In the rat liver, NF1 and CP1 bind to the major P2 promoter of the α1B adrenergic receptor gene to generate footprint II. Here we show that, in DDT1MF-2 smooth muscle cells, the major protein bound to footprint II is not NF1 but Sp1, which binds to the 5'-portion of the footprint II sequence (footprint IIb). Mutational analyses demonstrate that the CCCGCG sequence in footprint IIb is critical for Sp1 binding and P2 promoter activity. A second GC box in the P2 promoter also binds the Sp1 protein and contributes to the P2 promoter activity. Gel shift assays indicate that footprint II can bind Sp1, NF1, and CP1, and that the binding of these 3 proteins is mutually exclusive. This is also indicated by the results of functional cotransfection experiments, where transient overexpression of NF1 and Sp1 together caused a similar increase in the activity of a P2/CAT reporter construct as overexpression of either Sp1 or NF1 alone, indicating lack of additivity. The preferential interaction of footprint II with Sp1 in DDT1MF-2 cells and NF1 in liver appears to be due to low levels of NF1 expression in DDT1MF-2 cells and low levels of Sp1 in liver. These observations suggest that NF1 and Sp1 are the major transcription factors involved in controlling the P2 promoter in liver versus DDT1MF-2 cells, respectively, which may be one of the mechanisms responsible for the complex tissue-specific regulation of the expression of the α1B adrenergic receptor gene.

Expression of the α1B adrenergic receptor (α1BAR)1 gene is regulated by hormonal and developmental factors in a tissue-specific manner (1), best illustrated by differential regulation of the α1BAR gene in rat heart and liver under a variety of conditions. For example, hypothyroidism increases the level of α1BAR mRNA in the heart, but decreases it in the liver (2). In primary cultures of rat hepatocytes, high cell density prevents the decline in α1BAR expression observed at low cell densities (3), whereas in primary cultures of myocardial cells increasing cell density decreases α1BAR expression (4). The molecular mechanisms underlying such differential regulation remain unknown. As a first step toward understanding the molecular basis of tissue-specific transcriptional regulation, we have cloned and characterized the rat α1BAR gene (5–8). The gene is composed of two exons and a single large intron of at least 16 kb in length. Primer extension and reverse transcriptase-PCR studies using poly(A) RNAs from rat liver identified three transcription start points (tsp), located between 54 and 57 bp (tsp1), at ~443 bp (tsp2), and a cluster between ~1035 and ~1340 bp (tsp3) upstream from the translation start codon. Northern blot analyses have documented three α1BAR mRNA species of 3.3, 2.7, and 2.3 kb in length. The 3.3-kb species is preferentially expressed in rat liver and DDT1MF-2 cells (9–11), whereas the 2.7-kb species is dominant and widely expressed in many tissues (9, 10). The low abundance, 2.3-kb species is difficult to detect and has only been reported in rat liver (6), DDT1MF-2 cells (11), and rat medullary thyroid carcinoma 623 cells (12). The 3.3-, 2.7-, and 2.3-kb α1BAR mRNAs in rat liver and DDT1MF-2 cells are probably transcribed from tsp3, tsp2, and tsp1, which are directed by three distinct promoters: the distal promoter (P3), middle promoter (P2), and proximal promoter (P1), respectively (6).

DNase I footprinting using rat liver nuclear extracts identified three protected regions in the dominant P2 promoter: footprint I (~432 to ~452), footprint II (~490 to ~540), and footprint III (~609 to ~690). Footprint I contains a cAMP response element and a GC box, and the former was shown to bind CREB and to mediate basal as well as cAMP-induced P2 promoter activity.2 Footprint II interacts with NF1 and CP1 (8), and footprint III contains multiple sequence-specific transcription binding sites including an AP2 site, which binds purified AP2 protein and is also involved in mediating basal and cAMP-induced P2 promoter activity.2 Since the DDT1MF-2 hamster smooth muscle cell line expresses a similar pattern of three α1BAR mRNAs as rat liver (11) and a high level of P2 promoter activity, we wondered whether NF1 has a similar role in transcriptional control in these cells. Unexpectedly, we found that the major protein bound to footprint II in DDT1MF-2 cells was not NF1 but the Sp1 protein. Mutation of the GC region in footprint II abolished the formation of the Sp1 complex and resulted in a significant decrease of P2 promoter activity. We further demonstrated that a GC box in footprint I also bound Sp1 protein and contributed to basal P2 promoter activity. Finally, DNA mobility shift assay (DMSA) using ·32P-labeled consensus Sp1 or NF1 oligodeoxynucleotides (oligos) showed that rat liver contained high levels of NF1 but low levels of Sp1 binding, whereas the reverse was true in the case of DDT1MF-2 cells.

