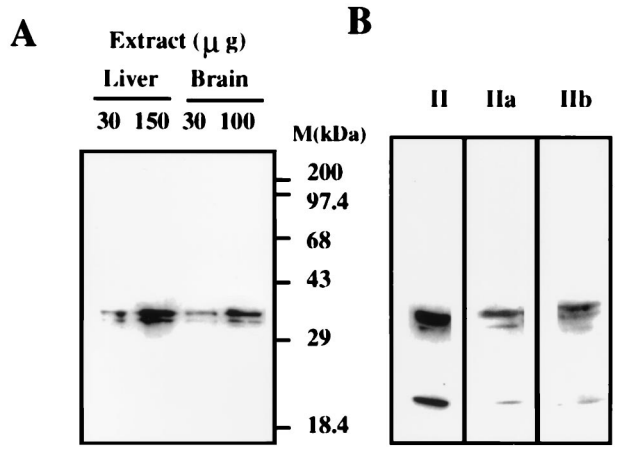

FIG. 1. Identification of the factor binding to footprint II by Southwestern blotting. (A) Samples contained the indicated amounts of nuclear proteins from the rat brain or liver and were suspended in loading buffer containing 2% SDS. The blot was hybridized with labeled oligonucleotide II. (B) Southwestern blots hybridized with labeled oligonucleotide II, IIa or IIb.

Partial hepatectomy. Adult male Sprague-Dawley rats were subjected to 2/3 partial hepatectomy (24). Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and the median and left lateral lobes of the liver were ligated at their stem and excised. Control rats were subjected to a sham operation, which consisted of laparotomy and a brief manipulation of the intestines but not the liver with a cotton swab prior to wound closure. The animals were allowed to recover and were sacrificed by decapitation at the indicated times after surgery.

Primary culturing of rat hepatocytes. For transfection into primary cultured rat hepatocytes, male Sprague-Dawley rats weighing 80 to 120 g were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and the portal vein was cannulated under aseptic conditions. Liver cells were isolated by a collagenase perfusion protocol previously described (48). The isolated cells were washed twice with hepatocyte wash medium (GIBCO) and plated onto polylysine-coated culture dishes in attachment medium (GIBCO). After 3 h, the medium was changed to Dulbecco modified Eagle medium containing 5% fetal bovine serum, 1×10^{-8} M dexamethasone, 10 ng of epidermal growth factor per ml, 5 μg of insulin per ml, 2.5 μg of amphotericin B per ml, 50 μg of gentamicin per ml, 67 μg of penicillin per ml, and 100 μg of streptomycin per ml, and cells were transfected with DNA by using the Lipofectin reagent (GIBCO/BRL), as described by the manufacturer. After 16 h, the medium was replaced with fresh medium without Lipofectin, and cells were grown for an additional 40 h after which they were used to measure CAT activity, as described above.

Northern blotting and nuclear run-on transcription assays. The isolation of total RNA and Northern analysis using a rat α_{1b}AR cDNA were described previously (19). For sequential hybridization of the blots with different probes, radioactivity was stripped from the blots by immersion in 0.1× Denhardt's solution containing 1 mM Tris Cl (pH 8.0) and 1 mM EDTA (pH 8.0) for 2 h at 75°C. Oligonucleotide probes for chicken β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 28S RNA (Clontech) were used as loading controls. Nuclear run-on transcription assays were done as described previously (59).

Statistical analyses. For multiple comparisons, one-factor analysis of variance followed by Tukey's post hoc test was used; $P < 0.05$ was taken to imply statistical significance.

RESULTS

αARTF is detectable by Southwestern blotting as 32- and 34-kDa polypeptides. Our earlier experiments suggested that a novel protein factor(s) binds to the α_{1b}AR gene middle (P2) promoter (20). To determine its molecular mass, we performed Southwestern blotting with nuclear extracts from rat livers and brains. The nuclear proteins were separated by SDS-PAGE, transferred onto a nitrocellulose filter, and hybridized with radiolabeled multimers of oligonucleotide II (see its sequence in Fig. 4B). As shown in Fig. 1, two major bands of 32 and 34 kDa and a weak band of 20 kDa were detected in rat liver and brain crude nuclear extracts.

Previous studies suggested that footprint II contains two binding sites for this factor and one for the nuclear protein CP1. In order to further define the domains within oligonucleotide II which bind the unknown protein, three equal aliquots of crude liver nuclear extract were size fractionated by SDS-PAGE and transferred onto nitrocellulose, and the lanes were separated and probed with multimers of oligonucleotides II, IIa, and IIb. The three probes were radiolabeled to the same specific activity. As shown in Fig. 1B, all three probes bind both the 32- and 34-kDa species. This suggests that the 32- and 34-kDa species are able to bind independently to the 3′ and 5′ portions of oligonucleotide II, which is consistent with our previous finding that oligonucleotide II contains two separate binding sites for αARTF (20). Binding of the 20-kDa band was very weak in Fig. 1A but was much stronger when the washing time was reduced (Fig. 1B).

Purification from rat liver of the protein binding to oligonucleotide II. Most transcription factors identified to date have been isolated by using multistep chromatography protocols, including combinations of ion-exchange, heparin binding, and DNA affinity chromatography (16, 30). A prerequisite for such purification is the availability of an easy way to monitor the target protein at every step of purification. The result of the Southwestern analysis illustrated above indicated that purification of αARTF by using an oligonucleotide II affinity column is feasible, and it also provided an estimate of the molecular mass(es) of the protein(s). Figure 2 illustrates the use of DMSA to monitor the active fraction during purification. Rat liver nuclear extracts were first applied to a DEAE-Sepharose column to remove nucleic acids. Then the flowthrough containing the oligonucleotide II binding activity was applied to a heparin-Sepharose column and eluted by a step gradient of KCl. Two-microliter aliquots of the 20-ml fractions collected were assayed by DMSA using ^{32}P-labeled oligonucleotide II as the probe (Fig. 2A). This step enriched the αARTF binding activity 12-fold and effectively separated it from a CP1-like protein, eluted at lower KCl concentrations (0.25 to 0.30 M). The separate identities of the proteins in these two bands were established by using unlabeled CP1 consensus oligonucleotide and oligonucleotide II as competitors in DMSA, respectively (Fig. 2B). Then the combined αARTF fractions were incubated with nonspecific competitor poly(dI-dC) (1 to 3 μg/ml) and applied to a DNA affinity column prepared by coupling multimers of the double-stranded oligonucleotide II to CNBr-activated Sepharose CL-4B, as described in Materials and Methods. The column was eluted by a stepwise gradient of KCl (150 to 600 mM), and 2-μl aliquots of the 0.5-ml fractions were used in DMSA. There was no significant activity detectable in the final flowthrough, and strong DNA binding activity could be eluted at between 0.25 to 0.35 M KCl (Fig. 2C). This step yielded an approximately 250-fold purification. In order to remove high-affinity nonspecific DNA-binding proteins, the active fractions were pooled, diluted to 0.1 M KCl, and passed over an oligonucleotide IIm (see below) affinity column. The flowthrough containing most of the oligonucleotide II binding activity was again passed through the oligonucleotide II affinity column and eluted with 0.25 to 0.35 M KCl. This step resulted in a further 2-fold purification, providing a 6,000-fold final purification over the crude extract. The pooled fractions recovered from the second oligonucleotide II affinity column were resolved by SDS-PAGE. Two major polypeptides of 32 and 34 kDa were identified by silver staining (Fig. 2D), which is consistent with the results of the Southwestern analysis.

