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Differences in Deoxyribonuclease I Hypersensitive Sites in Phenobarbital-Inducible and Constitutive Rabbit P450IIC Genes[†,‡]

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ABSTRACT: DNase I hypersensivity of nuclear chromatin near the rabbit cytochrome P450IIC genes was investigated by indirect end-labeling genomic Southern analysis. Major DNase I hypersensitive sites were observed in proximal (−200 base pairs) and distal (−2000 to −2200 base pairs) regions of the genes. The presence of the proximal site correlated well with the expression states of the individual genes. In contrast, the distal site was present in DNA from both liver and kidney nuclei and in untreated and phenobarbital-treated animals irrespective of the expression state of the genes. However, the distal site was present only in genes that respond to phenobarbital (cytochromes P450IIC1 and P450IIC2) and was not detected in the constitutive cytochrome P450IIC3 gene. The nucleotide sequences of 500 base pairs in the distal site regions of cytochromes P450IIC1 and P450IIC2 were 67% similar, and hepatocyte nuclear factor 1 like motifs were conserved in these two sequences. These results are consistent with the hypothesis that distal regions cooperate with the proximal promoter regions in the regulation of cytochrome P450IIC gene expression.

Cytochrome P450 (P450)[1] genes encode microsomal hemoproteins that are terminal monooxygenases involved in the metabolism of many xenobiotic and endogenous substances (Nebert & Gonzalez, 1987). On the basis of nucleotide and amino acid similarity, the P450s form a multigene superfamily containing 27 families of which 10 are present in mammalian species (Nebert et al., 1991). This classification roughly correlates with the induction of P450 by various compounds.

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[‡] The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers M76596 and M76597.
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[1] Abbreviations: P450, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kbp (kb in figures), kilobase pair(s); bp, base pair(s).

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Phenobarbital is an inducer for genes in the P450IIB[2] and P450IIC subfamilies and for the P450III family (Gonzalez, 1989). The induction of P450II genes by phenobarbital has been shown to occur at least in part at the level of gene transcription (Hardwick et al., 1983; Pike et al., 1985; Zhao et al., 1990). The molecular mechanism for phenobarbital induction of P450 genes is not known. A receptor for phenobarbital has not been identified, and the DNA sequences in the genes responsible for phenobarbital responsiveness have not been defined.

The rabbit P450IIC subfamily genes expressed in liver respond to phenobarbital in a heterogeneous fashion (Leighton et al., 1984; Tukey et al., 1985; Imai, 1987; Zhao et al., 1987). The mRNA of P450IIC1 is not detectable by Northern analysis in control animals, and expression is highly induced by phenobarbital treatment (Leighton & Kemper, 1984). P450IIC2, P450IIC3, P450IIC4, and P450IIC5 genes are expressed in untreated animals, and P450IIC2 and P450IIC4 mRNA concentrations are increased severalfold by phenobarbital treatment (Leighton & Kemper, 1984; Tukey et al., 1985; Zhao et al., 1987). Of these genes, only P450IIC2 is expressed in the kidney. To examine differences that might underlie the varied responses to phenobarbital and the tissue-specific expression, we have been studying the chromatin structure of the P450IIC genes. DNase I hypersensitive sites are often present near genes that are actively being transcribed and are thought to represent nucleosome-free regions of the genome that are accessible for binding of transcriptional regulatory proteins (Essienberg et al., 1985; Gross & Garrard, 1988; Elgin, 1988). Comparison of such regions in the P450IIC genes, therefore, may localize regions of the genes important for the regulation of their expression. In this paper, we report that P450IIC genes responsive to phenobarbital contain a major DNase I hypersensitive site 2 kbp to the 5′ side of the RNA initiation site which is not present in the constitutive P450IIC3 chromatin. A proximal site near the promoters was also detected, and its presence correlated with the expression states of the genes.

Materials and Methods

Materials. Restriction enzymes were purchased from Bethesda Research Laboratories, DNase I was from Sigma, *Escherichia coli* DNA polymerase I Klenow fragment was from Boehringer Mannheim, Sequenase and sequencing kits were from United States Biochemical Corp., and Hybond nylon membranes were from Amersham.