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‡ The abbreviations used are: AR, adrenergic receptor; oligo, oligodeoxynucleotide; CRE, cAMP response element; DMSA, DNA mobility shift assay; CAT, chloramphenicol acetyltransferase; CREB, CRE-binding protein; tsp, transcription start point; PCR, polymerase chain reaction; NF1, nuclear factor 1; CP1, CCAAT-binding protein 1; bp, base pair(s); kb, kilobase pair(s).

1 The abbreviations used are: AR, adrenergic receptor; oligo, oligodeoxynucleotide; CRE, cAMP response element; DMSA, DNA mobility shift assay; CAT, chloramphenicol acetyltransferase; CREB, CRE-binding protein; tsp, transcription start point; PCR, polymerase chain reaction; NF1, nuclear factor 1; CP1, CCAAT-binding protein 1; bp, base pair(s); kb, kilobase pair(s).

2 J. Chen, M. S. Spector, G. Kunos, and B. Gao, unpublished observation.
MATERIALS AND METHODS

Cell Culture—The DDT1MF-2 hamster smooth muscle cell line and the Hep3B human hepatocellular carcinoma cell line were obtained from the American Type Culture Collection (Rockville, MD) and cultured under conditions specified by the supplier.

Expression Vectors—pN1/L expression vector, which contains the N1/L coding region, was described previously (8). The P2Cp5-in expression vector (13), which contains the Sp1 coding region, was a generous gift from Dr. Robert Tjian (University of California, Berkeley).

Oligo Synthesis—The synthetic oligos were prepared on a Cyclone Plus DNA synthesizer (Milligen). After ammonium hydroxide deprotection, oligos were evaporated to dryness by vacuum centrifugation (Savant Speed-Vac) and purified by electrophoresis on a 15–19% polyacrylamide, 8 M urea gel (14).

Preparation of Nuclear Extracts—Nuclear extract from DDT1MF-2 cells was prepared as described by Dignam et al. (15). The preparation of nuclear extract from whole rat liver was described previously (7).

DMSA and DNA Mobility Supershift Assay—DMSA and nuclear extracts for DMSA were carried out as described previously (7). The oligos II, IIa, Ib, IImut1, IImut2, and IImut3 are described in the legend of Fig. 1. Oligo IIIm was described previously (8). The following oligos were double-stranded oligos were used in DMSA: oligo I 5′-GGTTCC-CGCCGCTGATGTG-3′ (432 to 456); oligo II 5′-GGCTTGATGAGTCAGCCGGAA-3′ (458 to 484); oligo III 5′-GGCTTGCTGACGTCTGCCAGC-3′ (483 to 516); and oligo IIb 5′-GGCTTGACGTCTGCCAGC-3′ (483 to 516). The double-stranded oligos were used in DMSA: oligo IIm 5′-GGCTTGATGAGTCAGCCGGAA-3′; AP1, 5′-GGCTTGATGAGTCAGCCGGAA-3′; CRE, 5′-GGAGTGCCGCTGGCTCAG-3′; Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′; NF1, 5′-TATTTTTGATGGAGCAAAATGATAGTA-3′. The purified Sp1 protein was purchased from Promega. The antibodies against Sp1 and NF1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Purified NF1 was obtained from rat liver by using sequential DEAE-Sepharose, heparin-Sepharose, and DNA affinity chromatography as described previously (8). Partially purified Sp1 was obtained from rat liver by using DEAE-Sepharose and heparin-Sepharose chromatography as described previously (8).

Construction of Plasmids—The P2/CAT, P2mut1/CAT, P2mut2/CAT, and P2mut3/CAT constructs were prepared by subcloning P2, P2mut1, P2mut2, and P2mut3 promoter regions into pCAT basic reporter vectors (Promega), respectively. The P2 promoter region was amplified by PCR using the rat α1B AR gene 5′-flanking region as a template, primer 1 (5′-TGCAGCTGCAAGACATCATCA-3′) containing a PstI site and primer 2 (5′-GATCGTACTCAGTCTGTCCAGTCG-3′) containing a HindIII site as 3′- and 5′-primers, respectively. The P2mut1 region was amplified by stepwise PCR (8). Briefly, partially overlapping Ib mut1 sense (543 to 516; 5′-GCAGCTAATGGTGCTGGCTGGCGGC-3′) and antisense (526 to 550; 5′-CCAGCGCCCATCTAGCTGGCGTGGA-3′) oligo 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 1 plus antisense primer 2. The final PCR product was purified and subcloned into the pCAT basic vector. The mutations were verified by sequencing. The P2mut2 and P2mut3 regions were amplified by sequential PCR using the above approach.

