

Functional interactions between an atypical NF- κ B site from the rat *CYP2B1* promoter and the transcriptional repressor RBP-J κ /CBF1

Sang Hyun Lee, Xiao-li Wang and Jeff DeJong*

Department of Molecular and Cell Biology, The University of Texas at Dallas, 2601 North Floyd Road, Richardson, TX 75080, USA

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ABSTRACT

The phenobarbital-inducible rat cytochrome P450 (*CYP*) 2B1 and 2B2 proteins are encoded by homologous genes whose promoters contain a mammalian-apparent long terminal repeat retrotransposon (MaLR). An NF- κ B-like site within the MaLR forms multiple protein–DNA complexes with rat liver and HeLa cell nuclear extracts. Using antibody supershift assays, we have identified these complexes as NF- κ B and RBP-J κ /CBF1. Competition assays using a series of single site mutant oligonucleotides reveal that the recognition sites for these two factors overlap. We also show that the *CYP2B1/2* NF- κ B element, but not the Ig κ NF- κ B element, can repress transcription *in vitro* when positioned upstream of the heterologous adenovirus major late core promoter. In addition, RBP-J κ over-expressed in COS-7 cells repressed expression *in vivo* from an SV40–luciferase reporter construct that contained the *CYP2B1/2* NF- κ B element. Finally, we observe similar levels of NF- κ B and RBP-J κ binding activities in nuclear extracts prepared from control and phenobarbital-induced rat livers. The results suggest that RBP-J κ /CBF1 binds an atypical NF- κ B site in the *CYP2B1/2* promoters and may help to maintain a low level of expression in the absence of inducer.

INTRODUCTION

Cytochrome P450s (*CYP*) are heme-containing membrane proteins that metabolize both foreign (xenobiotic) chemicals and endogenous compounds. Individual members of the *CYP450* gene superfamily are regulated in developmental-, tissue-, and gender-specific patterns and, in some cases, are activated by exposure to chemical inducers (1).

Transcription of the rat *CYP4502B1* and *2B2* genes (*CYP2B1/2*) is driven by liver-specific promoters that are ~96% identical within 2.35 kb upstream of the transcription initiation site. These promoters are notable for their ability to

be induced up to several hundred-fold by the barbiturate sedative phenobarbital (PB) or other ‘PB-like’ inducers. Several regions from the *CYP2B1/2* promoters are proposed to contain PB-responsive elements (PBRE) (reviewed in 2–5) and the most compelling evidence points to a distal element between –2318 and –2155 identified by assaying the activity of *CYP2B2* promoter constructs transfected into primary hepatocytes (6). Studies using transgenic mice confirm a role for this region (7,8) and *in vivo* footprinting shows PB-dependent alterations in chromatin structure (9). Genetic and biochemical characterization of the corresponding region from the homologous mouse *Cyp2b10* promoter suggests a role for NF-1 and the orphan nuclear receptor CAR (10–12). These sequences are distinct from the ‘barbie box’ element responsible for PB-induction of the *Bacillus megaterium CYP450_{BM-1}* and *CYP450_{BM-3}* genes (13).

In the absence of inducer, the rat *CYP2B1/2* and mouse *CYP2b10* genes are expressed at very low basal levels and may be maintained in a constitutively repressed state via negative regulatory elements (7,10,14–16; reviewed in 3). For instance, a construct that contained rat *CYP2B2* promoter proximal sequences (to –0.8 kb) was constitutively active in transgenic mice, whereas constructs that included additional upstream sequences (to –19 kb) suppressed basal activity (7). In addition, deletion analysis of the mouse *CYP2b10* promoter revealed that sequences between –971 and –775 reduced thymidine kinase promoter–CAT activity (10). Similarly, a 3-fold reduction in luciferase activity was seen with *CYP2B2* constructs containing sequences from –725 to –1400 that were transfected into HepG2 cells (16). Of additional interest is the fact that these reports focus on a region of the promoter that shares homology with a family of mammalian-apparent long terminal repeat retrotransposon (MaLR) elements (17).

In this report we identify and characterize an element within the *CYP2B1/2* MaLR that is recognized by both NF- κ B and the recombination signal sequence-binding protein RBP-J κ (18), also known as CBF1 (19). The dual NF- κ B/RBP-J κ sequence element, when fused to a heterologous promoter, can inhibit transcription *in vitro* and *in vivo*. The results support a role for RBP-J κ /CBF1 as a constitutively active nuclear repressor that can bind to a distinct class of NF- κ B sites.

*To whom correspondence should be addressed. Tel: +1 972 883 6882; Fax: +1 972 231 0628; Email: dejong@utdallas.edu

Present address:

Xiao-li Wang, Program in Cancer Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024, USA

