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Functional analysis of the phenobarbital-responsive unit in rat *CYP2B2*

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Abstract

An 163-bp fragment of the rat cytochrome P450 gene, *CYP2B2* has been shown to contain sequences that mediate phenobarbital (PB) responsiveness of this gene. In studies on this rat gene and the orthologous mouse gene, *Cyp2b10*, the minimal fragment required for near full PB responsiveness has varied from about 50 to 80 bp depending on the gene used and the number of copies of the PB responsive sequences assessed. Since there is a single copy of the *CYP* genes in the genome, we have evaluated deletion and block mutations across an 84-bp region of the PB responsive unit (PBRU), by *in situ* transfection in rat liver using single copies of the PBRU sequences. From the 5′ end, deletions to −2243 retained more than 50% responsiveness to PB compared to the 163-bp fragment. The fragment −2237 to −2155 retained less than 20% responsiveness even though it contained the nuclear receptor (NR)-1, NR-2, and NF-1 motifs which are present in the core of the PBRU. From the 3′ end, deletions from −2170 to −2194 eliminated PB responsiveness indicating that the 74-bp sequence from −2243 to −2170 is able to mediate full PB responsiveness. Block mutations within the NR-1 and NF-1 regions reduced responsiveness most dramatically, but did not abolish it, and mutations 3′ of the NF-1 site modestly reduced responsiveness. Protein binding was not affected by mutations in the NR-1 region as assessed by DNase I footprinting *in vitro* but mutations within the NR-2 region reduced binding to the NF-1 site. Mutations of the 5′ half or the 3′ half of the bipartite NF-1 site, resulted in loss of protection of the NF-1 site and new footprints to the 3′ or 5′ side, respectively, of the NF-1 site. These results indicate that sequences in addition to the NR-1 and -2 and the NF-1 sites are required for full responsiveness to PB and suggest that proteins which bind to these sites may interact.

Keywords: Cytochrome P450; Phenobarbital; Transcriptional regulation; Nuclear receptors; *In situ* transfection; DNase I footprinting

1. Introduction

Increased hepatic metabolism of xenobiotics after treatment with the classical inducer, PB was observed more than 40 years ago [1]. The effects of PB are pleiotropic, but key to the increase in drug metabolism is the increase in the amounts of cytochrome P450 in the liver which has been shown to be the result primarily of induction at the level of P450 gene expression [2,3]. There was little progress in understanding the molecular mechanism of PB induction until the recent observation that a 163-bp fragment at about −2300 in the *CYP2B2* gene had the properties of a PB-dependent enhancer in transfected hepatocytes in primary cultures [4]. This fragment exhibited similar properties in an *in situ* transfection assay in which DNA was injected directly into rat liver [5]. An analogous sequence was present in the murine *Cyp2b10* gene and was shown to mediate PB induction in mouse hepatic cells in primary culture [6]. The presence of this sequence at about −2300 in the *CYP2B2* gene is consistent with earlier studies in transgenic mice in which transgenes required *CYP2B2* sequences between −800 and −23,000 for normal hepatic expression dependent on PB [7]. These studies established that the 163-bp fragment contained a PB-dependent enhancer which was responsible for most of the PB induction.

Characterization of the 163-bp fragment has established that it is a complex enhancer that contains multiple regulatory elements. Progressive deletions from either end of the 163-bp fragment identified 5′ and 3′ endpoints required for a PB response, but a core fragment of about 30 bp with the identified 5′ and 3′ endpoints was not competent for PB induction [6,8,9]. Thus, the core sequence requires addi-

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Abbreviations: P450, cytochrome P450; PB, phenobarbital; *CYP*, P450 gene; NR, nuclear receptor; NF-1, nuclear factor-1; GRE, glucocorticoid response element; CAR, constitutive androgen receptor; RXR, retinoid X receptor; PBRU, PB response element

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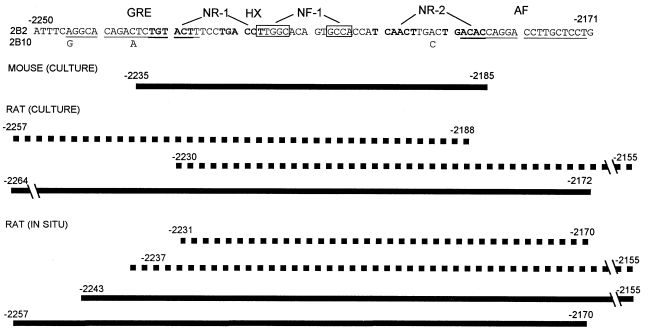

