Phenobarbital Alters Protein Binding to the CYP2B1/2 Phenobarbital-responsive Unit in Native Chromatin*

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Jongsook Kim and Byron Kemper†
From the Department of Molecular and Integrative Physiology and the College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Phenobarbital is a classical inducer of the drug metabolizing cytochrome P450 genes, but the molecular mechanism of induction has not been elucidated. Functional analyses have identified a phenobarbital-responsive unit in the rat CYP2B1/2 and mouse Cyp2b10 genes about 2.3 kilobase pairs from the transcriptional start site, but little or no changes in protein binding to this region were observed in vitro. To examine the role of chromatin structure, protein binding to the phenobarbital-responsive unit assessed by in vitro DNase I footprinting was compared with that assessed by DNase I in vivo footprints in native chromatin. A region centered on a putative nuclear factor-1 site was the major protected region in in vitro footprints, and there were no detectable differences in binding between extracts from control and phenobarbital-treated animals. In contrast, phenobarbital treatment dramatically altered the protection pattern in native chromatin. In control samples a core region of about 25 base pairs (bp) centered on the nuclear factor-1 site was protected. However, after phenobarbital treatment, the protection of this core region increased, and more dramatically the region of protection was extended 20 bp to either side so that a total of about 60 bp were protected. These results provide the first evidence that phenobarbital treatment alters the composition or architecture of proteins binding to the phenobarbital-responsive unit region and indicate that chromatin structure is important in this process. Because proteins are bound to the region in the untreated animal, the mechanism of induction involves the activation of proteins bound to the region and possibly recruitment of additional regulatory proteins rather than conversion of a closed chromatin structure to an open one that can bind regulatory factors.

Phenobarbital (PB)† has pleiotropic effects in the liver, affecting the mRNA levels of as many as 50 genes (1) as well as cellular morphology. The regulation of the microsomal drug metabolizing system by PB has been known for about 40 years (2), but the mechanism of transcriptional activation of the genes for cytochromes P450, the key enzymes in this system, is not understood. The rat CYP2B1 and CYP2B2 genes have nearly identical sequences in the proximal promoter and PBRU regions and are induced by PB by at least 200- and 40-fold, respectively (3). A PB-responsive enhancer region has been identified at about 2.3 kilobase pairs in the rat CYP2B1/2 (4, 5), and an analogous region is present in the mouse (6) Cyp2b10 genes. Multiple regulatory elements contribute to the PB effect so that this enhancer has been termed the PB response unit (PBRU) or module (6). Despite the functional data implicating the PBRU in the PB response, analysis of protein binding by either gel shift assays in rat (4) and mouse genes (6) or by in vitro footprinting in the mouse gene (6) has revealed little or no change in protein binding to this region after PB treatment. Here we show that a 25-bp core region of the PBRU DNA is protected from DNase I in native chromatin in rat liver nuclei from control animals and that PB treatment increases the protection from DNase I digestion in the core region and substantially extends the strongly protected region to 60 bp. These results indicate that PB treatment alters the composition or structure of the protein complex binding to the PBRU in native chromatin.

EXPERIMENTAL PROCEDURES

In Vitro Footprinting—Rats were injected intraperitoneally with either 100 mg/kg PB or isotonic saline and were sacrificed 6 h later. The livers were removed, and nuclear extracts were prepared as described (7). For in vitro DNase I footprinting analysis, end-labeled DNA fragments containing the PBRU were prepared by PCR using the labeled primers S3 or A3 (see Fig. 3) for the sense or antisense strand, respectively. Binding conditions were as described previously for gel shift assays (8). 40,000 cpm of the probes were incubated at 0 °C for 6 min in the absence or the presence of 30 μg of protein of rat liver nuclear extracts. 2.5–12.5 or 150 ng of DNase I for the naked DNA or DNA incubated with proteins, respectively. Deoxyribonucleotide DNA sequencing ladders were used as markers.

In Vivo Footprinting—Rats were treated as above, nuclei were isolated from the liver and incubated with 90–120 μg/ml DNase I for 10 min at 0 °C, and genomic DNA was isolated as described (9). Purified genomic DNA was incubated with 0.5–2.5 × 10−5 units DNase I/μg DNA at 37 °C for 5 min. 2–3 μg of the genomic DNA was subjected to ligation-mediated PCR as described (10). Annealing temperatures for the primers S1/A1, S2/A2, and S3/A3 were 56, 60, and 67 °C, respectively. A G-ladder as a marker was generated by ligation-mediated PCR of purified genomic DNA that had been incubated with 0.5% dimethylsulfate at room temperature for 2 min followed by piperidine treatment as described (10). Reproducible results were obtained with five to seven ligation-mediated PCR reactions for each strand from two sets of control and PB-treated rats.

RESULTS

In Vitro DNase I Footprinting of the CYP2B1/2 PBRU—Incubation in vitro of the CYP2B1/2 PBRU region with liver nuclear extracts from control and PB-treated rats resulted in DNase I protection patterns that were very similar (Fig. 1) as was also observed with the mouse Cyp2b10 gene (6). Strong protection was detected for both the antisense and sense strands from about −2180 to −2205, which includes an NF-1 motif that has been shown to bind to proteins that react with antisense to NF-1.‡ A second region of protection was observed...
from about −2221 to −2234. Several hypersensitive sites were detected in the regions from −2144 to −2173, from −2213 to −2217, and at −2237 flanking the protected regions. Similar footprints in the control and PB-treated samples suggest that PB does not change the concentration or affinity of proteins binding to the PBRU region, although binding of different proteins to the same site cannot be excluded.