Transient Transfections and CAT Assays—Transfections were performed by using the Lipofectin reagent, as recommended by the manufacturer (Life Technologies, Inc.) and described previously (6).

RESULTS

The Protein Bound to Footprint II in DDT1MF-2 Cells Is Sp1—Earlier experiments showed that NF1 is the major protein bound to the footprint II sequence in liver, and DDT1MF-2 cells are known for expressing high levels of α1B AR protein (11), α1B AR mRNA (11), and P2 promoter activity (6). To determine whether NF1 is also the major protein bound to footprint II in DDT1MF-2 cells, DMSA was performed using DDT1MF-2 cell nuclear extracts and 32P-labeled oligo II in the presence of various competitor oligos. As shown in Fig. 1B, 32P-labeled oligo II bound a major complex specifically (lane 2), as it was competed away by unlabeled oligo II (lane 3). However, this complex migrated much slower than NF1 and CP1 complexes formed in liver nuclear extracts (Fig. 1C), and it was not abolished by an NF1 consensus oligo (lanes 7 and 8 in Fig. 1B), suggesting that it did not contain NF1. Additionally, DMSA showed that this complex was competed away by an Sp1 consensus oligo (lane 11 in Fig. 1B) but not by consensus oligos for AP1, AP2, or C/EBP (Fig. 1B), suggesting that the major protein bound to oligo II in DDT1MF-2 cells was Sp1 or a related protein.

To further confirm that the protein bound to oligo II in DDT1MF-2 cells is Sp1, gel supershift assays were performed. The DDT1MF-2 cell nuclear extracts were incubated with anti-Sp1 or anti-NF1 antibody, and were then subjected to DMSA using 32P-labeled oligo II. As shown in Fig. 1D, the major complex formed by DDT1MF-2 cell nuclear extract and oligo II was supershifted by an Sp1 antibody but not by an NF1 antibody, indicating that oligo II binds the Sp1 protein in DDT1MF-2 cells. To verify that oligo II can bind the Sp1 protein, DMSA was carried out with purified Sp1 protein plus 32P-labeled oligo II. As shown in Fig. 1E, the pattern of binding of oligo II to purified Sp1 protein was similar to the pattern seen with crude DDT1MF-2 nuclear extracts. In competition DMSA (Fig. 1E), full inhibition of the Sp1 complex required a 50-fold excess of unlabeled oligo II or IIb but only a 10-fold...
excess of an Sp1 consensus oligo, which suggests that the Sp1 protein binds to oligo II with lower affinity than to the Sp1 consensus oligo.

**Distinct Patterns of Binding of Sp1, CP1, and NF1 Proteins to Footprint II—NF1 was shown to bind independently to both the 5′ (oligo Ib) and 3′ (oligo Ia) portions of footprint II (8). As shown in Fig. 1B, the Sp1 complex formed by DDT,MF-2 cell extract with oligo II was abolished by oligo Ib but not by oligo Ia, suggesting that Sp1 bound to the 5′-portion of oligo II only. To confirm this, DMSA was performed either with DDT,MF-2 cell nuclear extracts or purified Sp1 protein plus 32P-labeled oligo Ia or Ib. There was no indication of complex formation between oligo Ia and either DDT,MF-2 cell nuclear extract or purified Sp1 protein (lanes 1 and 2 in Fig. 2D). In contrast, a specific complex between oligo Ib and DDT,MF-2 cell nuclear extract was evident in DMSA, and the shifted band was self-competed by the unlabeled oligo Ib (Fig. 2B, lane 3). This complex was also competed away by an Sp1 consensus oligo (lane 9 in Fig. 2B) and oligo II (lane 9 in Fig. 2B), but not by oligo Ia, or consensus oligos for NF1, AP1, AP2 and C/EBP (Fig. 2B). Oligo Ia was able to bind purified NF1 and CP1 (lanes 3 and 4 in Fig. 2D; Ref. 7), and oligo Ib was able to bind purified Sp1 protein and NF1 but not CP1 (Fig. 2E). These results suggest that the Sp1 and CP1 proteins bind to the 5′- and 3′-portions (oligos Ib and Ia) of oligo II, respectively, whereas the NF1 protein binds to both oligos Ia and Ib.