MATERIALS AND METHODS

Oligonucleotides

For band shift and competition experiments: NF- κ B 2B1/2, 5'-ACTGTGGGAAATTCACACC-3'; NF- κ B I κ , 5'-CAGAGGGACTTTCCGAGAG-3'; AP-1 wt, 5'-GTGTCTGACTCATGCTT-3'; AP-1A, 5'-TCTCAAATGACTCTAGCTTG-3'; AP-1B, 5'-TCCATTTGACTCCTGAGCCT-3'; AP-1mut, 5'-CTCAAATTATTCTAGCTT-3'; AdML TATA, 5'-AAGGGGGGCTATAAAAGGGGGTGGG-3'; Sp1, 5'-TTCCGATCGGGGCGGGGCGAG-3'; 2B1/2DR, 5'-CTGATTCTTACAGAACCCAAGACTTCTTACAGAAGTCC-3'; 2B1/2 core, 5'-GTGGAGGGGCGGATTCAGCATAAAA-GATCCTGC-3'; C/EBP, 5'-TGCAGATTGCGCAATCTGCA-3'. For construction of transcription templates: NF- κ B I κ , 5'-CAGAGGGGACTTTCCGAGAGTACTGCATGCA-GAGGGGACTTTCCGAGAGTACTGCATG-3'; NF- κ B 2B1/2, 5'-CATGCAGTACTGTGGGAAATTCACACCGCATGC-AGTACTGTGGGAAATTCACACCG-3'. For construction of RBP-J κ expression vectors: RBP-myc5', 5'-CGCCGCGATCCAGTAATGCCCTCCGGTTTTCCT-3'; RBP-myc3', 5'-CCBCCBCTCGAGGGACACCACGGTTGCTGT-3'. For construction of pGL3-Pro-based reporter templates: I κ promoter, 5'-AGCGAGCTCGATATCAGAGGGGACTTTCGAGAGCTAGCCG-3'; *CYP2B1/2* promoter, 5'-AGCGAGCTCGATATCACTGTGGGAAATTCACACCGCTAGCTAG-3'. Oligos were obtained from Operon.

Preparation of nuclear extracts

Nuclear extracts were prepared essentially as described by Gorski *et al.* (20). Male Sprague–Dawley rats (~150–200 g; Zivic-Miller Laboratories) were given either no injection or i.p. injections of either saline (control) or 100 mg/kg PB (Sigma) 20 h prior to sacrifice. Livers were removed and homogenized in 10 ml/g ice-cold homogenization buffer (10 mM HEPES pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF and 1% Trasyol) through three passes in a glass–teflon homogenizer using a Glas-Col motor at a setting of 60. The resulting homogenate was layered onto 2 M sucrose cushions, centrifuged at 24 000 r.p.m. for 60 min and the cytoplasmic extract (supernatant) and pelleted nuclei were collected. Cytoplasmic extracts were dialyzed against dialysis buffer (25 mM HEPES pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol and 1 mM DTT) prior to use. Nuclei were resuspended in nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF and 1% Trasyol) and chromatin was pelleted by addition of 0.3 g/ml ammonium sulfate and centrifugation at 35 000 r.p.m. for 60 min. The upper aqueous nuclear extract was recovered and dialyzed. Extracts were aliquoted and snap frozen for storage at -70°C. Protein concentrations were typically 4–8 mg/ml and were determined using the Bradford method (Bio-Rad) with bovine serum albumin as the standard.

Electrophoretic mobility shift assays

Binding reactions (25 μ l) were performed in 10 mM HEPES, pH 7.9, 60 mM KCl, 2% PEG-8000 (w/v), 0.2 mM EDTA, 8% glycerol and 0.1 mg/ml poly(dI-dC) as described (21). Reactions contained 6–10 μ g of either nuclear or cytoplasmic proteins

prepared from untreated animals, from the livers of saline-treated (control) or PB-induced animals, or from HeLa cells. Oligonucleotides were typically purified by Sephadex G-25 gel filtration and annealed by incubating at 75°C for 5 min and cooling slowly to room temperature. Purified oligonucleotides were radiolabeled with T4 polynucleotide kinase and [γ -³²P]ATP and separated from unreacted nucleotides by purification with 1 ml Sephadex G-25 columns. Labeled oligonucleotides (~70 fmol) corresponding to 50 000–100 000 c.p.m. were added to reactions, which proceeded for 45 min at 4°C. The resulting protein–DNA complexes were loaded onto 0.5 \times TBE, 5% polyacrylamide gels and electrophoresed in 0.5 \times TBE running buffer. Unlabeled oligonucleotide competitors used in competition experiments were present in 100-fold excess over probe. In experiments where NF- κ B was post-translationally activated (22), various combinations of Nonidet P-40 (NP-40) (2.4%), formamide (27%) and sodium deoxycholate (DOC) (0.4%) were added to the reaction mixtures as indicated. Results were visualized by drying the gels and exposing to X-OMAT AR film (Kodak). Supershift reactions contained 0.5–2 μ l of antibodies specific for the p50, p52, or p65 subunit of NF- κ B (Santa Cruz), or for mouse RBP-J κ (K0043), and were added prior to the addition of probe.