Fig. 1. Sequence of the rat *CYP2B2* and mouse *Cyp2b10* PBRUs and summary of the activities of fragments of the PBRUs. The *CYP2B2* sequence is shown at the top. For the *Cyp2b10* sequence, only differences with the *CYP2B2* sequence are shown. Sequences that have been proposed as regulatory motifs are: NF-1 (boxed) [9], NR-1 and NR-2 (bold) [10], GRE and accessory factor (AF) (underlined) [8]. Below the sequence, deleted fragments are shown schematically. Solid bars represent fragments that are greater than 50% as active as the 163-bp full length fragment and dashed bars represent fragments less than 20% as active as the full length fragment. The species of origin of the fragments is indicated, and the fragments were assayed by either transient transfections in primary cultures of hepatocytes (CULTURE) or by transfection *in situ* by direct injection of DNA into rat liver (IN SITU). The data summarized are from Honkakoski et al. [10] for the mouse *Cyp2b10* gene in transfected cultured hepatocytes, from Stoltz et al. [8] for the rat *CYP2B2* gene in transfected cultured hepatocytes, from Liu et al. [9] and this paper for the *in situ* injections in rat. The nucleotide numbers of the endpoints of the *CYP2B2* fragments are given. For the mouse *Cyp2b10* fragment, the numbers shown are those that correspond to the rat numbering not the actual numbers in the mouse gene which are −2339 and −2289 [10].

tional sequence from either the 5′ or 3′ side for PB induction indicating that redundant elements are present in the flanking regions. Several potential regulatory motifs were present in the fragment mediating PB induction, including a NF-1 site, which was the primary site of protein binding *in vitro* [9], and two nuclear receptor-like binding elements, NR-1 and NR-2, flanking the NF-1 site (Fig. 1) [10]. Individual mutations of these sites only partially inhibited the PB response and none of a series of linker scanning mutations across 80 bp of the fragment eliminated the PB-response when three copies of the mutated fragments were assayed [6,8–10]. A sequence, designated as an accessory factor, to the 3′ side of the NR-2 site, and a putative GRE site which overlapped the NR-1 site were identified as contributing to the PB response [8]. These results indicate that multiple elements are required for the maximum PB response which is further supported by the observation of multiple protein-DNA complexes formed in gel shift assays in these same studies. Because of its complex nature, the PB enhancer has been designated a PB responsive unit (PBRU) in the rat genes or PB responsive module in the mouse genes.

NF-1 has been demonstrated to bind to the PBRU and mutation of this element reduces the PB response in transient transfections of hepatocytes cultured *in vitro* or liver *in situ* [6,8,9]. In addition to NF-1, proteins binding to the NR-1 motif at the 5′ side of the NF-1 site have been shown to be a heterodimer of RXR and CAR [11]. The heterodimer has been reported to bind to both NR-1 and to NR-2 sites, in the human gene [12]. The binding of CAR and RXR to the PBRU *in vitro* was increased with nuclear extracts from rats treated with PB, and co-transfected CAR and RXR could synergistically activate the PBRU in hepatic cell lines indicating that this nuclear receptor heterodimer plays a key role in the PB induction [11,12]. Mutation of the NR-1 site alone did not eliminate PB-induction so that binding of RXR/CAR or other proteins to motifs other than NR1 can also mediate PB induction.

Different minimal sequences in the PBRU required for full PB responsiveness have been identified for the mouse *Cyp2b10* and rat *CYP2B2* genes even though these genes are nearly identical (Fig. 1). In the mouse gene, a sequence of 51-bp which includes the NF-1 site and the two flanking nuclear receptor binding sites was sufficient for a full response [10]. In the rat gene, a fragment of 85 bp, which included the 51-bp region was only partially responsive in transfected primary rat hepatocytes [8]. A fragment containing the NR-1 and NF-1 sites and terminating 3 bp from the end of the 51-bp fragment was inactive in this system (Fig. 1). In the *in situ* assay with three copies of the rat PBRU fragments, nearly full activity was observed in a fragment of 64-bp which included the NR-1 and NF-1 sites but not the intact NR-2 site [9]. In contrast, with single copies of the PBRU fragments, this 64-bp fragment did not confer responsiveness and an 88-bp fragment was the minimal sized fragment tested which retained substantial PB responsiveness. A possible explanation for the difference in results with one or three copies is related to the apparent redundancy of elements in the PBRU. The effects of mutation or deletion of one element when assayed as a single copy may be compensated by multiple copies of the remaining unmutated elements when multiple copies are assayed. Overall, there is good agreement that a sequence of about 50 bp centered on the NF-1 site forms the core of the PBRU, but there is disagreement about whether and how much additional flanking sequences are required for full PB responsiveness depending on the gene, mouse *Cyp2b10* and rat *CYP2B2*, and the number of copies of the PBRU assayed.