Isolation of Nuclei. Adult male New Zealand rabbits were starved overnight, weighed, and injected intraperitoneally 4 h before sacrifice with 1 mL/kg of 10% phenobarbital, adjusted to pH <10.5 with 4 N NaOH. Rabbits were sacrificed by injection of 0.3 mL/kg Euthanasia T-61 through ear veins.

Isolation of nuclei was performed as described by Burch and Weintraub (1983) with minor modifications. All steps described below were carried out between 0 and 4 °C. Liver and kidney tissues were removed and immediately put into saline solution (0.9% NaCl). Five grams of each tissue was minced with a razor blade, and 40 mL of 1× SSC (0.15 M NaCl and 0.015 M sodium citrate)/10 mM Tris-HCl, pH 7.5, was added. After 5 min, the supernatant was decanted, and 20 mL of homogenization buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.05 mM EGTA, 0.5 mM EDTA, 0.15 mM spermidine, and 0.5 mM spermine), 0.5% NP40, and 1 mM phenylmethanesulfonyl fluoride were added. The tissues were homogenized with 5 strokes with a loose Dounce pestle, followed with at least 7 strokes with a tight pestle for liver tissues, and 2–4 strokes for kidney tissues until 90% of the cells were broken. The nuclear suspensions were filtered through nine layers of cheesecloth and washed 3 times with homogenization buffer containing 0.1% Nonidet-P40. The final pellets were resuspended in nuclease digestion buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM EGTA) and adjusted to a DNA concentration of about 1 mg/mL as estimated by the absorbance at 260 nm.

For DNase I digestion of isolated nuclei, DNase I solutions were serially diluted in DNase I dilution buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 23 mM $MgCl_2$, 12 mM $CaCl_2$, and 0.5 mg/mL bovine serum albumin) just before use. Eighty-eight microliters of the diluted DNase I solution was added to 880 μL of the nuclear suspensions, and the samples were incubated at 0 °C for 10 min. DNase I digestion was stopped by addition of 130 μL of 77 mM EGTA, 15 mM EDTA, 2 M NaCl, and 0.8 mg/mL proteinase K. The sample was incubated at 37 °C for 1 h, 0.01 volume of 10% sodium dodecyl sulfate solution was added, and the incubation was continued overnight. The DNA was isolated by extraction 1 time with phenol, 3 times with phenol/chloroform (1:1), 2 times with chloroform, and 2 times with 1-butanol and then precipitated with ethanol.

Indirect End-Labeling Genomic Southern Analysis. Purified genomic DNA was cleaved with 3 units/μg of a restriction enzyme (*Eco*RI, *Hin*dIII, *Pst*I, or *Xba*I) overnight (at least 8 h) according to the supplier's recommendations. At the end of the digestion, 2 μL of 10 mg/mL RNase A was added for 20 min. DNA was purified by extraction with phenol/chloroform (1:1) and chloroform followed by ethanol precipitation. DNase I digested genomic DNA was separated by electrophoresis, transferred to a Hybond nylon membrane (Amersham), and hybridized (Southern, 1975). DNA fragments used as probes were prepared from genomic DNA subcloned into pTZ18R or pTZ19R vectors (Mead et al., 1986). After cleavage by restriction enzymes, fragments were isolated by electrophoresis in low melting temperature agarose gels and labeled to a specific activity of at least 3×10^8 cpm/μg of DNA by the random hexamer primer labeling method (Maniatis et al., 1982).

Prehybridization and hybridization were carried out at 65 °C as described by the manufacturer (Amersham). Following hybridization, the filters were washed twice in 2× SSC and 0.1% sodium dodecyl sulfate at room temperature for 15 min, in 0.5× SSC and 0.1% sodium dodecyl sulfate for 15 min at 65 °C, and in 0.05× or 0.1× SSC and 0.1% sodium dodecyl sulfate at 65–67 °C for at least 15 min. Washed filters were exposed to Dupont Cronex X-ray film with intensifying screens for 2–7 days. To reuse filters, the probes were removed by gently shaking the membranes in 0.4 M NaOH at 45 °C for 30 min followed by washing with 0.1× SSC, 0.1% sodium dodecyl sulfate, and 0.2 M Tris-HCl, pH 7.5, at 45 °C for 30 min as described by the manufacturer (Amersham Corp.).