**Mutations of the GC Box in Oligo Ib Inhibit Sp1 Binding and P2 Promoter Activity—**Footprint Ib is GC-rich (85%) and contains an inverted one-mismatch GC box (CCCCGG). To define the domain responsible for Sp1 binding, we introduced substitution mutations into footprint Ib and used them as unlabeled competitors in DMSA. As shown in Fig. 2C, the Ib mut1, which contains a mutated GC box, did not abolish Sp1 binding (lane 2), whereas the oligos Ib mut2 and Ib mut3, which contain mutations in other GC-rich regions, did (lanes 3 and 4). This suggests that the GC box, but not other GC-rich domains in oligo Ib, is critical for Sp1 binding.

Deletions in the domain corresponding to oligo Ib were shown previously to abolish P2 promoter activity in DDT,MF-2 cells (7). To define the role of Sp1 in P2 promoter activity, several mutated P2/CAT constructs were prepared by introducing the mutated oligo Ib mut1, Ib mut2, and Ib mut3 as described under “Materials and Methods.” As shown in Fig. 2D, mutations in the GC box, but not in other GC-rich domains within oligo Ib, significantly reduced P2 promoter activity in DDT,MF-2 cells. Therefore, Sp1 is a positive regulator of the P2 promoter of the α1B AR gene in DDT,MF-2 cells, and this was further verified by cotransfection of the P2/CAT construct with an Sp1 expression vector. In DDT,MF-2 cells, such cotransfections resulted in weak stimulation of P2 promoter activity (1.7 ± 0.3-fold), which may have been due to the high abundance of endogenous Sp1 binding in this cell line (Figs. 1–3). Therefore, we next cotransfected these vectors into Hep3B cells, which contain low endogenous Sp1 binding (16). As shown in Fig. 2G, overexpression of Sp1 in Hep3B cells resulted in a 7.5 ± 0.7-fold stimulation of P2/CAT activity. To test the role in this stimulation of the CCCCCG sequence in oligo II, a mutated P2/CAT construct (P2mut1/CAT) was cotransfected with the Sp1 expression vector into Hep3B cells. As shown in Fig. 2G, P2mut1 activity is about 40% of wild type P2/CAT activity and Sp1 only stimulated the mutated P2mut1/CAT activity by 2.5 ± 0.6-fold, which is significantly lower than the 7.5 ± 0.7-fold stimulation of wild type P2/CAT. The residual stimulation of P2mut1/CAT by Sp1 is probably due to the presence of a second functional Sp1 binding site in the P2 promoter region (see above).

**Fig. 2. Sp1 binds to oligo Ib and mutations of the GC box in oligo Ib abolish Sp1 binding and inhibit P2 promoter activity.** A, the sequence of the wild type and mutated footprint Ib (~516 to ~543 bp) of the P2 promoter of the rat α1B AR gene. B and C, DMSA using 32P-labeled oligo Ia plus DDT,MF-2 cell extracts. One ng of labeled oligo Ia was incubated with 10 µg of DDT,MF-2 cell extract in the absence (first lane) or presence of a 100-fold excess of the indicated competitor oligos (second through tenth lanes in B and second through fourth lanes in C). D, DMSA using 32P-labeled oligo Ia plus DDT,MF-2 cell extracts, purified Sp1, CP1, or NF1. E, DMSA using 32P-labeled oligo Ib plus DDT,MF-2 cell extract in the absence (first lane) or presence of excess of the indicated competitor oligos (second through tenth lanes). F and G, mutations of the GC box within oligo Ib inhibit P2 promoter activity. The mutations in panel A were introduced into the P2 promoter construct to produce P2 mut1, P2 mut2, and P2 mut3 constructs as described under “Materials and Methods.” The mutated constructs were then transiently transfected into DDT,MF-2 cells, and CAT activities were measured and expressed as percent of the control P2 activity. Means ± S.E. from four experiments are shown. * Significant difference (p < 0.01) compared with the activity of the wild type P2 promoter. G, P2/CAT construct or P2mut1/CAT construct was cotransfected with Sp1 expression vector into Hep3B cells and CAT activities were measured and expressed as percent of the control P2 activity. Means ± S.E. from three experiments are shown. ** Significant difference (p < 0.05) compared with the activity of P2mut/CAT. & Significant difference (p < 0.05) compared with the activity of the P2mut1/CAT.
gests that NF1 and Sp1 contact different sites on oligo II.