DNA binding properties of the purified protein. As mentioned earlier, the DNA binding activity of αARTF was monitored throughout the purification procedure by DMSA using labeled oligonucleotide II. To further confirm the DNA bind-

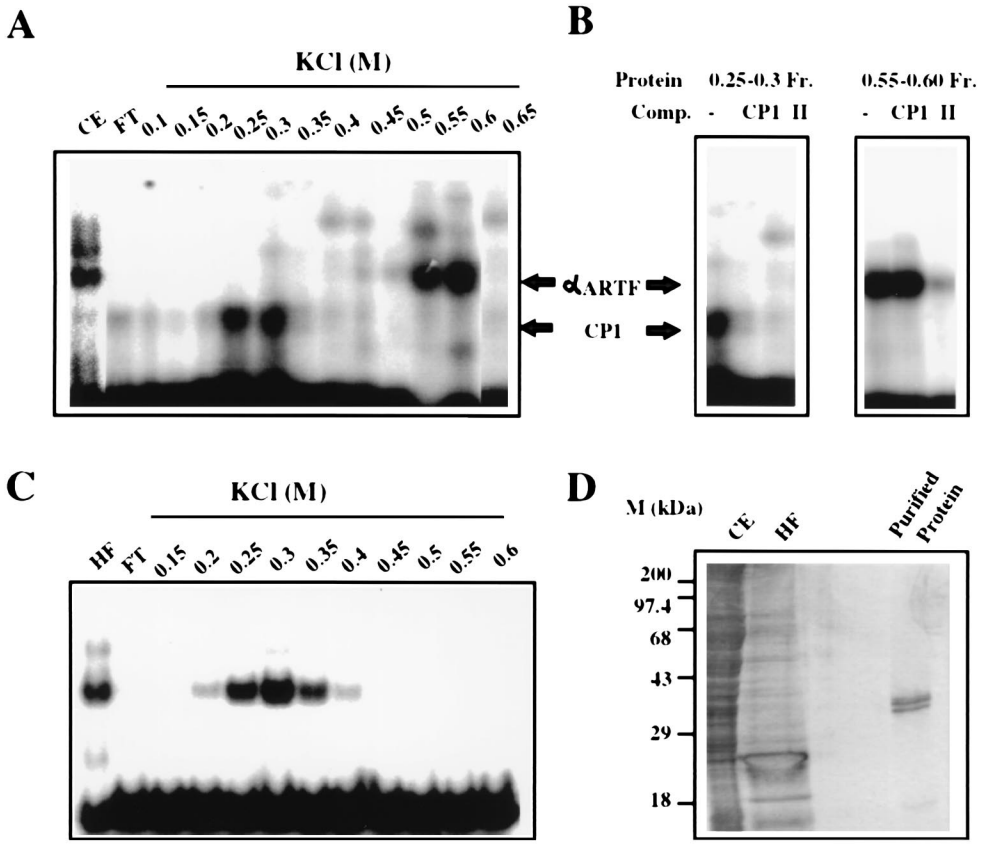

FIG. 2. Purification of the factor binding to footprint II. (A) DMSA of fractions eluted from the heparin-Sepharose column, with ^{32}P-labeled oligonucleotide II as the probe. CE, crude extract; FT, flowthrough from the heparin column. (B) Identification of the 0.25 to 0.35 M KCl fractions (Fr.) containing CP1 (left) and the 0.55 to 0.60 M KCl fractions containing αARTF (right) by DMSA, using labeled oligonucleotide II as the probe and unlabeled CP1 consensus oligonucleotide or oligonucleotide II as competitors (Comp.). (C) DMSA of KCl fractions eluted from the DNA affinity column. HF, 0.55 to 0.60 M KCl fractions from the heparin column of panel A; FT, flowthrough of the DNA affinity column. (D) SDS-PAGE analysis of the purified protein. Crude nuclear extract (CE), 0.55 to 0.6 M KCl fraction from the heparin column (HF), and purified protein (0.25 to 0.35 M KCl fraction eluted during the second run on the DNA affinity column) were resolved by SDS–8% PAGE and visualized by silver staining.

ing properties of the affinity-purified protein, DNase I footprinting experiments were performed with the ^{32}P-end-labeled DNA fragment C (20), which corresponds to the antisense strand between nucleotides −432 to −813 in the 5′-flanking domain of the α_{1b}AR gene. As shown in Fig. 3A, the purified protein (lane 3) generated a footprint on this template between nucleotides −490 and −540 that was identical to the one generated by crude liver nuclear extract (lane 2). This demonstrates that the purified protein retained its sequence-specific DNA binding property.

To further verify that the 32- and 34-kDa polypeptides represent the protein that binds to oligonucleotide II, the affinity-purified protein was UV cross-linked to the ^{32}P-labeled bromodeoxyuridine-containing double-stranded oligonucleotide II as described in Materials and Methods. As shown in Fig. 3B, the two labeled products obtained (lane 2) had the same sizes as the affinity-purified bands in the SDS-PAGE gel (Fig. 2D) or the bands identified by Southwestern analysis (Fig. 1). The specificity of the cross-linking reaction was indicated by the ability of excess unlabeled oligonucleotide II to protect the polypeptides from cross-linking to the labeled oligonucleotide II (Fig. 3B, lanes 3 and 4).

Methylation interference and mutational analyses. We previously demonstrated that αARTF binds to two separate sites on the oligonucleotide II region (20). To provide more specific information on the DNA-protein contacts involved in protein

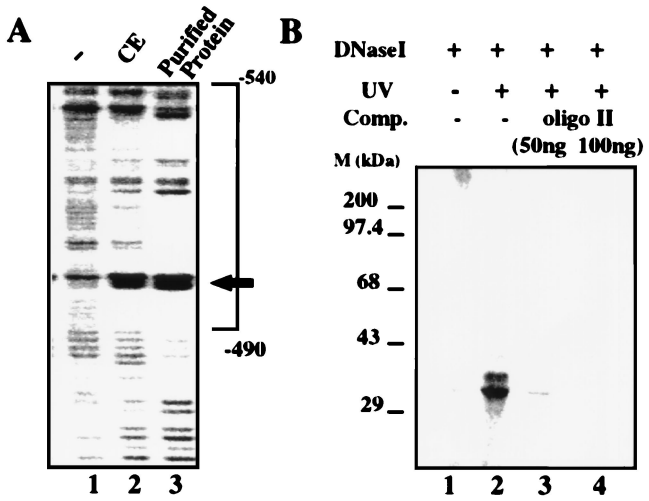

FIG. 3. DNA binding properties of the purified protein. (A) DNase I footprinting analysis of 40 μg of crude liver extract (CE; lane 2) and 50 ng of affinity-purified protein (0.25 to 0.35 M KCl fractions from Fig. 2C; lane 3). Lane 1 contained no protein (−). The probe, α_{1b}AR 5′-flanking fragment from −813 to −432, was end labeled at −432. The arrow indicates a DNase-hypersensitive site. (B) UV cross-linking analysis of the purified protein. The standard DMSA was performed with affinity-purified protein and ^{32}P-labeled oligonucleotide (oligo) II containing bromo-UTP, as described in Materials and Methods. The probe was cross-linked to the polypeptides by UV irradiation and analyzed by SDS–8% PAGE after digestion with DNase I and micrococcal nuclease. Comp., competitor.

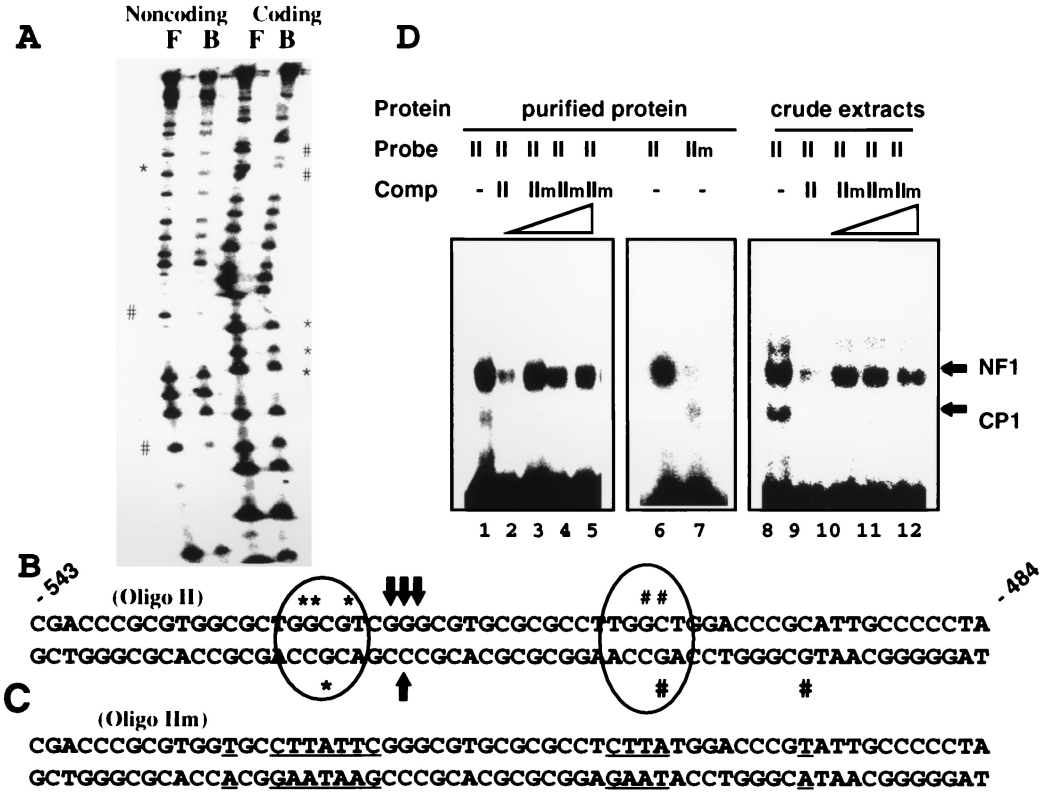

FIG. 4. Methylation interference and mutational analyses of the purified protein. (A) Coding and noncoding strand probes (oligonucleotide II) were end labeled and treated with dimethyl sulfate. DMSA were performed with affinity-purified protein. The free (F) and bound (B) probes were excised and cleaved with piperidine, and samples containing equal amounts of radioactivity were fractionated on an 8% polyacrylamide–6 M urea sequencing gel. Sites of strong (#) and weak (*) interference are indicated. (B) Sequence localization of the contact points of the purified protein. Arrows indicate sites of DNase I hypersensitivity. Oligo, oligonucleotide. (C) Sequence of the mutated oligonucleotide II (oligo IIm), with mutated residues underlined. (D) DMSA using purified protein (lanes 1 to 7) or crude liver nuclear extract (lanes 8 to 12). Oligo IIm was used as the unlabeled competitor (Comp; lanes 3 to 5 and 10 to 12) or as labeled probe (lane 7). −, no competitor.