In vitro transcription assays

Templates for *in vitro* transcription were based on pMLG4G, a G-free cassette reporter driven by the adenovirus major late (AdML) core promoter. Constructs were prepared by the insertion of synthetic double-stranded oligonucleotides containing either tandem NF- κ B 2B1/2 sites (pMLG4G-2B1/2) or tandem NF- κ B I κ sites (pMLG4G-I κ). *In vitro* transcription reactions (20,23) were performed in 25 mM HEPES, pH 7.6, 50 mM KCl, 6 mM MgCl₂, 0.6 mM ATP, 0.6 mM UTP, 35 μ M CTP, 1 μ l [α -³²P]CTP (800 Ci/mmol; ICN), 0.1 mM 3'-O-methyl-GTP (Pharmacia), 12% glycerol, 1 μ l RNasin (~30 U; Pharmacia). Reactions (50 μ l) also contained 20 μ g/ml *Hind*III-digested linear DNA template and ~70 μ g HeLa cell nuclear extract. After 45 min incubation at 30°C, the reactions were terminated by the addition 280 μ l stop mix (300 mM sodium acetate, 50 mM EDTA and 1.2% SDS). Proteins were removed by proteinase K treatment (10 μ l of 20 mg/ml proteinase K, 0.5 mg/ml tRNA) for 20 min at 55°C. After phenol/chloroform (1:1) extraction, labeled RNA was precipitated by addition of 350 μ l of 100% ethanol. The RNA pellets were rinsed with 80% ethanol and dried for 5 min under vacuum. Pellets were resuspended in 5 μ l of RNA loading dye (95% formamide, 1 mM EDTA and 0.05% bromophenol blue and xylene cyanol) and loaded onto 7 M urea–5% polyacrylamide sequencing gels. NF- κ B 2B1/2 oligonucleotide competitor was added to 0.28 μ M.

Transfections

The open reading frame of RBP-J κ was PCR amplified from the RBP-2 cDNA using the RBP-myc5' and RBP-myc3' oligonucleotides. The product was digested with *Xba*I and *Bam*HI and subcloned into pcDNA3.1/myc-His (Invitrogen). The insert produced by *Xho*I and *Bam*HI digestion of this plasmid was then subcloned into pCMV-Tag 2 (Stratagene) to generate pCMV-RBP. Reporter constructs were generated by subcloning synthetic NF- κ B binding sites from the *CYP2B1/2*

promoter or IgK enhancer into the pGL3 Pro vector (Promega) at the *SacI*-*NheI* site.

Transient transfections were performed using the calcium phosphate precipitate method (24). COS-7 cells were grown on 6-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone). When cells were 50% confluent they were co-transfected with 3 μ g pGL3-Pro-based reporter constructs together with pCMV-RBP. An aliquot of 200 ng of pCMV- β GAL was added as a control for transfection efficiency. Cells were harvested 48 h after transfection, and luciferase assays using 20 μ l of cellular extracts were performed as described by the manufacturer (Promega) using a Turner TD-20e luminometer. Experiments were performed in triplicate.

Measurement of RBP-J κ in COS-7 cells was performed with the cellular lysates used for the luciferase assay. Immunoblotting was performed using the ECL system (Amersham) and an anti-myc monoclonal antibody (25) prepared from mouse cell line MYC1-9E10.2 (ATCC).

Other techniques

Other molecular biology techniques were performed as described (24). Quantitation of autoradiographs was performed using an LKB Ultrascan XL laser densitometer or a Molecular Dynamics phosphorimager. Sequences were analyzed using the Lasergene software package (DNASTAR), BLAST sequence homology searches at NCBI (<http://www.ncbi.nlm.nih.gov/>) and the TRANSFAC and TESS programs (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html>) to predict binding sites for transcription factors.

RESULTS

Structure of the PB-inducible rat cytochrome P450 2B1 and 2B2 gene promoters

The direction and location of selected sequence elements in the *CYP2B1/2* promoters are summarized in Figure 1A. These include a PBRE between -2318 and -2155 (6,10,11), an MaLR long terminal repeat (LTR) between -1242 and -739 (17), a glucocorticoid response element (26), a core promoter region that contains sites for C/EBP (16,27), TBP and Sp1 and a region of repetitive CA dinucleotides. Regions upstream of -2.35 kb are not homologous, but include distinct truncations of LINE-1 repetitive elements and GA dinucleotide repeats of different lengths. The available sequence of the PB-inducible mouse *CYP2b10* (1.4 kb) promoter is ~83% identical to the *CYP2B1/2* gene (10).

Analysis of *CYP2B1/2* sequences between -1242 and -739 showed ~68% identity to 400 nt of the mouse MTa repetitive element (28). This region is thus a member of a large family (40 000–100 000 estimated copies) of retrotransposon-like MaLR repetitive elements often found as solitary, inactive LTRs (17). We tentatively divided the *CYP2B1/2* MaLR into U3, R and U5 regions oriented away from the direction of *CYP2B1/2* transcription (Fig. 1A). Computer analysis of the U3 region revealed that it contains a short direct repeat of 12 nt, as well as two adjacent sequences that resemble the GGGRNNYYCC recognition site for the inducible transcription factor NF- κ B (Fig. 1B). One of these sites (GGGAAATTCC) exactly matches a site found in the β -interferon enhancer (29).