Liver extract formed two complexes (lanes 2

control reactions were performed with DDT 1MF-2 or liver ex-
mixed and analyzed in DMSA using 32P-labeled oligo II. The

lane

the P2 promoter activity

Exclusive—

1

binding (8), was used as competitor in DMSA. As shown in Fig.

3

exclusive, three experiments were performed. First, mutated

binding of NF1 and CP1, and SP1, and the binding of NF1 and CP1 to

bind NF1, CP1, and SP1, and the binding of NF1 and CP1 to

A, DMSA using 32P-labeled oligo II with 10 μg of DDT, MF-2

cell extract alone (first lane), 10 μg of liver nuclear extract alone (fourth

lane) or both together (second and third lanes). B, DMSA using 32P-
labeled oligo II with 5 ng of purified Sp1 (pSp1) alone (first lane), 20 ng of

purified NF1 (pNF1) alone (second lane) or NF1 plus Sp1 (third lane).

C, DMSA using 32P-labeled oligo II with 2 μg of partially purified CP1

(pCP1) alone (first lane), or partially purified CP1 plus 1 ng (second

lane) or 5 ng of purified Sp1 (third lane), or 5 ng of purified Sp1 alone

(fourth lane). D, Sp1 and NF1 have no additive or synergistic effects on

the P2 promoter activity in vitro. Two μg of Sp1 and/or NF1/L were
cotransfected with 1 μg of P2mut/CAT construct into Hep3B cells. After

60 h, cells were harvested and used to measure CAT activity. CAT
activity is expressed as percent of control, which is the activity of the
P2mut/CAT construct alone. Data are the means ± standard errors
from three independent experiments. * significant difference (p < 0.01)
compared with the P2mut/CAT activity.

The Binding of Sp1, NF1, and CP1 to Oligo II Is Mutually Exclusive—Our findings so far have indicated that oligo II can
bind NF1, CP1, and Sp1, and the binding of NF1 and CP1 to footprint II is mutually exclusive (7, 8). To define whether the
binding of NF1 and Sp1 to footprint II was also mutually exclusive, three experiments were performed. First, mutated
oligo Ilm, which was shown previously not to abolish NF1 binding (8), was used as competitor in DMSA. As shown in Fig.

1B, oligo Ilm could eliminate Sp1 binding (lane 4), which suggests that NF1 and Sp1 contact different sites on oligo II.

Second, DDT, MF-2 cell extract and liver nuclear extract were mixed and analyzed in DMSA using 32P-labeled oligo II. The
control reactions were performed with DDT, MF-2 or liver extract alone. As shown in Fig. 3A, the mixture of DDT, MF-2 and
liver extract formed two complexes (lanes 2 and 3); the top

complex contained Sp1, as it had a similar pattern as the complex formed with DDT, MF-2 cell extract alone and it was
abolished by Sp1 oligo but not by NF1 oligo, whereas the bottom complex contained NF1, as it was inhibited by NF1 oligo (data not shown). The CP1 complex present in liver was absent when the mixed extract was used, suggesting that the binding of CP1 to oligo II was interfered with by Sp1 and NF1. Third, purified NF1 and Sp1 proteins were mixed and analyzed in DMSA using 32P-labeled oligo II. As shown in Fig. 3B, the mixture of Sp1 and NF1 formed two complexes: the top complex whose mobility was the same as that of the Sp1 complex alone was abolished by Sp1 oligo, and the bottom complex whose mobility was the same as that of the NF1 complex alone and was inhibited by NF1 oligo. There was no evidence for any band that could be competed away by both the NF1 and Sp1 oligos, indicating that no ternary complex containing NF1 + Sp1 + oligo II was formed. These data suggest that NF1 and Sp1 cannot bind simultaneously to oligo II; thus, their binding is mutually exclusive.

To test whether the binding of Sp1 and CP1 to oligo II is similarly mutually exclusive, DMSA was performed using 32P-
labeled oligo II with purified Sp1 protein and/or partially puri-

fied CP1 protein. As shown in Fig. 3C, the mixture of Sp1 and

CP1 proteins formed two complexes with oligo II. The top

complex contained Sp1 and the bottom one CP1, as deduced

from the position of the complexes formed by Sp1 alone (lane 4)
or CP1 alone (lane 1). Again, there was no band inhibited by

both Sp1 and CP1 oligos, which suggests that Sp1 and CP1
cannot bind simultaneously to oligo II and their binding is
mutually exclusive.