Representative autoradiograms are presented. Each experiment was repeated 3–5 times, and each DNase I hypersensitive site was observed consistently in at least three experiments.

Screening of the Rabbit Genomic Library. About 500 000 plaques from a rabbit genomic DNA library in λ phage EMBL 3 (Clontech, Inc.) were screened in duplicate by plaque filter hybridization according to Maniatis et al. (1982). The hybridization probe was the 0.8 kbp *Pst*I/*Nco*I fragment from

[2] P450s are named as previously described (Chan & Kemper, 1990; Zhao et al., 1990). According to the recently published nomenclature for P450s (Nebert et al., 1991), P450 is replaced by CYP and Roman numerals by Arabic numbers, e.g., P450IIC1 = CYP2C1.

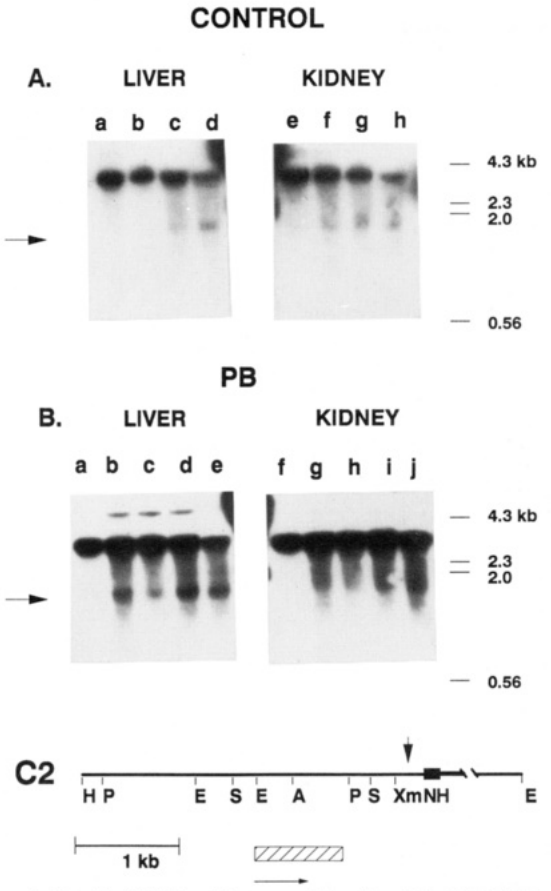

FIGURE 1: Proximal DNase I hypersensitive site of the P450IIC2 gene. Liver and kidney nuclei from a control rabbit (A) were digested with 0 (a, e), 4 (b, f), 8 (c, g), or 12 (d, h) μg/mL DNase I. Liver and kidney nuclei from a phenobarbital-treated rabbit (B) were incubated with 0 (a, f), 4 (b, g), 6 (c, h), 8 (d, i), or 12 (e, j) μg/mL DNase I at 0 °C for 10 min. The genomic DNA was cleaved with *Eco*RI and separated by electrophoresis on a 1.8% agarose gel. The *Eco*RI/*Pst*I fragment (hatched rectangle, arrow indicates direction of scan) was used as a probe to detect the major proximal DNase I hypersensitive site shown as thick arrows. At the bottom, a restriction map of the P450IIC2 5′-flanking region is shown with the solid rectangle representing exon 1. Abbreviations for restriction sites used in all figures are: H, *Hin*dIII; P, *Pst*I; S, *Sac*I; E, *Eco*RI; X, *Xba*I; B, *Bam*HI; N, *Nco*I; A, *Alu*I; Xm, *Xmn*I; Ev, *Eco*RV; Hp, *Hph*I; Bc, *Bcl*I.