In view of the differences in sequence requirements for the deleted fragments when three copies versus one copy were assayed and the differences in minimal sequences determined in the different systems, we have evaluated the effects of the linker scanning mutations across 88-bp of the rat *CYP2B2* PBRU on the activity of single copies of the mutated PBRU and have examined the effects of the mutations on protein binding. The assay of single copies, compared to multiple copies, of the PBRU should provide a better estimate of the relative importance of the motifs of the PBRU in its natural context *in vivo*. In the earlier studies, three copies of the PBRU were required to maximize the transcriptional response because of the inherent variability of the *in situ* assay. In the present studies, an internal standard has been included in the assays to reduce

the variability and permit assay of single copies of the PBRU. The results indicate that 74-bp of sequence centered on the NF-1 site are required for near full activity and that NF-1 and sequences to the 5′ side of the NF-1, which encompasses the NR-1 and putative GRE sites, contribute most to the PB-stimulated transcriptional activity in transfected hepatocytes *in situ*. In addition, protein binding to the flanking regions was observed with evidence for interactions between proteins that bind to the 3′ side of the NF-1 site and to the NF-1 site.

2. Materials and methods

2.1. Oligonucleotides

The following oligonucleotides were synthesized at the Genetic Engineering Facility, University of Illinois at Urbana-Champaign with an Applied Biosystems Model 380A DNA synthesizer:

LS8′:

5′ TGCCACCATCAA***AGATCT***TGACACCAGGAC 3′

LS9:

5′ CATCAACTTGAC***CCCGGG***CAGGACCTTGCT 3′

Lucseq2C1:5′ CAGTGTATGGTAGAT 3′

2.2. Plasmid constructions

Construction of linker scanning mutations, LS1-LS12, and NF-1 mutations have been described [9]. Single copies of linker scanning mutations (LS1-LS12) in the 163-bp *CYP2B1/2* PBRU region were inserted before the promoter region of *CYP2C1* (−272 to +1) in pTZ2C1 as described [9]. From these vectors, *Sma*I/*Hin*dIII fragments, which contain the PBRU-2C1 sequence, of each mutation were cloned into the *Sma*I/*Hin*dIII site of pA3LUC. Single stranded pTZPBRE2C1 [5], which contains a single copy of the *CYP2B2* PRBU was used as the template for site-directed mutagenesis using primers LS8′ and LS9′. Sequences for a *Bgl*II and a *Sma*I restriction site in LS8′ and LS9′, respectively, were substituted for PBRU sequence (bold italic in the oligonucleotide sequences above). The mutated nucleotides were confirmed by DNA sequencing. The *Kpn*I/*Hin*dIII fragments of the resulting plasmids were cloned into the *Kpn*I/*Hin*dIII site of pA3LUC.

Similarly, single copies of deletion mutations of the PBRU region were constructed in pTZ2C1, and *Kpn*I/*Hin*dIII fragments of the resulting plasmids were cloned into the *Kpn*I/*Hin*dIII site of pA3LUC. Since LS1 to LS6 mutations contain a *Kpn*I site at each mutation site, new deletional mutations, LS2d, LS3d, LS4d, LS5d and LS6d, were produced by isolation of the corresponding *Kpn*I/*Hin*dIII fragments from each mutation and then inserting the fragment into the *Kpn*I/*Hin*dIII site of pA3LUC. Plasmid DNA from each of the constructions in pA3LUC was sequenced using the Lucseq2C1 primer to confirm the mutations. The plasmid, pRL-SV40 (Promega), which contains the SV40 promoter and enhancer fused to the *Renilla* luciferase gene, was used as an internal standard.

2.3. Direct liver DNA injection

DNA was injected directly into rat liver *in situ* as described [5] except that DNA was purified with plasmid mega DNA isolation kits (Qiagen Corp.) and an internal standard of an SV40 promoter-enhancer/Renilla luciferase reporter was included. Ten μg of the SV40/Renilla plasmid and 300 μg of PBRU2C1 firefly luciferase DNA were co-injected into the liver. For each plasmid construction, three control and three PB-treated male Sprague-Dawley rats were used as described [5,9]. The activity of the firefly and Renilla luciferases were assayed in the same samples by the Dual-luciferase reporter assay according to the manufacturer's instructions (Promega Corp.). Approximately 1.5 g of liver, which included the three sites of injection, was homogenized on ice in 1.0 ml of 1X passive lysis buffer. The homogenates were centrifuged at 15,000 × g in a microfuge for 25 min. Twenty μl of the resulting supernatant was mixed with 100 μl of luciferase assay buffer II, the firefly luciferase activity was measured as arbitrary light units of chemiluminescence in a luminometer, 100 μl of stop and glow buffer was added, and the *Renilla* luciferase activity was measured. The firefly luciferase activities were normalized by dividing the light units measured for the firefly luciferase by those determined for the *Renilla* luciferase and then multiplying by 10^5 so that the normalized values are approximately the same magnitude as the actual light units measured.