We next examined the effects of NF1 and Sp1 on the activity of the P2 promoter in vivo. To eliminate interference by the
additional proximal Sp1 binding site present in the P2 pro-
moter (see below), we mutated this sequence to generate the
P2mut/CAT construct (see “Materials and Methods”), and co-
transfected these vectors into Hep3B cells. As shown in Fig. 3D,
overexpression of Sp1 or NF1 alone stimulated P2mut/CAT activity 5.1 ± 0.8-fold and 4.6 ± 0.7-fold, respectively. Cotransf-
ction of NF1 and Sp1 together stimulated P2mut/CAT activity to the same degree (4.8 ± 0.9-fold). This suggests that the
effects of Sp1 and NF1 on P2 promoter activity are not additive, which is consistent with their mutually exclusively binding
to oligo II.

Oligo II Binds NF1 in Rat Liver but Sp1 in DDT, MF-2

Cells—The above data clearly indicate that in the normal adult
rat liver oligo II binds NF1, but in DDT, MF-2 cells it binds the
Sp1 protein. A possible reason for this difference is low levels of
Sp1 binding activity in normal adult rat liver and low NF1
binding in DDT, MF-2 cells. To examine this possibility, DMSA
was carried out using 32P-labeled consensus Sp1 or NF1 oligos
plus adult liver nuclear extracts or DDT, MF-2 cell extracts. As
shown in Fig. 4A, equal amounts of a 32P-labeled consensus
Sp1 oligo formed a strong complex with DDT, MF-2 cell extracts
but a weak complex with adult rat liver extract. In contrast,
32P-labeled consensus NF1 oligo formed a strong complex with
adult liver nuclear extracts but a weak complex with DDT,
MF-2 cell extract.

Mutation of the GC Box at −445 to −451 Inhibits Sp1 Bind-
ing and P2 Promoter Activity in DDT, MF-2 Cells—The P2
promoter region contains a second GC box located at −445 to
−451. We wondered whether this GC box also bound Sp1
protein and influenced P2 promoter activity. Oligo IGC (Fig. 5A)
containing this GC box was synthesized and used in DMSA
with liver nuclear extract or DDT, MF-2 cell extract. Oligo IGC
formed a very weak band with liver extract (data not shown)
but a very strong complex with DDT, MF-2 cell extract (Fig. 5B,
within the P2 promoter binds Sp1 in DDT 1MF-2 cells. The
To further define the identity of this protein, a gel supershift
in Fig. 5D, lane 2). This complex was abolished by oligo IIb or by an Sp1 oligo, but not by mutated oligo IGCm. These results further demonstrate that the GC box in oligo IGC binds Sp1 protein.

The role of the GC box located at −455 to −451 in mediating P2 promoter activity was verified by mutational and transfection analyses. The P2Imut construct was prepared by introducing mutations of this GC box, incorporating it into the P2 promoter construct as described under “Materials and Methods,” and then transfecting the mutated construct into DDT1MF-2 cells. As shown in Fig. 5E, the activity of the P2Imut construct was approximately 35% of the wild type P2 promoter activity. These results indicate that the GC box located at −445 to −451 plays a positive role in the P2 promoter activity.

**DISCUSSION**

Our previous findings indicated that the 5′-upstream region from −490 to −540 bp (footprint II) within the P2 promoter of the rat α1B AR gene interacts with NF1 and CP1 in liver nuclear extracts, and that NF1 is a positive transcriptional regulator of the rat α1B AR gene in the liver (7, 8). The present findings demonstrate that in a different tissue, i.e. the DDT, MF-2 smooth muscle cell line, the same footprint region binds a different transcription factor, the Sp1 protein, which, much like NF1 in the liver, is required for basal transcription of the α1B AR gene in DDT, MF-2 smooth muscle cells. We also find that the affinity of Sp1 is lower for footprint II than for the consensus Sp1 sequence, and that Sp1 binds only to the 5′-portion (oligo IIb) of footprint II. In fact, the contact points for NF1 and Sp1 on oligo II are different. Nevertheless, the binding of NF1 and Sp1 to oligo II in vitro is mutually exclusive as indicated by the results of DMSA, which is consistent with the lack of additivity in the activation of the P2 promoter by these two factors in vivo. Our results further indicate that the reason for this striking tissue specificity in transcriptional control is due to the cell-specific expression or binding activity of the two transcription factors; Sp1 binding is low in the adult rat liver but high in DDT, MF-2 cells, whereas the opposite holds for NF1. Finally, a second GC box located at −445 to −451 bp was also found to bind Sp1 and to contribute to the activity of the P2 promoter.