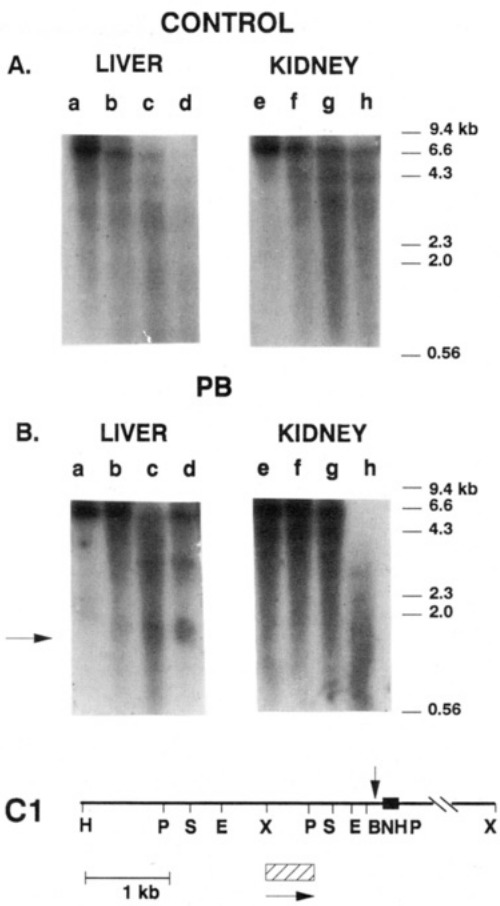

FIGURE 2: Proximal DNase I hypersensitive site of the P450IIC1 gene. Liver and kidney nuclei were isolated from control (A) and phenobarbital-treated (B) rabbits and digested with 0 (a, e), 4 (b, f), 8 (c, g), or 16 (d, h) μg/mL DNase I at 0 °C for 10 min. The genomic DNA was purified, cleaved with *Xba*I, and separated by electrophoresis on a 1.5% agarose gel. The *Xba*I/*Pst*I fragment shown as a hatched rectangle was used as a probe. The proximal DNase I hypersensitive site detected only in phenobarbital-treated liver samples is marked with an arrow. The restriction map of the 5′-flanking region of the P450IIC1 gene is indicated at the bottom. The sizes of fragments of *Hin*dIII-digested λ phage DNA are indicated. See Figure 1 for restriction site abbreviations.

the 5′-flanking region of the P450IIC2 gene which was labeled by random hexamer labeling to a specific activity of 5×10^8 cpm/μg. Hybridization was carried out at 65 °C for 18 h in the presence of hybridization solution. The positive λ phage DNA was restricted by *Hin*dIII and *Eco*RI enzymes, and Southern analysis (Southern, 1975) was carried out to find a fragment containing the 5′-flanking region of the P450IIC2 gene using the same *Pst*I/*Nco*I fragment as a probe. A 3.4 kbp *Hin*dIII fragment containing the major distal DNase hypersensitive site of the P450IIC2 gene was subcloned into pTZ18R (Mead et al., 1986), and sequencing was performed in the promoter region of the P450IIC2 gene to confirm the identity of the subcloned fragment.

Dideoxy Chain Termination Sequencing. Plasmid DNA was isolated by the boiling method (Holmes & Quigley, 1981) and was purified with glass milk as recommended by the manufacturer (Bio 101). The DNA sequence was determined by dideoxynucleotide sequencing on double-stranded DNA (Sanger et al., 1977).

RESULTS

DNase I Hypersensitive Sites near the RNA Initiation Site in Phenobarbital-Inducible P450IIC Genes. The P450IIC2 gene is constitutively expressed in both the liver and kidney, and phenobarbital treatment increases its expression about 3-fold (Leighton & Kemper, 1984). In active genes, a DNase I hypersensitive site is often present near the transcription initiation site. To analyze this region in the P450IIC2 gene, genomic DNA digested with *Eco*RI, which produces a 3.8 kbp fragment, was probed with an *Eco*RI/*Pst*I fragment (Figure 1). DNase I digestion produced a major new fragment of 1.6 kbp corresponding to a hypersensitive site at −200 bp. This site was seen in liver and kidney samples with and without phenobarbital treatment. These results correlate well with the expression pattern of P450IIC2.