2.4. Gel-shift assay and DNase I footprinting analysis

Male Sprague-Dawley rats (250–350 grams) were injected *i.p.* with PB (100 mg/kg of body weight) or isotonic saline and sacrificed 6 h later. Liver nuclear extracts were isolated as described earlier [9]. *Nco*I-*Kpn*I DNA fragments were isolated from LS4, LS5, and LS6 mutants and labeled by filling in the ends with *E. coli* DNA polymerase I, Klenow fragment, and $\alpha[^{32}P]$ATP. The probes (about 10,000 cpm) were incubated on ice for 15 min with 1 μg of liver nuclear protein. The binding conditions of gel-shift assays were as described [13]. DNA-protein complexes were separated by electrophoresis at 200 volts in 0.5 X TBE buffer. The gels were dried and exposed to X-ray film at −80°C. For DNase I footprinting, 460-bp *Eco*R1/*Hin*dIII fragments containing the PBRU mutants were labeled by filling in the *Eco*R1 end by incubation with *E. coli* DNA polymerase I, Klenow fragment and $\alpha[^{32}P]$ATP. The radioactive DNA fragments (40,000 to 50,000 cpm) were incubated with 30 μg of nuclear protein on ice for 15 min under

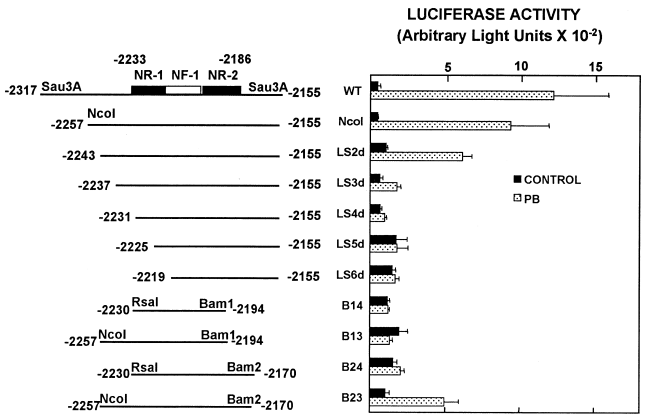

Fig. 2. Deletion analysis of the PBRU region. Single copies of fragments from the PBRU region were inserted in front of the CYP2C1 promoter fused to the firefly luciferase gene. Schematic diagrams of the PBRU fragments are shown at the left and the numbers refer to the endpoint of the wild type sequence in the fragment. Restriction sites used to generate the fragments are indicated. At the top of the schematic fragments, the solid and open boxes indicate the position of the NR-1, NF-1, and NR-2 motifs and the numbers denote the 5′ end of NR-1 and the 3′ end of NR-2. Bam1 and Bam2 are *Bam*HI sites generated by PCR. Three hundred μg of DNA of the plasmids were injected into rat liver with 10 μg of the SV40/Renilla luciferase plasmid as an internal standard. Rats were treated with isotonic saline (CONTROL) or 100 mg/kg PB injected *i.p.* 24 h before sacrifice. Luciferase activity is expressed as arbitrary light units normalized against the *Renilla* luciferase activity as described in Methods and materials. Each group consisted of three animals and the standard error is shown.

the same binding condition as for gel shift assays. Either 7.5–10 ng DNase I or 75–100 ng DNase I were added to the reactions without or with protein, respectively, for 5 min on ice. The DNA was purified by proteinase K digestion and phenol/chloroform extraction, the fragments were separated by electrophoresis on denaturing 6% polyacrylamide gels, and radioactivity was detected by autoradiography. Markers were generated by partial cleavage of fragments methylated with dimethyl sulfate and cleaved with piperidine [14].

3. Results and discussion

3.1. Monomer deletional analysis

Our previous studies had demonstrated that with three copies of PBRU fragments assayed, the minimal fragment for near complete PB responsiveness was −2257 to −2194 and a fragment from −2230 to −2170 was also nearly fully responsive [9]. In contrast, these fragments exhibited no responsiveness to PB when assayed as a single copy and the smallest fragment tested to exhibit full activity with one copy was −2257 to −2170. To further define the minimal size of the PB responsive fragment, additional fragments with progressive 5′ deletions were tested and the reproducibility of the assay was improved by the addition of an internal standard. Deletions from the 5′ end with a fixed 3′ end at −2155 exhibited a progressive loss (Fig. 2). About 50% responsiveness compared to the 163-bp fragment was retained in the deletion to −2243, but deletion of 6 additional residues reduced responsiveness to less than 20%. In fragments beginning at −2257 at the 5′ end, deletions from the 3′ end to −2170 remained partially active, but deletion to −2194 eliminated PB responsiveness.