binding, we performed methylation interference analysis using oligonucleotide II. The ^{32}P-end-labeled oligonucleotide II was partially methylated and used as the probe in a preparative DMSA with the purified protein. Both the free probe and the protein-DNA complex were excised from the gel; the DNA was isolated, cleaved with piperidine, and analyzed by denaturing SDS-PAGE. As shown in Fig. 4A, methylation of the guanine residues at positions −495, −504, −505, and −506 greatly reduced protein binding, whereas methylation at positions −524, −525, −526, and −527 caused a moderate reduction of protein binding to oligonucleotide II. As shown schematically in Fig. 4B, the purified protein strongly contacts TTGGCT and weakly contacts TGGCGT on the 3′ and 5′ portions of oligonucleotide II, respectively, which is in agreement with previous DMSA results showing that oligonucleotide II contains two separate binding sites (20).

To further confirm the specificity of the protein-DNA interaction, a mutated oligonucleotide II (IIm; Fig. 4C) was designed by using point mutations based on the results of the methylation interference analysis. As illustrated in Fig. 4D, oligonucleotide IIm failed to compete with protein binding to the ^{32}P-labeled oligonucleotide II when either the affinity-purified protein (lanes 3 to 5) or a crude liver nuclear extract (lanes 10 to 12) was used and did not bind the purified protein when used as the ^{32}P-labeled probe (lane 7). However, oligonucleotide IIm effectively competed for CP1 binding to oligonucleotide II (lanes 10 to 12), which indicates the specificity of the mutated residues as binding sites for the purified protein.

The purified protein binds to the NF1/CTF consensus sequence. The results of the methylation interference analysis described above indicated that the sequence domains containing the points of contact for the purified protein represented one-half of the consensus sequence motif for NF1, TGGCT and TGGCG (57). This led us to examine whether the purified protein may bind to the full NF1/CTF consensus sequence. Figure 5A illustrates that the purified protein binds to the NF1/CTF consensus oligonucleotide (lane 3), which suggests that it is NF1 or a related factor. However, in previous experiments, a 100-fold excess of the same NF1 consensus oligonucleotide failed to abolish protein binding to oligonucleotide II with a crude liver nuclear extract (20). Therefore, we repeated the competition assay using the purified protein and different relative concentrations of the NF1 consensus oligonucleotide. When tested in a DNase I footprinting assay (Fig. 5B), a 100-fold excess of the NF1 oligonucleotide weakly inhibited the protection (lane 3), but full competition was observed in the presence of a 200-fold excess of the NF1 oligonucleotide (lane 4). A similar 200-fold excess of consensus oligonucleotides for SP1 AP1, AP2, AP3, NF-κB, and CREB (not shown) did not modify the footprint. To further explore the relationship between the purified protein and NF1/CTF, we compared the abilities of various unlabeled oligonucleotides to compete with the purified protein for binding to the ^{32}P-labeled oligonucleotide II. As shown in Fig. 5C, complex formation by the labeled oligonucleotide II is effectively inhibited by the unlabeled oligonucleotide II, but not by the mutated oligonucleotide IIm.

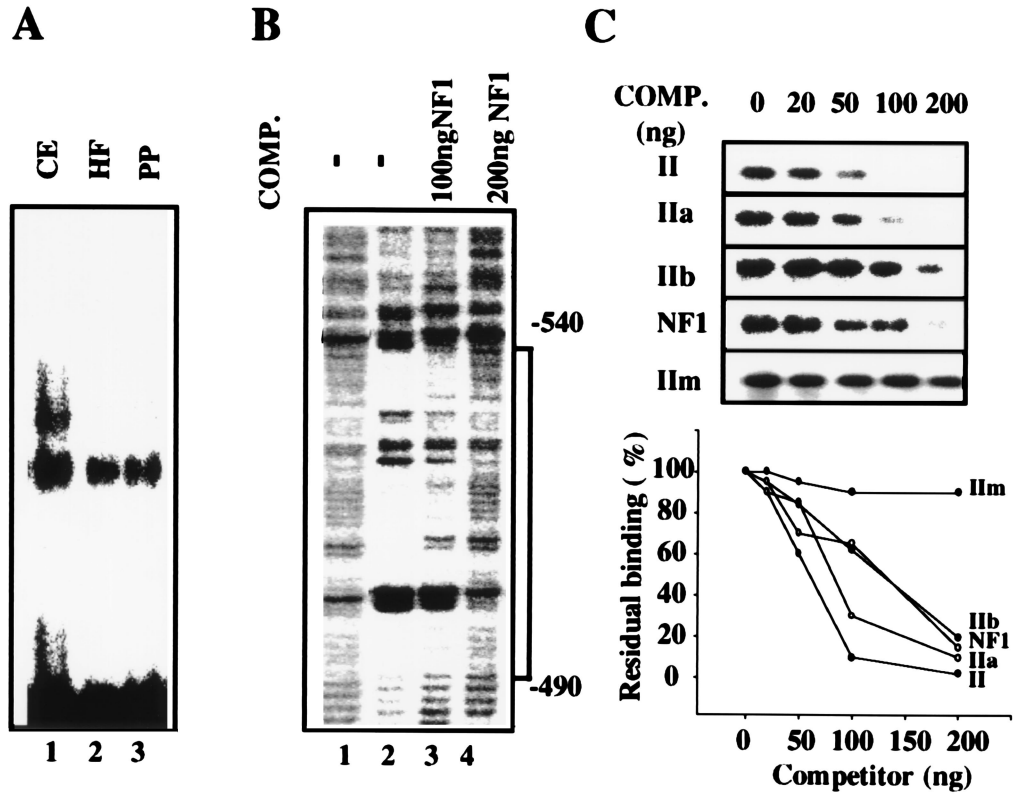

FIG. 5. Competition binding analysis of the purified protein. (A) Affinity-purified protein binds to the NF1/CTF consensus sequence. DMSA were performed with 10 μg of crude liver extract (CE; lane 1), 1 μg of the 0.55 to 0.6 M KCl heparin fraction (HF; lane 2), or 50 ng of purified protein (PP; lane 3) and ^{32}P-labeled consensus oligonucleotide for NF1/CTF (see Materials and Methods). (B) NF1/CTF consensus oligonucleotide is able to abolish footprint II on the labeled P2 fragment from −813 to −432. Lane 1, no protein; lanes 2 to 4, 50 ng of purified protein with no (−) competitor (Comp.) or 100- or 200-fold excess of NF1/CTF consensus oligonucleotide. (C) DMSA competition analysis of the purified protein. Various amounts of the indicated competitor (Comp.) oligonucleotides were added to a standard DMSA performed with 50 ng of purified protein and ^{32}P-labeled oligonucleotide II. The amounts of radioactivity in the shifted bands were quantified with a PhosphorImager and plotted as percentages of the control in the absence of competitor.

The other oligonucleotides competed with a potency order of IIa > NF1/CTF oligonucleotide > IIb. Comparisons of the amounts of unlabeled oligonucleotides required to displace 50% of the labeled probe reveal that oligonucleotide II is two to five times more potent than either oligonucleotide IIa or the NF1/CTF1 consensus oligonucleotide. This may be due to the fact that oligonucleotide II contains two TGGC binding sites. The finding that oligonucleotide IIa competed more effectively than did oligonucleotide IIb is consistent with the results of the methylation interference analysis, which indicated that the purified protein strongly contacted the 3′ half (oligonucleotide IIa) and weakly contacted the 5′ half (oligonucleotide IIb) of the oligonucleotide II sequence domain.