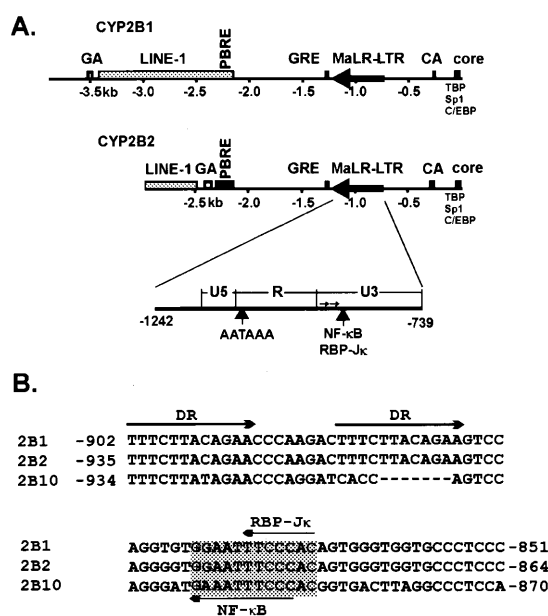


Figure 1. (A) Schematic diagram of the rat *CYP450 2B1* and *2B2* promoters. Core promoter binding factors, including TBP, C/EBP and Sp1, are indicated at the far right. CA refers to a repetitive CA sequence of five (2B1) or 19 (2B2) reiterations. The black arrow (-1242 to -739) pointing away from the core promoter indicates a solitary LTR from a family of mammalian apparent LTR retrotransposons (MaLR). The predicted boundaries between the U3, R and U5 regions of the LTR are shown in the expansion below, along with the position of putative NF- κ B and polyadenylation (AATAAA) sites. GRE indicates the position of a glucocorticoid response element. The dark box centered around -2300 (PBRE) represents a distal enhancer that confers responsiveness to PB. Sequences further upstream consist of distinct regions of LINE-1 elements, as well as reiterated GA dinucleotide sequences. See text for references. (B) Comparison of selected sequences within the MaLR from the rat *CYP2B1* and *2B2* and mouse *CYP2b10* promoters. The rat *CYP2B1* and *2B2* sequences are identical within the region shown and include a direct repeat (DR) of 12 nt. The putative NF- κ B and RBP-J κ sites are shaded and indicated with arrows. The MaLR element from the mouse *CYP2b10* promoter lacks the DR, but contains NF- κ B and RBP-J κ sites.

These sequences were of interest because little is known as to whether MaLR elements regulate expression of adjacent cellular genes and because of the possibility of a connection between *CYP2B1/2* induction and the responsiveness of rel (NF- κ B) family transcription factors to chemical stress (30).

The NF- κ B site in the *CYP2B1/2* LTR is recognized by multiple proteins in rat liver nuclear extracts

To determine if rat liver nuclei contained factors that would selectively recognize the NF- κ B element within the *CYP2B1/2* MaLR, we tested the ability of oligonucleotides that span this region (denoted NF- κ B 2B1/2) to form complexes in electrophoretic mobility shift assays (Fig. 2A). For comparison we used a control oligonucleotide that contains the NF- κ B site from the Ig κ enhancer (denoted NF- κ B Ig κ) (31). The control probe detected up to three complexes (C_2 - C_4) in this experiment (lane 1). The NF- κ B 2B1/2 probe revealed complexes of approximately similar mobility, but also formed an abundant,

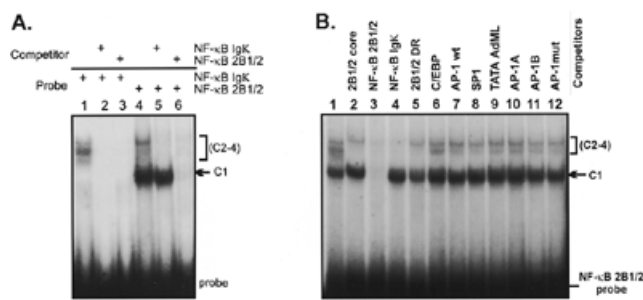


Figure 2. (A) The NF- κ B element from the rat *CYP2B1/2* MaLR LTR forms several shifted complexes in rat liver nuclear extracts. Band shift reactions contain probes for NF- κ B Ig κ (lanes 1–3) or NF- κ B 2B1/2 (lanes 4–6), as shown. One major complex (C_1) and up to three minor complexes (C_2 – C_4) were observed. Addition of unlabeled oligonucleotide competitors NF- κ B Ig κ (lanes 2 and 5) and NF- κ B 2B1/2 (lanes 3 and 6) indicate that the C_1 complex is competed by the NF- κ B 2B1/2 site, but not the NF- κ B Ig κ site. (B) C_1 complex formation is specific for the NF- κ B 2B1/2 element. Reactions were performed using the NF- κ B 2B1/2 probe as described in (A). Oligonucleotide competitors include none (lane 1), 2B1/2 core –43 to –13 (lane 2), NF- κ B 2B1/2 (lane 3), NF- κ B Ig κ (lane 4), 2B1/2 DR (lane 5), C/EBP (lane 6), AP-1 (lane 7), Sp1 (lane 8), AdML TATA (lane 9), AP-1-like site A (lane 10), AP-1-like site B (lane 11) and an AP-1 mutant (lane 12). The upper C_2 – C_4 complexes are competed by both the NF- κ B 2B1/2 and NF- κ B Ig κ oligonucleotides (lanes 3 and 4), while competition for the C_1 complex is seen only with NF- κ B 2B1/2 (lane 3).

faster migrating species (C_1) (lane 4). Competition experiments using unlabeled oligonucleotides showed that the upper set of complexes were efficiently competed by both NF- κ B 2B1/2 and NF- κ B Ig κ competitors (lanes 2, 3, 5 and 6), while formation of the major C_1 complex was abolished only in the presence of the NF- κ B 2B1/2 competitor (lane 6). To further establish that these results were not due to recognition of the NF- κ B 2B1/2 probe by a non-specific factor, additional oligonucleotide competitors were tested for their ability to eliminate C_1 complex formation (Fig. 2B). The results show that formation of the C_1 complex was abolished only in the presence of NF- κ B 2B1/2 competitor (lane 3).