At the 5′ end, deletion to −2230 had been shown to result in loss of responsiveness to PB in transfected rat hepatocytes and in rat liver directly injected with DNA (Fig. 1) [8,9]. The present studies are consistent with these earlier studies, and define more precisely the region between −2243 and −2237 as critical for PB responsiveness. Deletion to −2230 removed sequence in both the putative GRE site and the NR-1 site (Fig. 1) so that inactivation of either could explain the loss of responsiveness. Deletion to −2237, however, leaves the NR-1 intact but deletes part of the GRE suggesting that this motif is important. The loss of responsiveness when −2243 to −2237 was deleted is consistent with studies in which residues −2241 to −2239 were mutated and PB responsiveness was reduced to 10% of wild type in transfected rat hepatocytes [8].

These results differ from studies of the mouse *Cyp2b10* gene transfected into primary mouse hepatocytes in which a fragment from −2235 to −2185 was shown to confer full PB responsiveness [10]. The sequences of the mouse *Cyp2b10* and rat *CYP2B2* genes between −2243 and −2167 differ at only two positions, −2236 and −2193 (Fig. 1). Neither change appears to be within a critical binding site, so that differences in the sequences of the two species are unlikely to explain the different requirements for PB responsiveness. The reasons for the differences in results is not known but could be related to differing concentrations of transcription factors in the mouse and rat cells, which might alter the requirements for the individual elements.

3.2. Monomer mutational analysis

We previously reported a linker scanning study of the PBRU region in which three copies of the mutated PBRU fragments were assayed [9]. Since there are significant differences in the requirements for PB responsiveness in the deletional studies if one rather than three copies of the PBRU fragments are used, we felt it was important to analyze the effects of these mutations with single copies of the mutated PBRU. In LS8 and 9, the use of the *Kpn*I site as the scanning mutational sequence changed only 4 of 6 bp so that new mutations, LS8′ and LS9′, were used at these position which changed all six (Fig. 3).

The results with the one copy of the PBRU generally agreed with the earlier studies using three copies but there were some differences. As might have been expected, deleterious mutations had greater effects in general with one copy rather than three. As before, the most dramatic decreases in response were observed with LS4, LS5, and NF-1 mutations (Fig. 3). The LS3 and LS6 mutations modestly reduced responsiveness with single copies while little effect

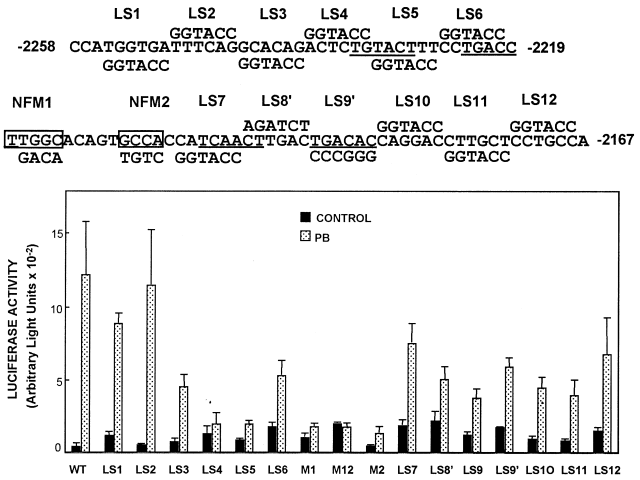

Fig. 3. Linker scanning mutagenesis of the PBRU region. The sequence of the rat *CYP2B2* PBRU region is shown at the top and the changes introduced by the linker scanning (LS) mutations and the mutations in the NF-1 site are indicated. Single copies of the mutated fragments were inserted in front of the CYP2C1 promoter fused to the firefly luciferase gene. Luciferase activity was assayed as described in the legend to Fig. 2.

had been observed with three copies. None of the mutations completely eliminated PB responsiveness although a double mutation of the two parts of the NF-1 site eliminated detectable responsiveness.

Mutations with the most severe effects were those of NF-1 (Fig. 3), which is consistent with results in both transfected mouse and rat primary hepatocytes [6,8]. The *in vivo* role of NF-1 was recently called into question, since mutation of the NF-1 site did not reduce expression after PB treatment in three independent transgenic strains compared to two wild type transgenic strains [15]. The mutated transgenes, however, were expressed at higher levels in the untreated animal so that the fold-increase was reduced in the mutants. These data would suggest that binding of proteins to the NF-1 site repress transcription in the untreated animal, but do not make a significant positive contribution to expression in the PB treated animal, which would be in conflict with the transient transfection experiments. The binding of NF-1 to the PBRU and its function in PB induction of the gene might be dependent on the gene being in its natural chromatin state as it is in the transgene in contrast to the transiently transfected gene. In support of this idea the binding of NF-1 to its motifs has been shown to be reduced in nucleosomes compared to naked DNA [16]. An alternative explanation is that the transgenic effects are specific to the mutation made, i.e. a site for a different regulatory protein might have been inadvertently created, or to the sites of insertion which lead to higher expression relative to the sites into which the wild type transgenes inserted. The role of NF-1, thus, remains unclear and will require additional study.