The purified protein is NF1 or a related factor. The evidence discussed above strongly suggests that the purified protein is NF1 or a closely related factor. To obtain more conclusive evidence, we analyzed the purified protein by supershift assay and Western blotting, using a polyclonal anti-CTF-1 antibody (N. Tanese, New York University Medical Center). NF1 represents a family of sequence-specific DNA-binding proteins with very high levels of sequence homology over the first 240 amino acids at their N-terminal ends and lower levels of homology for their C-terminal portions. CTF-1 and NF1/L are 98% homologous in their N-terminal 175 residues, and both are recognized by the antibody mentioned above (1), which probably recognizes other related members of the NF1 family of proteins as well. The purified protein was incubated with CTF-1 antiserum or with preimmune serum prior to incubation with ^{32}P-labeled NF1/CTF oligonucleotide (Fig. 6A, lanes 1 to 5) or oligonucleotide II (lanes 6 to 11) in the DMSA. With both probes, incubation with the antiserum but not with the preimmune serum resulted in a complete supershifted band. The specificity of the supershifted band was demonstrated by the lack of a similar effect of the same antibody on complexes formed between oligonucleotide II and the partially purified CP1 factor (Fig. 6A, lanes 12 to 15).

Figure 6B illustrates the results of Western blotting experiments. With either crude liver nuclear extracts or the purified protein, two immunoreactive species with molecular masses of 32 and 34 kDa were visualized, whereas higher-molecular-weight bands were seen only with the crude extract, suggesting that the latter were the result of nonspecific interactions. This provides strong evidence for the identity of the purified protein(s) with NF1.

Role of NF1 in the regulation of α_{1b}AR gene transcription. In earlier experiments, deletion of the 5′-flanking domain of the rat α_{1b}AR between −490 and −540 bp abolished the promoter activity of P2 (20). This suggested that αARTF and CP1 together are positive transcriptional regulators of the α_{1b}AR gene, since this region contains binding sites for both factors. To identify the role of αARTF more selectively, a mutated P2 promoter-pCAT construct (P2m) was prepared by using the mutated oligonucleotide II (IIm) and transfecting the construct into Hep3B liver tumor cells. As illustrated above, this mutation eliminated αARTF binding without affecting CP1 binding to oligonucleotide II. The significant decrease in CAT

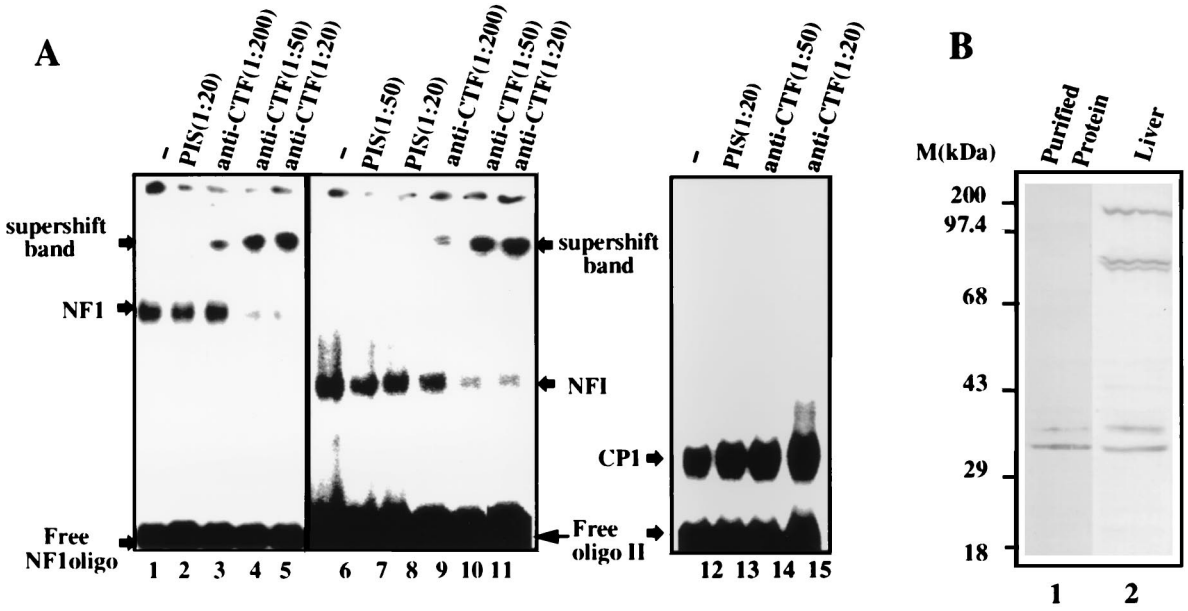

FIG. 6. The purified protein is NF1 or a related factor. (A) Supershift analysis of the purified protein using anti-CTF serum. DMSA were performed with 5 ng of purified protein plus ^{32}P-labeled NF1 oligonucleotide (oligo; lanes 1 to 5) or ^{32}P-labeled oligonucleotide II (lanes 6 to 11) or 2 μg of heparin-purified CP1 plus ^{32}P-labeled oligonucleotide II (lanes 12 to 15). The purified protein or CP1 was preincubated with preimmune serum (PIS) or the anti-CTF serum as indicated. −, no preincubation. (B) Western blotting analysis of the purified protein using anti-CTF serum. Purified protein (lane 1) or crude nuclear extracts from livers (lane 2) were separated by SDS-PAGE, and Western blots were incubated with the anti-CTF serum as described in Materials and Methods.

activity obtained with this P2m construct (Fig. 7A) more specifically defines the positive transcriptional regulatory role of NF1 in the control of the expression of the α_{1b}AR gene in the rat liver.

The transactivating role of NF1 in α_{1b}AR gene expression was further verified by cotransfection into Hep3B cells of the P2 promoter-pCAT construct with NF1/L, NF1/Red1, or NF1/X expression vectors containing cDNAs encoding these three NF1 proteins, which were originally identified and purified from the liver (1, 21, 47). As shown in Fig. 7A, coexpression of NF1/L, NF1/Red1, or NF1/X caused approximately three-, five-, and fivefold increases in P2 promoter activity, respectively. P2m-pCAT constructs had lower levels of promoter activity than did P2-pCAT constructs, and this activity was only minimally affected by cotransfection of NF1 variants.

In the experiments discussed above, the P2 promoter was inserted into a simian virus 40 (SV40) enhancer-containing CAT vector. In order to rule out the potentially confounding effect of a strong viral enhancer, we prepared P2b-pCAT by subcloning the P2 promoter into the enhancerless and promoterless pCAT basic vector. In the absence of the SV40 enhancer, the promoter (CAT) activity in Hep3B cells is only 51% of the activity observed with constructs containing the viral enhancer (Fig. 7A). Figure 7A also shows that cotransfection of NF1/L, NF1/X, and NF1/Red1 with P2b-pCAT caused the same three-, five-, and threefold increases in promoter activity, respectively, observed in the presence of the enhancer.

To test whether the positive transcriptional regulatory role of NF1 is also present in the normal rat liver, cotransfection experiments similar to those described above were done with primary cultured rat hepatocytes. As illustrated in Fig. 7B, cotransfection of any one of the three NF1 variants with the P2-pCAT construct resulted in similar increases in transcription; these effects were evident in both the presence and absence of the SV40 enhancer.

Partial hepatectomy decreases α_{1b}AR gene transcription, NF1 expression and NF1 regulation of the P2 promoter. Partial hepatectomy causes a rapid change from α_1- to β_2-adrenergic glycogenolysis in the residual liver that precedes the regenerative response and is associated with downregulation of α_{1b}AR and upregulation of β_2AR readily detectable within 24 h of the surgery (2, 55). In order to define the level at which the regulation of α_{1b}AR gene expression occurs, we quantified the steady-state levels of α_{1b}AR mRNA as well as the rate of transcription of this receptor gene in the partial-hepatectomy model. Total RNA extracted from rat livers at various time points after partial hepatectomy or sham operation was analyzed by Northern (RNA) blotting. As shown in Fig. 8A, the α_{1b}AR cDNA probe hybridized with a major mRNA species of 2.7 kb and two minor species of 2.3 and 3.3 kb in length, in agreement with our earlier findings (19). Partial hepatectomy resulted in marked reductions in the amounts of all three mRNA species, which were evident at 6, 16, and 24 h after surgery. In this experiment and the three repeat experiments with similar results, radioactivity in the 2.7-kb mRNA band decreased by 60 to 70% between 6 and 24 h, as quantified with a PhosphorImager. The Northern blots were subsequently stripped and reprobed with oligonucleotide probes for β-actin or constitutively expressed 28S RNA. The amount of the 2.2-kb β-actin mRNA did not change within the first 2 h after partial hepatectomy, whereas at 6, 16, and 24 h, there were marked increases (data not shown), which is in agreement with several published reports (17, 45). The unchanged intensity of the 28S RNA band in each lane (not shown) further indicated that the observed decrease of α_{1b}AR mRNA after partial hepatectomy was real and not the result of uneven loading or transfer of mRNAs to the membranes.