Factors that recognize the NF- κ B 2B1/2 element show differential localization and responsiveness to protein-dissociating reagents

Binding activities present in nuclear and cytoplasmic extracts prepared from rat livers were compared in the presence of dissociating agents such as NP-40, DOC and formamide that release active NF- κ B from its cytoplasmic inhibitor I- κ B (22). As shown in Figure 3, band shift experiments with the NF- κ B 2B1/2 probe demonstrate that the upper complexes could be activated by various dissociating agents (for example lanes 5 and 12). In contrast, the C_1 complex was restricted to the nuclear fraction and was unaffected by addition of protein-dissociating agents (Fig. 3B, lanes 1–7). These results suggest that the C_1 complex contains a distinct nuclear factor that recognizes this particular NF- κ B site, or an adjacent or overlapping sequence.

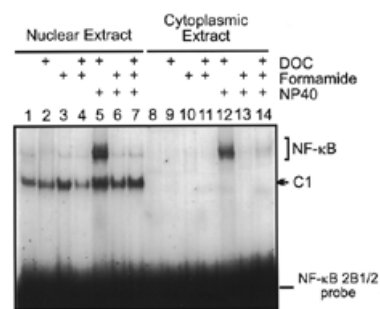


Figure 3. Localization and inducibility of protein–DNA complexes formed with the NF- κ B 2B1/2 element. Extracts were prepared from rat liver nuclei (lanes 1–7) and cytoplasm (lanes 8–14) and tested for complex formation using the NF- κ B 2B1/2 element as probe. Inducibility of complex formation was tested by the addition of DOC, formamide or NP-40, as indicated. The C_1 complex (lanes 1–7) was restricted to nuclear extracts and was unaffected by treatment with dissociating reagents.

The *CYP2B1/2* NF- κ B element is recognized by NF- κ B and RBP-J κ

Interestingly, the NF- κ B 2B1/2 sequence (but not the NF- κ B Ig κ control oligonucleotide) contains an overlapping site (GTGGGAA) that matches a consensus ag/cCGTGGGAAActa/t RBP-J κ recognition sequence (32; see Fig. 1B). To verify whether these proteins are present in the upper and lower complexes, we performed band shift experiments in the presence of factor-specific antibodies. As shown in Figure 4, addition of antibodies for the p50 (lanes 2 and 7) and p65 (lanes 3 and 8) subunits of NF- κ B, but not for the p52 subunit (lanes 4 and 9), resulted in disappearance of the upper complex and formation of slower migrating complexes. Likewise, addition of a monoclonal antibody (K0043) specific for mouse RBP-J κ resulted in disappearance of the lower C_1 complex and formation of an upper complex (lanes 5 and 10) that migrated just above the position of the NF- κ B complex (lane 1). The antibodies themselves do not shift the probe (lanes 11–14). This experiment demonstrates that the upper complexes contain NF- κ B-like proteins, whereas the lower C_1 complex is due to RBP-J κ /CBF1. Interestingly, the corresponding sequence from the mouse *Cyp2b10* gene (Fig. 1B) is also recognized by both NF- κ B and RBP-J κ (data not shown).

The *CYP2B1/2* NF- κ B element contains overlapping NF- κ B and RBP-J κ recognition sequences

To investigate the NF- κ B 2B1/2 recognition sequence in more detail, we synthesized a series of oligonucleotides that contained mutations in the putative RBP-J κ or NF- κ B recognition sites (Fig. 5A). These oligonucleotides were used as competitors in band shift assays using the NF- κ B 2B1/2 probe. The results (Fig. 5B) show that oligonucleotides with mutations in underlined positions of ACTGTGGGAAATTCCACACC competed poorly for NF- κ B complex formation, consistent with the idea that these positions are important for NF- κ B binding. Likewise, oligonucleotides with mutations in the underlined positions of

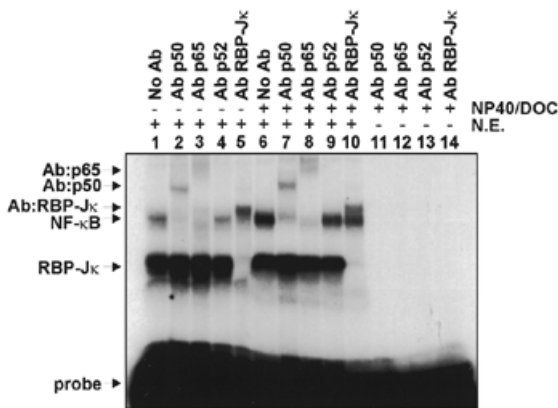


Figure 4. Supershift assays using antibodies against NF- κ B subunits p50, p52 and p65 and against RBP-J κ . Band shift reactions contain HeLa cell nuclear extract (N.E., lanes 1–10) and a kinase-labeled NF- κ B 2B1/2 probe. Reactions were carried out in the absence (lanes 1–5) or presence (lanes 6–14) of DOC and NP-40. Lanes 11–14 contain antibody alone. The results indicate that Ab-p50 (lanes 2 and 7) and Ab-p65 (lanes 3 and 8) supershift the upper complex, while Ab-RBP-J κ (lanes 5 and 10) supershifts the lower complex. The positions of RBP-J κ , NF- κ B and supershifted complexes are indicated to the left.