LS4 and LS5 mutations, which had effects similar to the NF-1 mutations, are within the GRE and the NR-1 sites (Fig. 1). The effects of these mutations are consistent with previous studies indicating that these two motifs are important for maximal PB induction [8,11]. Other mutations within the GRE region, LS2 which alters 2 nucleotides and LS3 which alters 6 nucleotides had relatively modest effects on responsiveness suggesting that the modification of the NR-1 site by LS4 and LS5 was most important for their effects. LS6, however, which changes two nucleotides in the NR-1 motif, had only modest effects which may reflect continued binding of CAR-RXR to this motif.

The remaining linker scanning mutations, LS7-12, all appear to have small negative effects on PB responsiveness, suggesting that elements in this region may contribute to the overall responsiveness but only modestly. This region includes NR-2 and the accessory factor region.

None of the mutations completely eliminated PB responsiveness, suggesting that multiple elements are PB responsive. This is in agreement with studies in which mutation of the NR-1, NR-2, NF-1, AF, and the GRE sequences reduced but did not eliminate PB responsiveness [6,8,9,11]. LS3 alters the GRE sequence, LS6 alters the NR-1 sequence, LS7-9 alter the NR-2 sequence, and LS10–12 alter the AF element, but only modestly reduced responsiveness. Notably mutation of both NR-1 and NR-2 eliminated the response in the mouse gene [11].

3.3. Gel shift assays

Our previous studies indicated that *in vitro*, NF1 binds strongly to the PBRU area but several protein complexes were still observed when NF1 sites were mutated [9] and several binding complexes were observed when a fragment from the *Nco*I site to the *Kpn*I site in LS6 (−2257 to −2226 of wild type sequence) was used as a probe. Since LS4 and LS5 mutations had the strongest effects on PB responsiveness, the binding of proteins to fragments with 5′ ends at −2257 generated by progressive 3′ deletions from the LS6 to LS4 *Kpn*I sites was examined. Deletion to the *Kpn*I site in LS5 (−2231) had little effect on the binding of proteins to the fragment (Fig. 4). This lack of change in protein binding contrasts with the substantial decrease in responsiveness of the LS5 mutant. The most likely functional effect of the LS5 mutant is the disruption of the NR-1 site and the binding of CAR-RXR to that site. However, even though CAR has been reported to be present in nuclear extracts from PB-treated animals [17], its differential binding to the NR-1 site within the PBRU has not been detected by gel-shift assays or DNase I footprinting nor have any other differences between control and PB-treated samples been detected [6,8,18]. The lack of a major change in protein binding between the deletions to LS6 and LS5 is consistent with these observations. Nevertheless, multiple complexes of protein with DNA are observed with this fragment and deletion to the *Kpn*I site in LS4 eliminated most of the binding complexes (Fig. 4). These results indicate that one or more proteins bind to the region −2237 to −2231 which potentially may modulate the action of CAR-

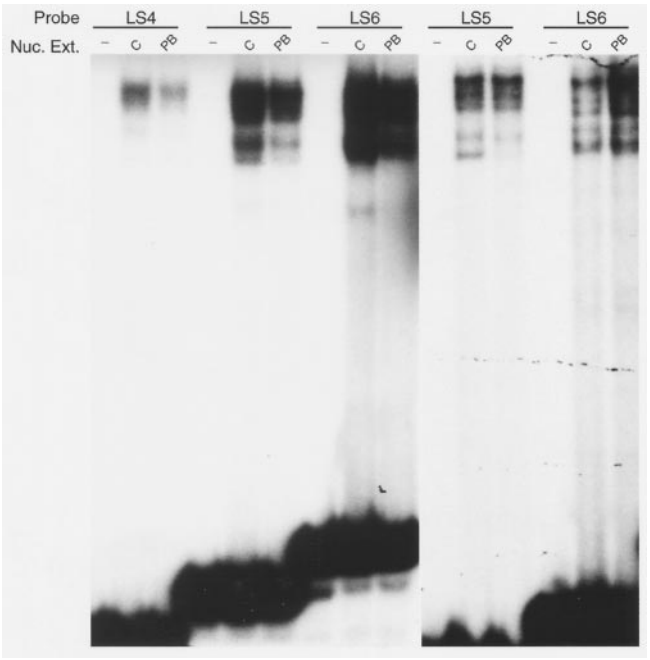

Fig. 4. Gel shift analysis of protein binding to the PBRU region 5′ of the NF-1 site. DNA fragments from the *Nco*I site (−2257) at the 5′ side to *Kpn*I sites in LS4 (−2238), LS5 (2232), or LS6 (−2226) were used as probes for gel shift assays with nuclear extracts isolated from livers of rats treated with isotonic saline (C) or 100 mg/kg PB for 6 h. The left panel is with one extract and the right is a second extract which is a shorter exposure to illustrate the five bands of protein-DNA complexes formed for the LS5 and LS6 fragments.