Figure 8B illustrates the changes in the rate of gene transcription, as measured by nuclear run-on experiments using nuclei isolated from residual liver tissue at 0, 2, or 6 h after partial hepatectomy and at 6 h after sham operation. The

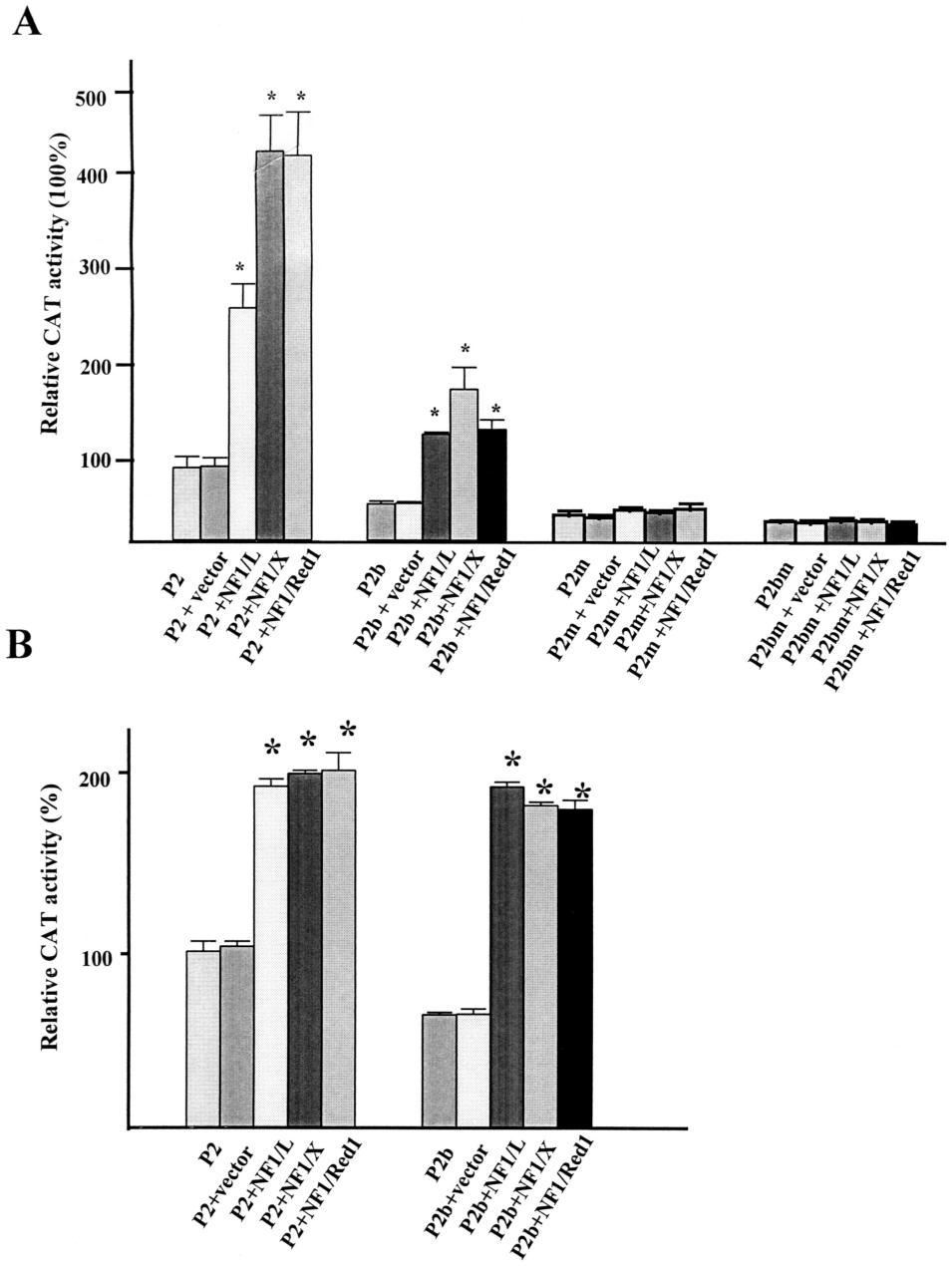

FIG. 7. Cloned variants of NF1 increase P2 transcriptional activity in Hep3B cells (A) or primary cultured rat hepatocytes (B). P2-, P2m-, P2b-, or P2bm-pCAT constructs were cotransfected with the expression vector pcDNA3 with no insert or harboring the cDNA for NF1/L, NF1/X, or NF1/Red1, as described in Materials and Methods. In all experiments, 1 μg of β-galactosidase vector (Promega) was cotransfected to allow for adjustments of transfection efficiency (19). CAT activity is expressed as a percentage of the control, as established by using P2-pCAT alone. Data are the means ± standard errors of the means from four independent experiments. *, significant difference ($P < 0.05$) from the result for corresponding controls.

transcriptional rate of the α_{1b}AR gene was progressively reduced after partial hepatectomy, whereas the rate of transcription of the control GAPDH gene remained essentially unchanged, with the latter result being in agreement with a previous report (9). Similar results were obtained in three additional experiments.

In order to examine whether the decreased rate of α_{1b}AR gene transcription after partial hepatectomy correlates with NF1 binding to the P2 promoter and with the concentration of NF1 protein, nuclear extracts were prepared from sham remnant livers as well as posthepatectomy remnant livers and analyzed by DMSA and Western blotting. As shown in Fig. 8A, NF1 binding to oligonucleotide II was reduced between 2 and 16 h after partial hepatectomy compared with after the sham operation. The decrease in NF1 binding was selective, as there was no difference in the binding of the same protein extracts to an OCT-1 consensus oligonucleotide (not shown). The level of the OCT-1 transcription factor is known to be unaffected by partial hepatectomy (39). Figure 8A shows that the 32- and 34-kDa species detected in a Western blot by anti-NF1/CTF serum were significantly reduced in intensity between 2 and 16 h posthepatectomy, compared with time-matched sham

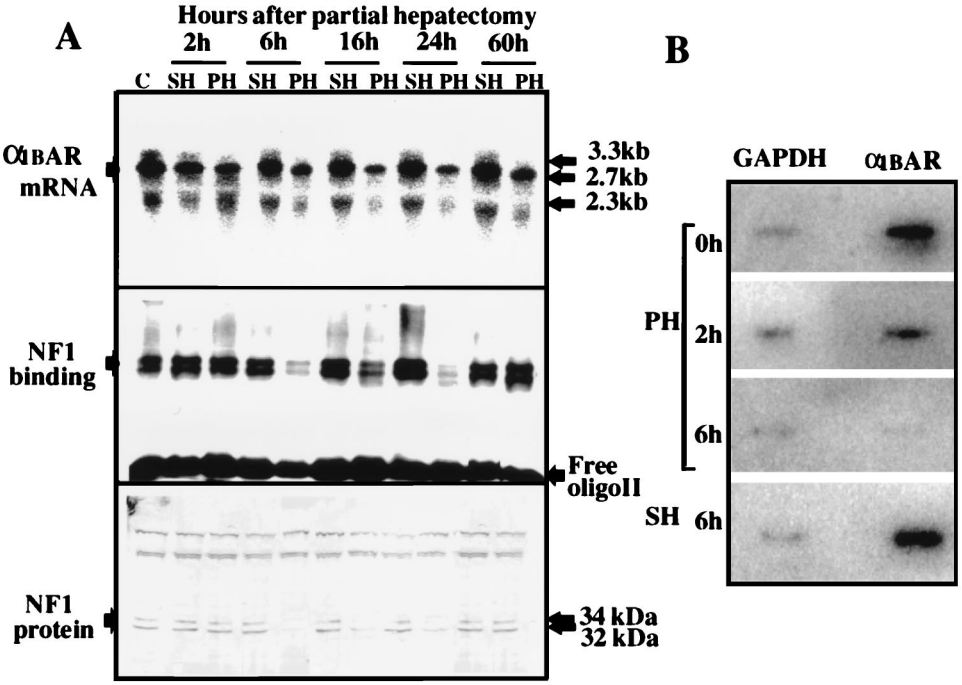

FIG. 8. Parallel decline in the expression of α_{1b}AR and NF1 in the regenerating liver. (A) The effect of partial hepatectomy (PH) on α_{1b}AR mRNA (top), NF1 binding to oligonucleotide II (oligoII) quantified by DMSA (middle), and tissue levels of NF1 detected by Western blotting in the rat liver (bottom). C, control. (B) Relative rates of transcription of the α_{1b}AR and GAPDH genes in livers from partially hepatectomized (PH) and sham-operated (SH) rats, as measured by nuclear run-on assays. Equal amounts of radioactive mRNA transcribed in vitro by nuclei isolated from the livers of PH or SH rats were hybridized to the indicated cDNA probes (α_{1b}AR, 10 μg; GAPDH, 1 μg) immobilized on nitrocellulose membranes. The radioactivities on blots were quantified with a PhosphorImager.

preparations. The decline in the amount of the NF1 protein was real, as the staining patterns of major protein bands visualized by Coomassie blue during SDS-PAGE of the same nuclear extracts (not shown), as well as the high-molecular-weight bands present in all lanes, were not different for sham and remnant livers. This suggests that the decrease of NF1 binding to the P2 promoter is due to a selective decline in the concentration of NF1 protein after partial hepatectomy. Taken together, these findings strongly suggest that the decrease in α_{1b}AR gene expression in the regenerating liver is due to a parallel decline in the nuclear concentration of the NF1 protein.