In Vivo DNase I Footprinting of the CYP2B1/2 PBRU—In contrast to the in vitro footprints, binding of proteins to the PBRU in native chromatin was dramatically altered by 6 h after PB treatment. In liver nuclei from control rats, strong protection of both the antisense and sense strands was observed from about −2180 to −2210, and hypersensitive sites were observed at −2162/−2170 and −2225/−2227 (Fig. 2). The protected region, centered on the NF-1 site, was very similar to the protection observed in the in vitro footprints, and the hypersensitive sites were within regions of hypersensitivity observed in vitro, although the protected region from −2221 to −2234 observed in the in vitro experiment was not present (Fig. 3). In the footprints from nuclei of PB-treated rats, the level of protection within the control footprint region was increased, and more dramatically the footprints for both the antisense and sense strands were extended about 20 bp to either side of the control footprints (Fig. 2). The total protected region was 60 bp from −2163 to −2222 (Figs. 2 and 3). The extended protection was particularly evident for the region from about −2163 to −2182. Interestingly, this region exhibited DNase I hypersensitivity in both control and PB-treated in vitro footprints, which is suggestive of protein interaction. Reproducible results were obtained in samples from two sets of rats analyzed independently as shown.

DISCUSSION

Protein binding to a region centered on the NF-1 site is consistent with functional analysis of the region. In CYP2B1/2, a core sequence of 37 bp centered on the NF-1 site was necessary but not sufficient for PB induction. Sequence from either side of the NF-1 region was able to confer PB inducibility extending to either −2258 on the 5′ side or to −2170 on the 3′ side. Likewise, in the mouse Cyp2b10 gene, a corresponding 32-bp core sequence was defined by transcriptional analysis, and an additional flanking sequence from either side of the core was required to confer maximal PB induction (6). The region from −2170 to −2258, defined functionally, largely overlaps the protected region in chromatin in the PB-treated samples from −2161 to −2223. In agreement with the functional studies, protein is bound to the CYP2B1/2 core region in both the control and PB-treated samples in native chromatin, whereas the binding extends further into the flanking regions in the PB-treated samples.

Because purified NF-1 protects a region of about 25 bp (11), the protection of 24–29 bp in the control native chromatin samples may be primarily the result of NF-1 binding. The similarity of this binding with that in vitro and the observation that the affinity of NF-1 for DNA in nucleosomal structures was decreased (12) suggests that the DNA in the chromatin at the NF-1 site is in an open conformation. This conclusion is also supported by the detection of DNase I hypersensitivity in PBRU chromatin of both control and PB-treated animals (13). The protection of 60 bp in the PB-treated samples, which differs from the in vitro binding, indicates that protein binding alters the chromatin structure, which then influences the binding. The extended protection after PB treatment compared with both control native chromatin and in vitro footprints suggests that PB-induced changes in the chromatin structure facilitate additional binding of proteins to the PBRU either di-
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directly or by binding to NF-1 to form a larger complex. Although evidence has been presented for PB-responsive elements in the proximal promoter regions of CYP2B1/2 (14, 15), most recent data favor a PB-responsive enhancer at about −2.3 kilobase pairs as the primary mediator of the response to PB. The enhancer region from the rat CYP2B1/2 genes or the homologous region from the mouse Cyp2b10 gene has been shown to mediate the response in transfected primary hepatocytes or after transfection of rat liver cells or after transfection of rat liver genes or the homologous region from the mouse Cyp2b10 gene has been shown to mediate the response in transfected primary hepatocytes or after transfection of rat liver cells or after transfection of rat liver.

Two basic models have been proposed for ligand-mediated transcriptional activation. In first model, proteins are not bound to the regulatory region until a ligand-induced change in the chromatin structure enables the binding of positive regulatory factors (17, 18). Interestingly, in the two cited studies, NF-1 is a key protein that binds to chromatin in response to ligand treatment. In the second model, regulatory proteins are bound in the uninduced state but are either inactive or suppressed by negative coregulators. The ligand activates the factors directly or indirectly by altering chromatin conformation, so that binding of positive coregulators is favored over that of negative coregulators (19). The results presented in this paper are most consistent with the second of these mechanisms, because strong binding of protein, possibly NF-1, to the PBRU in chromatin is observed in the untreated animal, in which liver expression of CYP2B1 is undetectable and that of CYP2B2 is very low (20). The observation that binding of proteins to the PBRU in vitro is not affected by PB treatment is also consistent with the second mechanism, in which activity or conformation of proteins already bound to the DNA is affected. The alteration of the protected region in native chromatin by PB treatment suggests that either the conformation of the binding proteins is changed or additional proteins are recruited to the regulatory protein complex and that the change is dependent on the chromatin structure. The most straightforward interpretation of these results is that NF-1 is bound in the untreated animal in an inactive state. PB treatment results in a change in chromatin structure, which results in recruitment of coregulators and/or additional DNA binding regulatory factors producing a transcriptionally active regulatory complex. This process may be accompanied by further modifications of chromatin such as histone acetylation, which could result from recruitment of activator coregulators or their exchange for suppressor coregulators, which have histone acetylase and deacetylase activities, respectively (21–23). Characterization of the complex of proteins that bind to the PBRU will be required to establish the mechanism.

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