RXR which has been shown to bind to the NR-1 site extending from −2233 to −2219 [11].

3.4. DNase I footprinting analysis

To further assess the effects of mutations on protein binding to the PBRU region, protein binding to fragments with LS4 to LS10 and the NF-1 mutants was analyzed by DNase I footprinting. As shown previously [18], in the wild type fragment strong protection was observed in the region from −2200 to −2224, which largely corresponds to the NF-1 site and to the Hx/NR-1 half site to the 5′ side of the NF-1 (Fig. 5, WT, marked by a bar). A second protected region is observed at −2238 to −2242. The LS4, LS5, and LS6 mutations did not have detectable effects on the protection pattern except that LS5 and LS6 mutations altered the DNase I cleavage pattern in purified DNA so that new bands were present in the −2224 to −2234 region. The LS5 and LS6 results are consistent with the gel shift assays since the complexes formed with these two mutations were similar. The LS4 mutation, however, did not detectably alter the footprint even though binding as assayed by the gel shift assay was decreased. The reason for this discrepancy is not clear. It is clear, however, that the dramatic effects of the mutations of LS4 and LS5 do not correlate with dramatic changes in protein binding detected *in vitro* which is consistent with the lack of effect of PB treatment on footprints on the wild type PBRU.

The mutation of the 5′ part of the bipartite NF-1 site (−2214 to −2217) or both the 5′ and 3′ (−2205 to −2208) NF-1 bipartite sites eliminated the protection from −2207 to −2224 which corresponds to the NF-1 and HX/NR-1 half site binding regions. No protection was observed in the HX/NR-1 region even though the mutations did not alter this nuclear receptor half site. In contrast, a new footprint in the region of −2192 to −2204 is observed which corresponds to the NR-2 sequence. Mutation of only the 3′ part of the NF-1 bipartite site (Fig. 5, NFM2), eliminated protection in the −2207 to −2214 region, which indicated that NF-1 binding was lost, but in contrast to mutation of the 5′ part of the NF-1 motif, protection was observed between −2218 and −2224 within the HX/NR-1 half site and protection was present in the region −2192 to −2204 corresponding to the NR-2 site. This latter result is consistent with continued binding in the HX/NR-1 half site when the 3′ part of the NF-1 bipartite site was mutated in the mouse gene [6]. Binding to the HX/NR-1 site has also been reported after mutation of both parts of the NF-1 site in the rat *CYP2B2* gene, but in this case the 5′ part was a point mutation of residue −2217 [8]. The identity of the proteins that bind in the NR-1 and NR-2 regions after mutation of the NF-1 site is not known, but there is no difference in the binding in the control and PB-treated samples suggesting that it is not a CAR-RXR heterodimer, which is enriched in extracts from PB-treated animals [17]. This suggests that other proteins may compete for the binding of CAR-RXR to these regions which could be important in suppressing transcription in the untreated animal or modulating the function of CAR-RXR after PB treatment.

The LS8, LS9 and LS10 mutations had little effect on the binding in the PBRU region. The most obvious effect of mutation LS7, and to a lessor effect with LS8, was a decrease in protection of bands within the NF-1 site at −2107 and −2218 (Fig. 5, LS7, asterisks). These results suggest an interaction between proteins interacting with the LS7/LS8 regions, which includes the NR-2 sequence, and the NF-1 region. Similarly as noted above, altered binding in the NR-2 region when NF-1 binding is disrupted would be consistent with an interaction between proteins binding at NF-1 site and in the NR-2 region as well.

3.5. Conclusions

A core sequence which includes an NF-1 site flanked by two nuclear receptor sites, NR-1 and NR-2, is critical for PB induction. The NF-1 site is not essential for PB induction since mutation of this site does not eliminate induction in transient transfections or transgenic mice and the human PBRU has a mutated NF-1 site [6,8,9,12,15]. NR-1 and NR-2 appear to be redundant sequences since both must be