DISCUSSION

We have previously isolated and characterized the rat α_{1b}AR gene and found multiple sequence-specific factors that interact with the dominant P2 promoter, including the CCAAT binding factor CP1 and an unidentified ubiquitous transcription factor, tentatively named αARTF (18–20), both of which bind to the footprint II sequence within the P2 promoter. The binding of CP1 to footprint II was weak (Fig. 4D, lane 8) or undetectable (Fig. 8A) in the presence of αARTF, i.e., when a crude extract was used, but it was much stronger in the absence of αARTF, such as after the deletion of the αARTF binding site from footprint II (20) or after the removal of αARTF from the nuclear extract (Fig. 2A and B). This suggests that the binding of αARTF and the binding of CP1 to footprint II are mutually exclusive, although the points of contact differ (see below).

αARTF was further purified from the rat liver by sequential ion-exchange, heparin-Sepharose, and DNA affinity chromatography and identified as 32- and 34-kDa protein(s). Several lines of evidence indicate that the purified 32- and 34-kDa polypeptide species represent the original αARTF protein identified by DNase I footprinting using crude liver nuclear extracts. First, the 32- and 34-kDa polypeptides eluted from the second affinity column constitute more than 90% of the protein in purified samples and they coelute with the peak of DNA binding activity (not shown). Second, the footprint of the purified protein is identical to the original footprint II generated by crude nuclear extract in the region from −490 to −540 of the P2 promoter. Third, the molecular weight of the purified protein is identical to the molecular weight of the DNA-binding proteins detected in crude nuclear extracts by Southwestern analysis. Finally, cross-linking analysis using affinity-purified samples yielded only these two polypeptides.

A weak, 20-kDa band was observed in SDS-PAGE analysis of affinity-purified protein (Fig. 2D) as well as in Southwestern analysis of crude extract (Fig. 1B), suggesting that this polypeptide also binds to footprint II. Although the 20-kDa polypeptide was not recognized by anti-CTF antiserum in Western blotting, we cannot rule out the possibility that it is a breakdown product of NF1, which may not contain the epitopes recognized by the antiserum. However, this 20-kDa polypeptide in the purified preparation is definitely not CP1, since CP1 was separated from NF1 by heparin affinity chromatography (Fig. 2A and 2B) and the molecular mass of CP1 is between 27 and 38 kDa (32).

Although microsequencing of a purified protein is the only way to unambiguously ascertain its identity, we provide several lines of evidence that the purified 32- and 34-kDa polypeptides are NF1 or a closely related factor. First, SDS-PAGE of the purified protein yielded two bands with molecular masses of 32 and 34 kDa. The pattern is very similar to that observed with NF1 identified in hamster (21), chicken (52), and rat (1, 47)

livers. Second, a 200-fold excess of an NF1/CTF consensus oligonucleotide was able to compete with the purified protein for binding to oligonucleotide II in DNase I footprinting assays and DMSA. Third, the purified protein binds to the NF1/CTF consensus sequence. Fourth, methylation interference analysis indicates that the purified protein binds to oligonucleotide II via contacting TTGGCT and TGGCGT sequences, which represent the NF1 binding motif (57). Fifth, the results of supershift assays using an antiserum against the NF1/CTF protein indicate that immunoreactive NF1 is present in the protein-oligonucleotide II complex. Finally, the strongest evidence is that the purified 32- and 34-kDa species are recognized by an antiserum against the NF1/CTF protein in Western blots.

In earlier studies, only a single major 32-kDa polypeptide was purified from the rat liver by DNA affinity chromatography using the human albumin promoter sequence, which contains a TGGCA site (47), or the peripherin negative regulator element, which contains GGCAGGGCGCC (1), and the polypeptide was identified as NF1/L (1, 47). However, when the 3-hydroxy-3-methylglutaryl-coenzyme A reductase promoter sequence (which contains $TGGN_7CCA$) was coupled to the affinity column, two NF1 factors, including NF1/L and NF1/Red1, were purified from the hamster liver (21). Northern analyses have shown that NF1/L and NF1/Red1 are highly enriched, whereas NF1/X is barely detected in the liver (21, 47). In the two studies in which only NF1/L was purified from the rat liver, the affinity probes used represented half of the NF1 binding site (TGGCA) or a mismatch NF1 binding site (GGCA), with low binding affinities. In contrast, using the high-affinity perfect NF1 site ($TGGN_7CCA$) as the probe yielded both NF1/L and NF1/Red1 from the hamster liver (21). The 32- and 34-kDa purified proteins in the present study match the pattern observed with NF1/L and NF1/Red1 in the hamster liver (21), which strongly suggests that these two polypeptides represent NF1/L and NF1/Red1. The footprint II sequence we used for affinity purification contains multiple NF1 half binding sites and has a higher affinity for binding the NF1 protein than does the NF1/CTF consensus sequence (Fig. 5C), which may explain why we have obtained two NF1 polypeptides from the rat liver.

On the basis of more limited information, in an earlier study we proposed that the protein binding to oligonucleotide II may be an as-yet-unidentified factor distinct from NF1 (20). This conclusion was based mostly on the inability of an excess (up to 100-fold) of an NF1 consensus oligonucleotide to compete with the binding of liver nuclear proteins to the labeled oligonucleotide II (20). The present observations provide an explanation for this anomalous finding in that the purified protein displayed higher affinity for binding to oligo II than to the NF1/CTF consensus sequence, which was only effective as a competitor when applied at a 200-fold excess (Fig. 5C). As discussed above, this may be due to the fact that two of the TGGC binding sites in oligonucleotide II are involved in binding NF1, as demonstrated by methylation interference analysis and competition DMSA, which results in increased binding affinity. However, we cannot rule out the possibility that the purified protein is an NF1-related factor which has a higher affinity for oligonucleotide II than for the NF1/CTF1 consensus sequence.

Methylation interference analysis and competition DMSA demonstrated that the purified factor has differential affinities for the different TGGC sites in oligonucleotide II; it binds with higher affinity to the site on the 3′ half of oligonucleotide II (−504 to −507) than to one on the 5′ half (−525 to −528) and does not appear to bind at all to a third TGGC site closer to the 5′ end of oligonucleotide II (−531 to −534) (Fig. 4B). This suggests that the nucleotides flanking the TGGC sequence must influence protein binding affinity and specificity.

NF1 represents a family of sequence-specific DNA-binding proteins which includes heterogeneous species resulting from either alternative splicing events of a single gene (34, 56) or transcription of multiple genes (21, 27, 52). Western blotting using an anti-NF1 antiserum that recognizes more than one member of the NF1 family (1) visualized multiple immunoreactive bands in crude liver nuclear extracts, including 32- and 34-kDa species, whereas only these latter two species were visualized in Western blots of the purified protein. This suggests that the 32- and 34-kDa species represent NF1, whereas the higher-molecular-weight species in the crude extract are likely nonspecific. This is further supported by the findings that in Southwestern blots, oligonucleotide II did not recognize these larger proteins and partial hepatectomy resulted in sharp declines in the amounts of the 32- and 34-kDa bands, whereas the higher-molecular-weight bands remained unaffected (Fig. 8A).

NF1 has been shown to serve as a *trans*-acting factor in adenovirus DNA replication (22, 42, 44) and in eukaryotic class II gene transcription. NF1 has also been reported to act as a silencer for some genes (58), such as the genes encoding retinol-binding protein (12), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (21), AP1 (5), growth hormone (51), mouse α2(I) collagen (5, 50), and peripherin (1), but as a transcriptional activator for other genes, including the α-globin gene (29), human hepatitis B virus S gene (57), and the gene encoding myelin basic protein (27). Our present findings indicate that NF1 is a positive transcriptional regulator of the rat α_{1b}AR gene via binding to the footprint II sequence in its dominant promoter. Mutations of a few key nucleotides representing the contact points for our purified protein resulted in a marked reduction in the basal promoter activity of P2-pCAT constructs, which implies that the purified protein is a constitutive transcriptional activator at this promoter. In accordance with this protein being NF1, cotransfection of various NF1 variants into either primary hepatocytes or Hep3B tumor cells (Fig. 7) markedly increased CAT activity in both the presence and absence of the SV40 enhancer in the construct. Furthermore, transcriptional activation was observed with the wild-type P2 but not with the mutated P2 (Fig. 7A).