Although the role of NF1 and CP1 in generating footprint II within the P2 promoter of the rat α1B AR gene could be clearly demonstrated in the adult rat liver (7, 8), several lines of evidence presented here suggest that in DDT, MF-2 cells the major protein bound to oligo II is Sp1. First, the gel mobility of the complex formed by oligo II in DDT, MF-2 cell extract was slower than in liver extract (Fig. 1, B and C). Second, the complex formed in DDT, MF-2 cell extract is abolished by an Sp1 consensus oligo but not by an NF1 consensus oligo even

![Image](99x561 to 257x729)

**FIG. 4. Low Sp1 binding in liver nuclear extracts and low NF1 binding in DDT, MF-2 cell extracts.** Figure shows DMSA using 10 μg of DDT, MF-2 cell extract and 10 μg of liver nuclear extract with 32P-labeled consensus oligo Sp1 (A) or NF1 (B).

![Image](352x402 to 520x729)

**FIG. 5. The GC box located at −445 to −451 bp binds Sp1 and contributes to P2 promoter activity.** A, DNA sequences of oligo IGC and mutated oligo IGCmut between −439 to −456 bp in the P2 promoter of the rat α1B AR gene. B, DMSA using 32P-labeled oligo IGC plus DDT, MF-2 cell extract. One ng of labeled oligo IGC was incubated with 10 μg of DDT, MF-2 cell extract in the absence (first lane) or presence of a 100-fold excess of the indicated competitor oligos (second through fifth lanes). C, supershift analysis of the specific protein binding to oligo IGC using anti-Sp1 or anti-NF1 antibodies. DMSA were performed with 10 μg of DDT, MF-2 cell extract plus 32P-labeled oligo IGC. The nuclear extract was preincubated with anti-Sp1 or anti-NF1 antibodies for 30 min at 25 °C, no preincubation. D, DMSA using 32P-labeled oligo IGC plus purified Sp1 protein. One ng of labeled oligo IGC was incubated with 10 ng of purified Sp1 protein in the absence (first lane) or presence of the indicated competitor oligos (second through fifth lanes). E, mutations of the GC box within oligo IGC inhibit P2 promoter activity. The mutations in oligo IGC in panel A were introduced into the P2 promoter construct to produce P2Imut construct as described under “Materials and Methods.” The mutated constructs were then transiently transfected into DDT, MF-2 cells, and CAT activities were measured and expressed as percent of the wild type P2 activity. Means ± S.E. from four experiments are shown. *, significant difference (p < 0.01) compared with wild type P2 promoter activity.
when present at a 200-fold excess (Fig. 1B). Third, mutations in oligo II that were shown to abolish NF1 binding (8) did not affect Sp1 binding (Fig. 1B), which suggested that NF1 and Sp1 contact different sites on oligo II. Methylation interference (8) and mutational analyses (Fig. 2) indicated that NF1 contacts a “TGGCGT” motif, whereas Sp1 interacts with a “CCCGCG” motif in oligo II. Fourth, whereas NF1 binds to both the 3′- and the 5′-portions of footprint II, Sp1 only binds to the 5′-portion (oligo IIb, Figs. 1B and 2). Fifth, the pattern of binding of oligo II to purified Sp1 protein or to crude DDT, MF-2 extracts was similar (Fig. 1E). Finally, the strongest evidence for the presence of Sp1 in the oligo II complex is the ability of an antibody against Sp1 but not of an NF1 antibody, to cause supershift in DMSA (Fig. 1D).

The results of competition DMSA indicated that Sp1 only bound to the 5′-portion (IIb) of the oligo II sequence, which is G+C-rich (85%) and contains a one-mismatch GC box (CCCGCG) sequence. This motif has been shown to mediate the basal transcription of several genes (17, 18), and the finding that mutations of this motif but not of other GC-rich domains within the oligo IIb sequence abolished the formation of Sp1 complex and inhibited the P2 promoter activity indicate the critical role of Sp1 in the transcription of the α1BAR gene in DDT, MF-2 cells (Fig. 2D). The observed lower affinity of Sp1 for oligo IIb than for a consensus Sp1 oligo is probably due to the presence of one mismatch in the oligo IIb sequence to the consensus GC box sequence.