Compared to P450IIC2, mRNA of P450IIC1 is not detected by Northern analysis in liver from control rabbits, and expression is highly induced by phenobarbital treatment (Leighton & Kemper, 1984). To examine DNase I hypersensitivity near the promoter region for the P450IIC1 gene, genomic DNA digested with *Xba*I was probed with a *Xba*I/*Pst*I fragment. In the liver samples from phenobarbital-treated animals, a major subfragment of 1.5 kbp was observed which corresponds to a DNase I hypersensitive site at about −200 bp (Figure 2). This site was not detected in the kidney or control liver samples, which is consistent with the lack of expression of the P450IIC1 gene under these

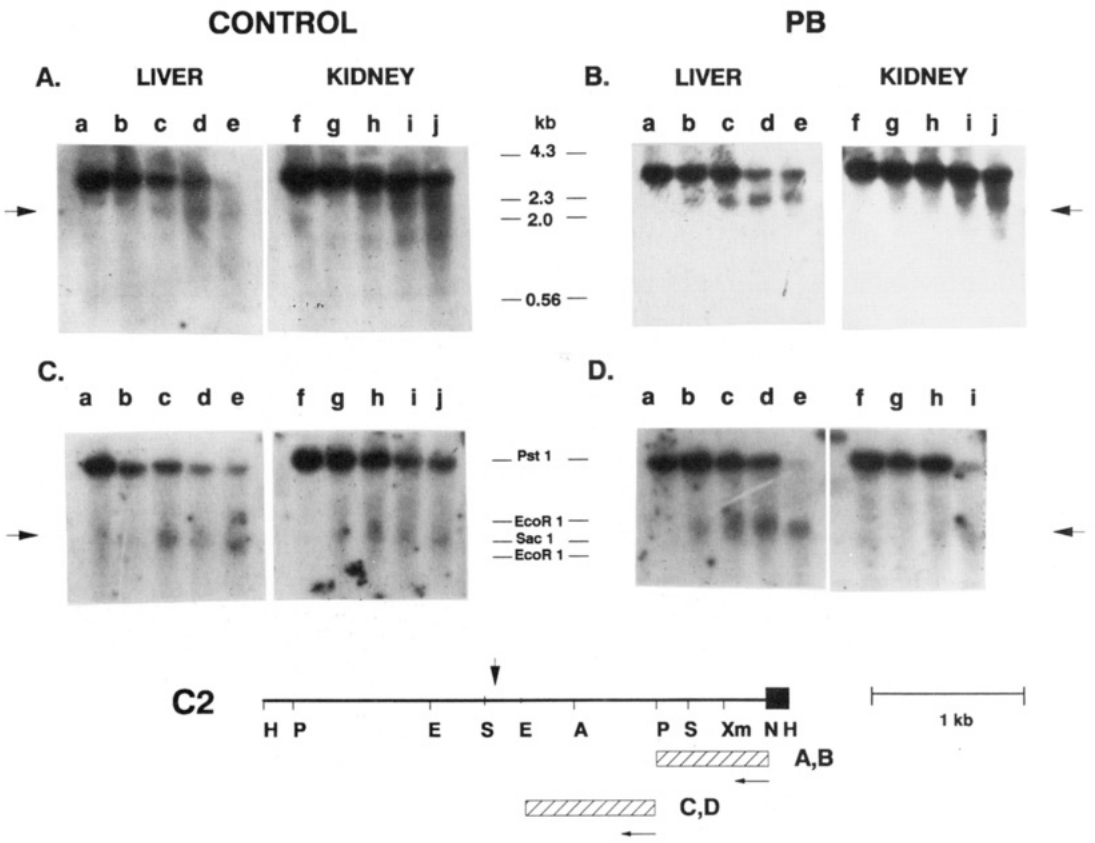

FIGURE 3: Distal DNase I hypersensitive sites in the 5′-flanking region of the P450IIC2 gene. The thick arrows correspond to major DNase I hypersensitive sites, and the thin arrows indicate the direction of the scan. A restriction map of the P450IIC2 5′-flanking region is shown at the bottom. See Figure 1 for restriction site abbreviations. (A, B) Liver and kidney nuclei were isolated from control (A) and phenobarbital-treated (B) rabbits and incubated with 0 (a, f), 4 (b, g), 8 (c, h), 12 (d, i), or 16 (e, j) μg/mL DNase I. Genomic DNA was cleaved with *Hin*dIII, and 5 μg of each sample was separated by electrophoresis on a 1% agarose gel. DNase I hypersensitive sites were detected using a *Pst*I/*Nco*I probe, indicated as a hatched rectangle (A, B) at the bottom with the arrow indicating the direction of the scan. The positions of fragments from λ phage DNA digested with *Hin*dIII are shown. (C, D) For analysis at moderate resolution, liver and kidney nuclei were incubated at 0 °C for 10 min with 0 (a, f), 4 (b, g), 8 (c, h), 12 (d, i), or 16 (e, j) μg/mL DNase I for the control (C) samples and with 0 (a, f), 4 (b, g), 8 (c, h), 12 (d), or 16 (e, i) μg/mL DNase I for the phenobarbital-treated (D) rabbits. The DNase I treated genomic DNA was purified and digested with *Pst*I, and 10 μg of DNA was separated by electrophoresis on a 1.5% agarose gel. The *Eco*RI/*Pst*I fragment nearer to the major distal DNase I hypersensitive site was selected as a probe to improve the resolution of Southern analysis [hatched rectangle (C, D)]. Internal size markers were prepared by digestion with *Sac*I, and partial digestion with *Eco*RI of the 2.4 kbp *Pst*I fragment and their positions are shown.

conditions (Leighton & Kemper, 1984). Larger subfragments were also detected after DNase I treatment which corresponded to hypersensitive sites in intron 1 of the gene. The significance of these sites, which did not correlate with gene expression, is not known.