More than a dozen NF1 isoforms have been cloned to date (34). They all have nearly identical NH_2-terminal regions containing the DNA binding domains, which implies similar DNA binding properties. The functional role for the divergence of the C-terminal region remains unknown. It has been proposed (21) that the C terminus may be involved in protein-protein interactions, such as the one recently observed to occur on the negative regulator element of the peripherin gene (1). An observation we made during our purification procedure may also suggest the interaction of NF1 with another protein. DMSA with oligonucleotide II and crude liver nuclear extract yielded two shifted bands, of which only the lower one remained after the heparin-Sepharose step (0.55 to 0.60 M KCl fractions) (Fig. 2) (20). This could suggest that crude extracts contain a factor(s) that does not bind to oligonucleotide II directly but can contact NF1 through protein-protein interaction to form the top band. This factor is not CP1, since mixing of the NF1 protein with the CP1-containing fraction didn't reconstitute the top shifted band (data not shown).

NF1/L, NF1/Red1, and NF1/X were all able to activate transcription via P2 to more or less the same degree (Fig. 7). This suggests that the C termini of all three of these NF1 proteins are able to activate gene transcription directly or indirectly.

The expression of these isoforms may be tissue specific, as illustrated by the high levels of NF1/Red1 but low levels of NF1/X in the hamster liver (21), and they may also be differentially regulated by hormonal and other factors. Whether such a mechanism may account for the tissue-specific regulation of the expression of the rat α_{1b}AR gene by hormonal (38) and other factors (35) remains to be determined.

Activation of the α_{1b}AR is one of the first events that initiates regenerative DNA synthesis after partial hepatectomy (13, 14, 43). However, the number of α_{1b}AR is downregulated when hepatocytes dedifferentiate, such as in the fetal liver, after partial hepatectomy or in malignantly transformed hepatocytes (28, 35, 36). We have previously shown that the transcription of the hepatic α_{1b}AR gene is controlled by three promoters that generate three mRNA transcripts, with the 2.7-kb mRNA being the major one (18, 19). Here we show that partial hepatectomy results in a rapid and marked reduction in all three mRNA species, which can be attributed to a corresponding decrease in the rate of transcription of the α_{1b}AR gene (Fig. 8). We also demonstrate for the first time that the tissue level of the NF1 protein and its binding to the P2 promoter are downregulated in the remnant liver. An analogous decrease of NF1 binding to the P2 promoter was also observed in primary hepatocytes after several hours of culture (unpublished observation). Since NF1 enhances P2 promoter activity in Hep3B cells and primary hepatocytes (Fig. 7), the decline in the tissue level of the NF1 protein is very likely one of the factors responsible for the decreased expression of the α_{1b}AR gene during liver regeneration.

Multiple factors, including transforming growth factor β (50), several proto-oncogenes (4, 40), and cell-cell contact (23), are known to regulate the activity of NF1. Whether these or other factors are responsible for the downregulation of the NF1 protein after partial hepatectomy is not known. Since the Ha-ras proto-oncogene is activated after partial hepatectomy (15) and activation of this gene has been shown to destabilize NF1 mRNAs (46), Ha-ras may be one of factors that downregulates NF1 expression in the regenerating liver.

What is the possible biological significance of the suppression of α_{1b}AR expression after partial hepatectomy? Norepinephrine acting at hepatic α_{1b}AR is a strong comitogen for hepatocytes (13). The decline in α_{1b}AR expression in the early stages of the regenerative response may therefore serve to turn off a mitogenic signal and limit the extent of hepatocyte proliferation. A failure to suppress the expression of hepatic α_{1b}AR after partial hepatectomy may promote abnormal liver growth and aberrant differentiation. Indeed, in an experimental model, the overexpression of α_{1b}AR induced agonist-dependent focus formation and disordered growth (3). Thus, the decline in the concentration and transactivator function of the NF1 protein may be part of a tightly regulated cellular program aimed to ensure a brief burst of proliferative activity immediately followed by differentiation.

In summary, we identified NF1 as one of the nuclear proteins that can activate the transcription of the rat α_{1b}AR gene in the liver via the P2 promoter. Partial hepatectomy downregulates the hepatic expression of the NF1 protein, and this may be one of the factors responsible for the inhibition of the expression of the rat α_{1b}AR gene during liver regeneration. Whether an analogous change in the concentration of NF1 occurs under other conditions associated with hepatocyte dedifferentiation, such as fetal state and malignant transformation, remains to be established.

ACKNOWLEDGMENTS

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REFERENCES

1. **Adams, A. D., D. M. Choate, and M. A. Thompson.** 1995. NF1-L is the DNA-binding component of the protein complex at the peripherin negative regulatory element. J. Biol. Chem. **270:**6975–6983.
2. **Aggerbeck, M., N. Ferry, E. S. Zafrani, M. C. Billon, R. Barouki, and J. Hanoune.** 1983. Adrenergic regulation of glycogenolysis in rat liver after cholestasis. Modulation of the balance between α1 and β2 receptors. J. Clin. Invest. **71:**476–486.
3. **Allen, L. F., R. J. Lefkowitz, M. G. Caron, and S. Cotecchia.** 1991. G-protein-coupled receptor genes as proto-oncogenes: constitutively activating mutation of the α_{1B}-adrenergic receptor enhances mitogenesis and tumorigenicity. Proc. Natl. Acad. Sci. USA **88:**11354–11358.
4. **Andres, A. C., M. A. van der Valk, C. A. Schonenberger, F. Fluckiger, M. LeMeur, P. Gerlinger, and B. Groner.** 1988. Ha-ras and c-myc oncogene expression interferes with morphological and functional differentiation of mammary epithelial cells in double and single transgenic mice. Genes Dev. **2:**1486–1495.
5. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. Imbra, H. Rhamsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1988. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49:**729–739.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. More, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Short protocols in molecular biology. John Wiley & Sons, New York.
7. **Barnhart, K. M., C. G. Kim, S. S. Banerji, and M. Sherrery.** 1988. Identification and characterization of multiple erythroid cell proteins that interact with the promoter of the murine α-globin gene. Mol. Cell. Biol. **8:**3215–3226.
8. **Bevilacqua, M., G. Norbiato, E. Chebat, G. Baldi, P. Bertora, E. Regalia, G. Colella, L. Gennari, and T. Vago.** 1991. Changes in α1 and β2 adrenergic receptor density in human hepatocellular carcinoma. Cancer **67:**2543–2551.
9. **Cairo, G., and A. Pietrangelo.** 1994. Transferrin receptor gene expression during rat liver regeneration. J. Biol. Chem. **269:**6405–6409.
10. **Chodosh, L. A., A. S. Baldwin, R. W. Carthew, and P. A. Sharp.** 1988. Human CCAAT-binding proteins have heterologous subunits. Cell **53:**11–24.
11. **Chodosh, L. A., R. W. Carthew, and P. A. Sharp.** 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol. **6:**4723–4733.
12. **Colantuoni, V., A. Pirrozi, C. Blance, and R. Cortese.** 1987. Negative control of liver-specific gene expression: cloned human retinol-binding protein gene is repressed in HeLa cell. EMBO J. **6:**631–636.
13. **Cruise, J. L., K. A. Houck, and G. K. Michalopoulos.** 1985. Induction of DNA synthesis in cultured rat hepatocytes through stimulation of α1 adrenergic receptor by norepinephrine. Science **227:**749–751.
14. **Cruise, J. L., S. J. Knechtle, R. R. Bollinger, C. Khun, and G. K. Michalopoulos.** 1987. α1-Adrenergic effects and liver regeneration. Hepatology **7:**1189–1194.
15. **Fausto, N., and J. E. Mead.** 1989. Regulation of liver growth: proto-oncogenes and transforming growth factors. Lab. Invest. **60:**4–13.
16. **Frain, M., G. Swart, P. Monaci, A. Nicosia, S. Stampfli, R. Frank, and R. Cortese.** 1989. The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell **59:**145–157.
17. **Friedman, J. M., E. Y. Chung, and J. E. Darnell, Jr.** 1984. Gene expression during liver regeneration. J. Mol. Biol. **179:**37–53.
18. **Gao, B., and G. Kunos.** 1993. Isolation and characterization of the gene encoding the rat α_{1B} adrenergic receptor. Gene (Amsterdam) **131:**243–247.
19. **Gao, B., and G. Kunos.** 1994. Transcription of the rat α_{1B} adrenergic receptor gene in liver is controlled by three promoters. J. Biol. Chem. **269:**15762–15767.
20. **Gao, B., M. S. Spector, and G. Kunos.** 1995. The rat α_{1B} adrenergic receptor gene middle promoter contains multiple binding sites for sequence-specific proteins including a novel ubiquitous transcription factor. J. Biol. Chem. **270:**5614–5619.
21. **Gil, G., J. R. Smith, J. L. Goldstein, C. A. Slaughter, K. Orth, M. S. Brown, and T. F. Osborne.** 1988. Multiple genes encode nuclear factor 1-like proteins that bind to the promoter for 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Proc. Natl. Acad. Sci. USA **85:**8963–8967.
22. **Gounari, F., R. de Francesco, J. Schmidt, P. Van der Vliet, C. Cortese, and H. G. Stunnenberg.** 1990. Amino-terminal domain of NF1 binds to DNA as a dimer and activates adenovirus DNA replication. EMBO J. **9:**559–566.
23. **Hartig, E., B. Nierlich, M. Sigrun, G. Nebl, and A. C. B. Cato.** 1993. Regulation of expression of mouse mammary tumor virus through sequences located in the hormone response element: involvement of cell-cell contact and a negative regulatory factor. J. Virol. **67:**813–821.
24. **Higgins, G. M., and R. M. Anderson.** 1931. Experimental pathology of liver