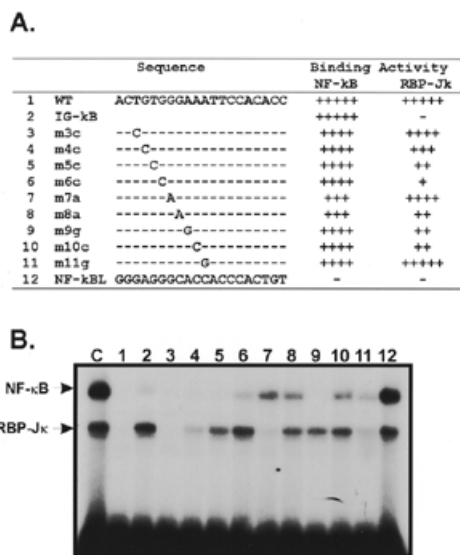


Figure 5. (A) Sequences of *CYP2B1/2* NF- κ B mutant oligonucleotides. Sequences (top strand) of NF- κ B 2B1/2 (oligo 1) and NF- κ B Ig κ (oligo 2) oligonucleotides, and various point substitutions (oligos 3–12) are aligned. The relative ability of these oligos to compete for NF- κ B and RBP-J κ binding activity is indicated to the right (100%, +++++; 80–100%, ++++; 60–80%, +++; 40–60%, ++; 20–40%, +; <20%, -/+; no binding, -). (B) Competition for NF- κ B and RBP-J κ complex formation using mutant oligonucleotides. Oligonucleotides 1–12 were used as competitors in band shift reactions containing HeLa cell nuclear extracts and a labeled NF- κ B 2B1/2 probe (lanes 1–12). Lane C shows a control experiment performed in the absence of competitor.

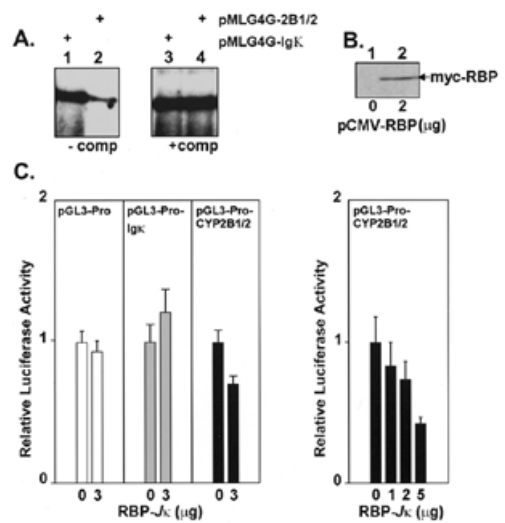


Figure 6. (A) The NF- κ B element from the *CYP2B1/2* promoter represses transcription from the adenovirus major late promoter *in vitro*. Adenovirus major late (AdML) promoter-G-free cassette *in vitro* transcription templates containing two NF- κ B Ig κ (pMLG4G-Ig κ) (lanes 1 and 3) or NF- κ B 2B1/2 (pMLG4G-2B1/2) (lanes 2 and 4) sites were tested for activity in *in vitro* transcription experiments using HeLa cell nuclear extracts. Reactions were performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the NF- κ B 2B1/2 oligonucleotide competitor. (B) A myc-tagged RBP-J κ construct (pCMV-RBP-J κ) overexpresses RBP-J κ in COS-7 cells. RBP-J κ protein (68 kDa) was detected in extracts prepared 48 h post-transfection using anti-myc antibodies. (C) Overexpressed RBP-J κ protein reduces expression from an SV40 promoter construct that contains the *CYP2B1/2* NF- κ B site. The left panel shows that a control vector (pGL3-Pro) and an Ig κ NF- κ B site vector (pGL3-Pro-Ig κ) are not affected by co-transfection and overexpression of RBP-J κ *in vivo*, whereas a construct with the *CYP2B1/2* NF- κ B site (pGL3-Pro-*CYP2B1/2*) is reduced. The right panel shows that repression of pGL3-Pro-*CYP2B1/2* is dependent on the amount of co-transfected pCMV-RBP-J κ .

ACTGTGGGAAATTCCACACC competed poorly for binding of RBP-J κ , indicating that these positions are important for RBP-J κ binding. The results are consistent with the idea that the recognition sequence for RBP-J κ (GTGGGAA) overlaps that for NF- κ B (GGGAATTCCC). This analysis, and the fact that slower migrating complexes that contain both NF- κ B and RBP-J κ proteins were not observed (data not shown), also suggests that binding of either factor is exclusive.