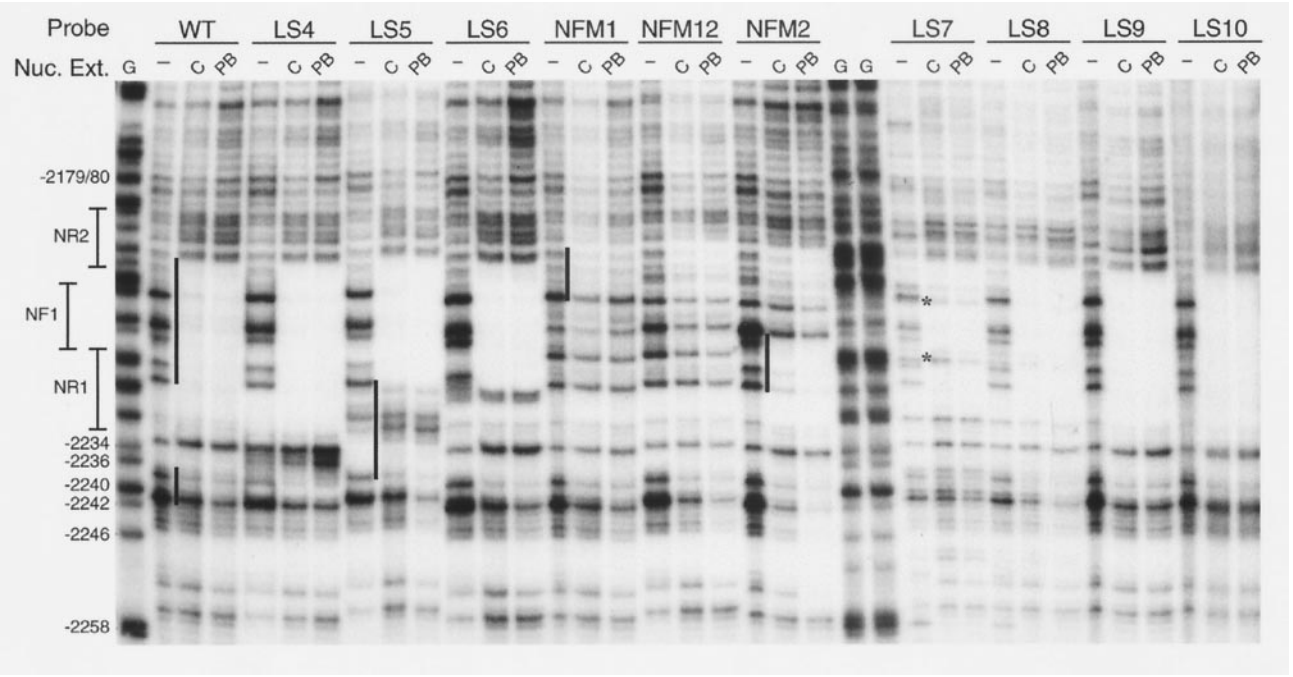

Fig. 5. DNase I footprinting analysis of mutated PBRU fragments. *Eco*R1/*Hin*dIII fragments, ^{32}P-labeled at the *Eco*R1 site to the 5′ side of the PBRU were incubated with liver nuclear extracts from rats treated with isotonic saline (C) or 100 mg/kg PB for 6 h, digested with DNase I and separated by electrophoresis in denaturing polyacrylamide gels as described in Materials and methods. Each fragment was also digested with DNase I in the absence of nuclear proteins (free). The wild type fragment partially cleaved at G residues (G) served as a size marker and the positions of some of the G-residues (C's on the sense strand) are indicated at the left. The positions of the NR-1, NF-1 and NR-2 sites are indicated. Bars within the autoradiogram indicate footprint regions for wild type (WT), NFM1, and NFM2, and a region of new DNase I cleavage sites introduced by the mutations in LS5. Asterisks in LS7 indicate the positions of decreased susceptibility to DNase I in the NF-1 region.

deleted to eliminate PB responsiveness [11]. Binding of CAR/RXR heterodimers to NR-1 and the presence of CAR only in nuclear extracts from PB-treated provide compelling evidence for a critical role for these factors in the response to PB [11]. However, the present studies indicate that other proteins may bind to the NR-1 and NR-2 regions as well and potentially, therefore, could contribute to the regulation by the PBRU. In the rat gene, additional sequence flanking the NR-1, NF-1, and NR-2 core is required for full PB responsiveness [8,9, this study]. Further, in contrast to the partial loss of PB induction when NR-1 and NR-2 are mutated individually, fragments derived by 5′ or 3′ deletions become nonresponsive when the NR-1 from the 5′ side or NR-2 from the 3′ side are partially deleted. This suggests that sequences flanking the central core are important to the PB responsiveness and loss of these flanking regions accentuates the loss of the NR-1 or NR-2 binding sites. In contrast, in the mouse, full activity is observed with a fragment containing only the core of NF1, NR-1, and NR-2 [10] and multiple copies of NR-1 alone are able to mediate PB induction in HepG2 cells stably expressing CAR [12]. In spite of these differences, both the protein binding studies and the functional assays are consistent with a multi-protein complex at the PBRU mediating the increase in transcription with the effects of PB on CAR-RXR translocation to the nucleus triggering the activation.

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