restoration of liver of the white rat following partial surgical removal. Arch. Pathol. **12:**186–202.
25. **Hu, Z. W., and B. B. Hoffman.** 1993. Cycloheximide induces the α_{1B} adrenergic receptor gene by activation of transcription in DDT_1 MF-2 smooth muscle cells. Mol. Pharmacol. **44:**1105–1112.
26. **Hu, Z. W., X. Y. Shi, M. Sakaue, and B. B. Hoffman.** 1993. Prolonged activation of protein kinase C induces transcription and expression of the α_{1B} adrenergic receptor gene in DDT_1 MF-2 cells. J. Biol. Chem. **268:**3610–3615.
27. **Inoue, T., T. A. Tamura, T. Furuichi, and K. Mikoshiba.** 1990. Isolation of complementary DNAs encoding a cerebellum-enriched NF1 family that activates transcription from the mouse myelin basic protein promoter. J. Biol. Chem. **265:**19065–19070.
28. **Ishac, E. J. N., E. Lazar-Wesley, and G. Kunos.** 1992. Rapid inverse changes in α_{1B}- and β_2-adrenergic receptors and gene transcripts in acutely isolated rat liver cells. J. Cell. Physiol. **152:**79–86.
29. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eucaryotic transcription and DNA replication. Cell **48:**79–89.
30. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA-binding proteins. Proc. Natl. Acad. Sci. USA **83:**5889–5893.
31. **Kanasaki, M., H. Matsubara, S. Murasawa, H. Masaki, Y. Nio, and M. Inada.** 1994. cAMP responsible element-mediated regulation of the gene transcription of the α1B adrenergic receptor by thyrotropin. J. Clin. Invest. **94:**2245–2254.
32. **Kim, C. G., S. L. Swendeman, K. M. Barnhart, and M. Sheffery.** 1990. Promoter elements and erythroid cell nuclear factors that regulate α-globin gene transcription in vitro. Mol. Cell. Biol. **10:**5958–5966.
33. **Kost, D. P., M. C. DeFrances, C. R. Lee, and G. K. Michalopoulos.** 1992. Patterns of α1 adrenergic receptor expression in regenerating and neoplastic hepatic tissue. Pathobiology **60:**303–308.
34. **Kruse, U., and A. E. Sippel.** 1994. The genes for transcription factor NF1 give rise to corresponding splice variants between vertebrate species. J. Mol. Biol. **238:**860–865.
35. **Kunos, G., and E. J. N. Ishac.** 1987. Mechanism of inverse regulation of alpha1- and beta-adrenergic receptors. Biochem. Pharmacol. **36:**1185–1191.
36. **Kunos, G., E. J. N. Ishac, B. Gao, and L. Jiang.** 1995. Inverse regulation of hepatic α_{1B}- and β_2-adrenergic receptors: cellular mechanisms and physiological implications. Ann. N. Y. Acad. Sci. **757:**261–270.
37. **Latchman, D. S.** 1993. Transcription factors. IRL Press, Oxford.
38. **Lazar-Wesley, E., J. R. Hadcock, C. C. Malbon, G. Kunos, and E. J. N. Ishac.** 1991. Tissue-specific regulation of α_{1B}, β_1 and β_2-adrenergic receptor mRNAs by thyroid state in the rat. Endocrinology **129:**1116–1118.
39. **Liu, Y. H., A. B. Beedle, L. Lin, A. W. Bell, and R. Zarnegar.** 1994. Identification of a cell-type-specific transcription repressor in the promoter region of the mouse hepatocyte growth factor gene. Mol. Cell. Biol. **14:**7046–7058.
40. **Lubon, H., and L. Henninghausen.** 1988. Conserved region of the rat lactalbumin promoter is a target site for protein binding in vitro. Biochem J. **256:**391–396.
41. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
42. **Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NF1 is distinct from the replication and DNA binding domain. Cell **58:**741–753.
43. **Michalopoulos, G. K.** 1990. Liver regeneration: molecular mechanisms of growth control. FASEB J. **4:**176–187.
44. **Nagata, K., R. A. Guggenheimer, and J. Hurwitz.** 1983. Specific binding of a cellular DNA replication protein to the origin of the replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA **80:**6177–6181.
45. **Nakamura, H., K. Hirate, K. Yamshiro, K. Hiranuma, K. Shibata, K. Higashi, T. Morita, and H. Hirano.** 1994. Increase of hepatic mRNAs of prolifin, actin and extracellular matrix proteins after carbon tetrachloride treatment and partial hepatectomy in rats. Biochem. Biophys. Res. Commun. **198:**568–573.
46. **Nebl, G., N. Mermod, and A. C. B. Cato.** 1994. Post-transcriptional downregulation of expression of transcription factor NF1 by Ha-ras oncogene. J. Biol. Chem. **269:**7371–7378.
47. **Paonessa, G., F. Gounari, R. Frank, and R. Cortese.** 1988. Purification of a NF1-like DNA-binding protein from rat liver and cloning of the corresponding cDNA. EMBO J. **7:**3115–3123.
48. **Preiksaitis, H. G., W. H. Kan, and G. Kunos.** 1982. Decreased α1-adrenoceptor responsiveness and density in liver cells of thyroidectomized rats. J. Biol. Chem. **257:**4321–4327.
49. **Rossby, S. P., and L. E. Cornett.** 1991. Steady state levels of hepatic α1 and β2 adrenergic receptors and gene transcripts during development of the male rat. J. Cell. Physiol. **147:**55–61.
50. **Rossi, P., G. Karsenty, A. B. Roberts, N. S. Roche, M. Sporn, and B. de Crombrugghe.** 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-β. Cell **52:**405–414.
51. **Roy, R. J., and S. L. Guerin.** 1994. The 30-kDa rat liver transcription factor NF1 binds the rat growth-hormone proximal silencer. Eur. J. Biochem. **219:**799–806.
52. **Rupp, R. A. W., and A. E. Sippel.** 1987. Chicken liver TGGCA protein purified by preparative mobility shift electrophoresis shows a 36.8 to 29.8 kDa microheterogeneity. Nucleic Acids Res. **15:**9707–9726.
53. **Sakaue, M., and B. B. Hoffman.** 1991. Glucocorticoids induce transcription and expression of the α_{1B} adrenergic receptor gene in DDT_1 MF-2 smooth muscle cells. J. Clin. Invest. **88:**384–389.
54. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Sandnes, D., T. E. Sand, G. Sager, G. O. Brnstad, M. R. Refsnes, I. P. Gladhaug, S. Jacobsen, and T. Christoffersen.** 1986. Elevated level of β-adrenergic receptors in hepatocytes from regenerating rat liver. Time study of [^{125}I]iodocyanopindolol binding following partial hepatectomy and its relationship to catecholamine-sensitive adenylate cyclase. Exp. Cell Res. **165:**117–126.
56. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334:**218–224.
57. **Shaul, Y., R. Ben-Levy, and T. De-Medina.** 1986. High affinity binding site for nuclear factor I next to the hepatitis B virus S gene promoter. EMBO J. **5:**1967–1971.
58. **Spandau, D. F., and C. H. Lee.** 1992. Repression of the hepatitis B virus enhancer by a cellular factor. J. Gen. Virol. **73:**131–137.
59. **Szentendrei, T., E. Lazar-Wesley, T. Nakane, M. Virmani, and G. Kunos.** 1992. Selective regulation of β_2-adrenergic receptor gene expression by interleukin-1 in cultured human lung tumor cells. J. Cell. Physiol. **152:**478–485.
60. **Wellstein, A., A. F. Dobrenski, M. N. Radonovich, J. F. Brady, and A. T. Riegel.** 1991. Purification of PO-B, a protein that has increased affinity for the pro-opiomelanocortin gene promoter after dephosphorylation. J. Biol. Chem. **266:**12234–12241.