The *CYP2B1/2* NF- κ B element is a *cis*-acting repressor of transcription

To determine whether binding of RBP-J κ to the NF- κ B 2B1/2 element is responsible for a functionally distinct effect on gene expression compared to the NF- κ B Ig κ element, we performed *in vitro* transcription assays. Tandem NF- κ B 2B1/2 or NF- κ B Ig κ sites were cloned into pMLG4G, a template that drives expression of a G-free cassette under the control of the AdML core promoter (-49 to +10). As shown in Figure 6A, transcription from pMLG4G-2B1/2 (lane 2) was ~6-fold lower compared to the pMLG4G-Ig κ template (lane 1). This result suggests that the NF- κ B site from the *CYP2B1/2* gene, but not the NF- κ B Ig κ site, is a negative *cis*-acting regulatory element. Addition

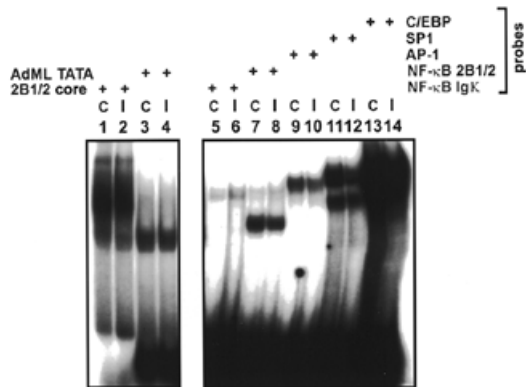


Figure 7. Formation of NF- κ B, RBP-J κ and several other protein–DNA complexes does not differ in rat hepatic extracts prepared from control (C) or PB-induced animals (I). Oligonucleotides spanning the *CYP2B1/2* core (–45 to –13) (lanes 1 and 2) and the AdML TATA element (lanes 3 and 4) were used to normalize activity of control (C) and PB-induced (I) extracts with respect to TATA element binding activities. Oligos tested include NF- κ B Ig κ (lanes 5 and 6), NF- κ B 2B1/2 (lanes 7 and 8), AP-1 (lanes 9 and 10), Sp1 (lanes 11 and 12) and C/EBP (lanes 13 and 14).

of an NF- κ B 2B1/2 oligonucleotide competitor had no effect on pMLG4G-Ig κ transcription (lane 3), but restored transcription of pMLG4G-2B1/2 to normal levels (lane 4). The selective reversal of pMLG4G-2B1/2 inhibition by this oligonucleotide and the protein–DNA interaction assays presented in Figures 2 and 4 suggest that RBP-J κ is responsible for repression.

To confirm that the *CYP2B1/2* element, but not the Ig κ element, would down-regulate a heterologous promoter *in vivo* (as predicted by the experiment in Fig. 6A), *CYP2B1/2* and Ig κ NF- κ B sites were positioned upstream of the SV40 promoter–luciferase reporter in the pGL3-Pro vector. The parental vector and each of the derived constructs were transfected into COS-7 cells with or without 3 μ g of a pCMV-RBP expression vector that encoded a 68 kDa myc-tagged RBP protein (Fig. 6B, lane 2). The results in Figure 6C show that the pGL3-Pro-*CYP2B1/2* promoter activity, as measured by luciferase activity, was repressed in the presence of pCMV-RBP. In contrast, the pGL3-Pro and pGL3-Pro-Ig κ constructs, which do not contain RBP recognition sequences, were unaffected. The right hand panel in Figure 6C shows that repression increased (17, 28 and 57%) with increasing amounts of co-transfected pCMV-RBP (1, 2 and 5 μ g).

Formation of NF- κ B, RBP-J κ and other protein–DNA complexes does not differ in control and PB-treated extracts

A unique characteristic of the *CYP2B1/2* promoters is their transcriptional up-regulation in response to PB treatment. For this reason we wanted to test whether NF- κ B or RBP-J κ binding activities were altered in hepatic extracts prepared from saline- or PB-treated rats. To do so, we first normalized the amounts of each extract preparation on the basis of their ability to form complexes with oligonucleotide probes that spanned the *CYP2B1/2* core promoter (–45 to –13) or with an AdML TATA element, as shown in Figure 7 (lanes 1–4). We then tested a panel of synthetic oligonucleotides that contained

binding sites for several transcription factors present in adult rat liver. Extracts prepared from control (C) and induced (I) animals 20 h post-treatment showed ~2-fold or less change in the binding of RBP-J κ (lanes 7 and 8), NF- κ B (lanes 5–8), AP-1 (lanes 9 and 10), Sp1 (lanes 11 and 12) or C/EBP (lanes 13 and 14). Each of the shifted complexes observed in this assay was competed specifically (data not shown). It has been suggested elsewhere that NF- κ B (33,34) and AP-1 (35,36) activities increase in hepatic nuclear extracts following PB treatment. Although the basis for these differences is not known, we conclude here that RBP-J κ is a constitutive negative factor.

DISCUSSION

In this study we have identified an atypical NF- κ B site within the rat *CYP2B1/2* MaLR element that is recognized by the inducible NF- κ B and constitutive RBP-J κ /CBF1 transcription factors. Assuming a consensus RBP-J κ recognition sequence of G/ATGGGAA (32), the occurrence of dual NF- κ B/RBP-J κ elements is predicted to occur at ~12.5% of NF- κ B sites that contain the GGGAA NF- κ B half site. This element, together with similar sites in the IL-6 (37,38) and NF- κ B-2 genes (39), defines a functionally distinct subclass of NF- κ B recognition sequences. Interestingly, the PB-inducible mouse *CYP2b10* gene also contains a putative RBP-J κ element that occurs in the context of a NF- κ B site (Fig. 1B). However, a search for NF- κ B and RBP-J κ sites in the promoter regions of additional *CYP450* genes did not reveal other examples of sites that overlap. It is interesting to note that another class of atypical NF- κ B sites are G-C rich and are proposed to be the site of an interplay between NF- κ B and Sp1 (40). The data also make the important point that a widely distributed family of repetitive elements, MaLRs (17), can contain sites that bind *trans*-acting transcriptional regulatory factors.