Our findings clearly indicate that the reason for the tissue-specific difference in protein binding to oligo II is the low Sp1 binding activity found in liver and low NF1 binding activity detected in DDT, MF-2 nuclear extracts (Fig. 4). The absence of Sp1 binding to oligo II in the adult rat liver may be due to the presence in liver of the phosphorylated form of Sp1, which has been shown to have much reduced binding affinity (Ref. 18; Fig. 4), to the low level of expression of the Sp1 protein in the rat liver, or to a combination of these two possibilities. Indeed, treatment of liver nuclear extract with phosphatase was reported to significantly increase Sp1 binding (18), and the adult rat liver was found to express very low levels of Sp1 protein and mRNA (20). Whether the high level of Sp1 binding in DDT, MF-2 cells is due to the presence of dephosphorylated Sp1, to high levels of Sp1 expression, or both remains to be determined.

Sp1 has been shown to activate the transcription of many viral and cellular genes via its glutamine-rich domain (21–26). For a large molecule (105 and 95 kDa), Sp1 is highly conserved, suggesting that it might play an important role in interacting with other transcription factors. Indeed, there is emerging evidence to show that Sp1 interacts with a whole range of transcription factors, including OTF-1 (27), CREB (28), NF-kB (29), Ets (30) YY1 (31), CP1 (32, 33), and Sp1 (34–36). The molecular mechanisms underlying these interactions are not well defined. In the case of an interaction of Sp1 with CP1 in the promoters of the major histocompatibility complex class II-associated invariant chain gene (32) or the murine Thy-1 gene (33), CP1 and Sp1 mutually stabilized the binding of one another, resulting in a synergistically activated transcription (32, 33). As illustrated in our previous paper (8) and the present study, oligo II is able to bind NF1, CP1 as well as Sp1. The binding of NF1 and CP1 to oligo II in liver nuclear extracts is mutually exclusive, probably as a result of their overlapping binding sites (7, 8). Although NF1 and Sp1 have different contact points on oligo II (NF1 contacts two TGGCT motifs (8), whereas Sp1 contacts a GC box within oligo IIb (Fig. 2)), the binding of these two factors to oligo II is mutually exclusive, as demonstrated by the absence of a NF1+Sp1+oligo II complex on DMSA. This is consistent with the observed lack of additivity in the in vivo activation of the P2 promoter by NF1 and Sp1.

The GC box located at −445 to −451 bp was also found to bind Sp1 protein and to contribute to P2 promoter activity. Interestingly, this GC box is adjacent to a CRE motif. We have found previously that an oligo that contained this CRE motif and part of the GC box (oligo I) bound CREB protein in either DDT, MF-2 cell extract or liver nuclear extract. The present results show that oligo I GC, which contains the GC box and part of the CRE, binds Sp1. The oligo I CG-CRE, containing both the GC box and the full CRE, was able to bind both Sp1 and CREB, but their binding was unstable, suggesting that the binding was mutually exclusive, probably as a result of overlapping binding sites. The binding of Sp1 around the transcription start site in the promoter of the human Ha-ras gene was shown to mediate the initiation of transcription of that gene (37). Similarly, the GC box located at −445 to −451 bp is adjacent to the tsp2 found at −443 bp of the rat α1BAR gene. Whether binding of Sp1 to this GC box is involved in transcription initiation from tsp2 requires further evaluation.

Our previously published observations (6–8) and the present data allow the following conclusions to be made about the structure and transcriptional control of the rat α1BAR gene, as schematically illustrated in Fig. 6. The rat α1BAR gene is composed of two exons and a single large intron. Transcription of the α1BAR gene utilizes three tsp and is controlled by three promoters in the liver. The dominant P2 promoter binds many transcription factors, including AP2, CRE, NF1, CP1, and Sp1. There is clear evidence that the major factor controlling the transcription of the α1BAR gene is NF1 in the liver, but Sp1 in DDT, MF-2 cells, which may account for the well documented tissue-specific regulation of α1BAR gene expression. For example, exposure to a phorbol ester significantly decreased the expression of the α1BAR gene in isolated hepatocytes (38) but increased it in DDT, MF-2 cells (11). The rat heart expresses high levels of α1BAR binding sites and of the 2.7-kb α1BAR mRNA (9, 10), but little if any NF1 binding (7) or mRNA (39) was detected. Whether Sp1 is the major transcription factor controlling α1BAR gene expression in the heart remains to be established.

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