RBP-J κ was originally isolated based on its recognition of an immunoglobulin κ gene recombination signal probe (18) and functions either as a cofactor-dependent positive regulator or, in the absence of additional proteins, as a negative regulator. For instance, RBP-J κ recruits Epstein–Barr virus nuclear antigen-2 (EBNA-2) protein to sites in the viral latent membrane protein, *BamHI* C, terminal protein and cellular CD23 promoters (41–47), where it up-regulates transcription. The *Drosophila* counterpart of RBP-J κ , *Suppressor of Hairless*, mediates transcriptional activation by *Notch* through a similar mechanism (reviewed in 48) and both EBNA-2 and mammalian Notch1 interact directly with RBP-J κ to activate transcription (49). By itself, however, RBP-J κ is able to repress gene expression. For instance, the adenovirus pIX (50), human IL-6 (37,38) and NF- κ B-2 (39) promoters are repressed by RBP-J κ . Moreover, Gal4–RBP-J κ fusion proteins inhibit transcription from a herpes simplex virus thymidine kinase core promoter construct that contains upstream Gal4 sites (51). The mechanism of repression by RBP-J κ repression at sites near the TATA element may involve interference with the function of the general transcription factor TFIID bound to TBP (52). RBP-J κ is also associated with a histone deacetylase that could negatively modulate transcription factor accessibility to chromatin (53,54).

Induction of the *CYP2B1/2* genes by chemical inducers involves a positively acting PBRE that binds a transcriptional activator(s) responsive to PB (reviewed in 2–5). The function

of such factors may be to overcome, or derepress, the effects of negatively acting sequences that appear to maintain these genes in a relatively inactive state (reviewed in 3). Repression is strikingly illustrated by the ability of sequences between -971 and -775 of the mouse *CYP2b10* promoter to reduce the activity of a heterologous thymidine kinase promoter (10). As the NF- κ B/RBP-J κ site in the rat *CYP2B1/2* (-888 to -882) and mouse *CYP2b10* (-894 to -888) promoters is within this negative regulatory region, our studies suggest a role for RBP-J κ in down-regulating *CYP2B1/2* gene expression. This idea is supported by several observations. First, RBP-J κ is an abundant factor whose levels, as shown in this report, are not affected by PB treatment (Fig. 3). Moreover, subcellular fractionation (Fig. 3) and immunolocalization experiments (data not shown) show that RBP-J κ is a nuclear factor. Second, the *CYP2B1/2* site represses transcription from the heterologous AdML core promoter in a cell-free *in vitro* transcription system (Fig. 6). The degree of repression observed (~6-fold) is comparable to that observed with the adenovirus pIX promoter and a partially reconstituted *in vitro* transcription system to which purified RBP-J κ was added (50). Third, the *CYP2B1/2* NF- κ B element also represses transcription *in vivo* from the heterologous SV40 promoter in COS-7 cells, an effect not seen with a similar NF- κ B site from the IgK enhancer that does not bind RBP-J κ (Fig. 6). Similarly, a related NF- κ B/RBP-J κ element from the IL-6 gene interleukin response element repressed expression *in vivo* when positioned ~1.2 kb away from the initiation site (37). Of course, it is possible that additional transcriptional regulatory proteins that bind the MaLR may also help to down-regulate *CYP2B1/2* gene expression.

Several additional questions arise from our findings. For instance, are the *CYP2B1/2* promoters activated by interactions between the mammalian Notch transcriptional activator or EBNA-2 proteins and promoter-bound RBP-J κ ? Also, are the *CYP2B1/2* or other genes that contain dual NF- κ B/RBP-J κ elements controlled by an interplay of the cognate binding factors, or does competition from RBP-J κ help mainly to re-establish repressed transcription levels more rapidly on dual NF- κ B/RBP-J κ sites? While the current study is limited to a demonstration that the *CYP2B1/2* element is a negative regulatory element, in the case of the IL-6 gene RBP-J κ is able to diminish the NF- κ B-mediated response to TNF α and IL-1 (37). Displacement of RBP-J κ by NF- κ B would be facilitated by an increase in nuclear NF- κ B concentrations following induction and by the higher affinity of NF- κ B-DNA interactions (37,39). Indeed, Figure 3 also shows that the intensity of the C₁ complex and the upper NF- κ B complexes in nuclear extracts is ~11:1 in the absence of dissociating reagents (e.g. lane 1), but is nearly 1:1 following activation (lane 5). If these results reflect the abundance of NF- κ B binding activities in the nucleus following the response to physiological inducers, they suggest the potential for an interplay between these factors on the *CYP2B1/2* NF- κ B site. However, future work will be required to investigate these issues in more detail.

In conclusion, the results here show that RBP-J κ /CBF1 acts as a constitutively active nuclear repressor whose binding site can in some instances overlap sites for the NF- κ B transcriptional activator. The activity of RBP-J κ /CBF1 at such elements may be to constitutively diminish transcription from nearby core promoters, for instance the uninduced *CYP2B1/2* genes, or to modulate the effects of signaling pathways that activate NF- κ B.

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