DNase I Hypersensitivity in the Distal Region of the 5′-Flanking Regions of Phenobarbital-Inducible P450IIC Genes. (*A*) *P450IIC2*. To examine DNase I hypersensitivity in the distal 5′-flanking region of the P450IIC2 gene, rabbit genomic DNA was digested with *Hin*dIII which produces a 3.4 kbp fragment extending from +100 bp to about −3.3 kbp. An 800 bp *Nco*I/*Pst*I fragment which hybridizes to the downstream end of the *Hin*dIII fragment was used as a probe. Mild DNase I digestion of nuclei resulted in one major subfragment which corresponded to cleavage at a DNase I hypersensitive site at −2.0 kbp (Figure 3A,B). This hypersensitive site was present in both liver and kidney tissue from untreated and phenobarbital-treated animals (Figure 3A,B).

To define more precisely the location of the major hypersensitive site at −2.0 kbp, the *Pst*I-digested genomic DNA was probed with an *Eco*RI/*Pst*I fragment that binds closer to this upstream site. A major subfragment of about 1.2 kbp was observed which is consistent with a hypersensitive site at −2.0 kbp (Figure 3C,D). A subcloned *Pst*I fragment of the P450IIC2 gene that was digested with *Sac*I and partially with *Eco*RI was used as an internal marker. The internal markers established that the major DNase I hypersensitive site is near the *Sac*I site (Figure 3, bottom). The general patterns for DNA isolated from either liver or kidney tissues from control and phenobarbital-treated animals were similar.

(*B*) *P450IIC1*. To analyze the hypersensitive sites in the distal regions of the P450IIC1 gene, genomic DNA was digested with *Hin*dIII, which produces a fragment of 3.8 kbp. This fragment can be distinguished in size from the 3.4 kbp *Hin*dIII fragment of the P450IIC2 gene, demonstrating that the hybridization probes are specific for each of these two genes. Digestion of nuclei with DNase I resulted in the appearance of a major subfragment of 2.3 kbp (Figure 4A,B). Since the 5′ end of the hybridization probe is at +100 bp, this band corresponds to a hypersensitive site at −2.2 kbp. Similar to the observations with P450IIC2, this band was detected in liver and kidney samples with and without phenobarbital treatment. Minor subfragments were also observed, most prominently in the kidney samples, and are of unknown significance.

To better map the major site at −2.2 kbp, the genomic DNA digested with *Hin*dIII was probed with a *Hin*dIII/*Pst*I fragment that binds to the 5′ end of the *Hin*dIII fragment. A major subfragment of 1.4 kbp was observed which is consistent with the hypersensitive site at −2.2 kbp (Figure 4C,D). The subcloned *Hin*dIII fragment of P450IIC1, digested with several restriction enzymes, was used as an internal marker, and the

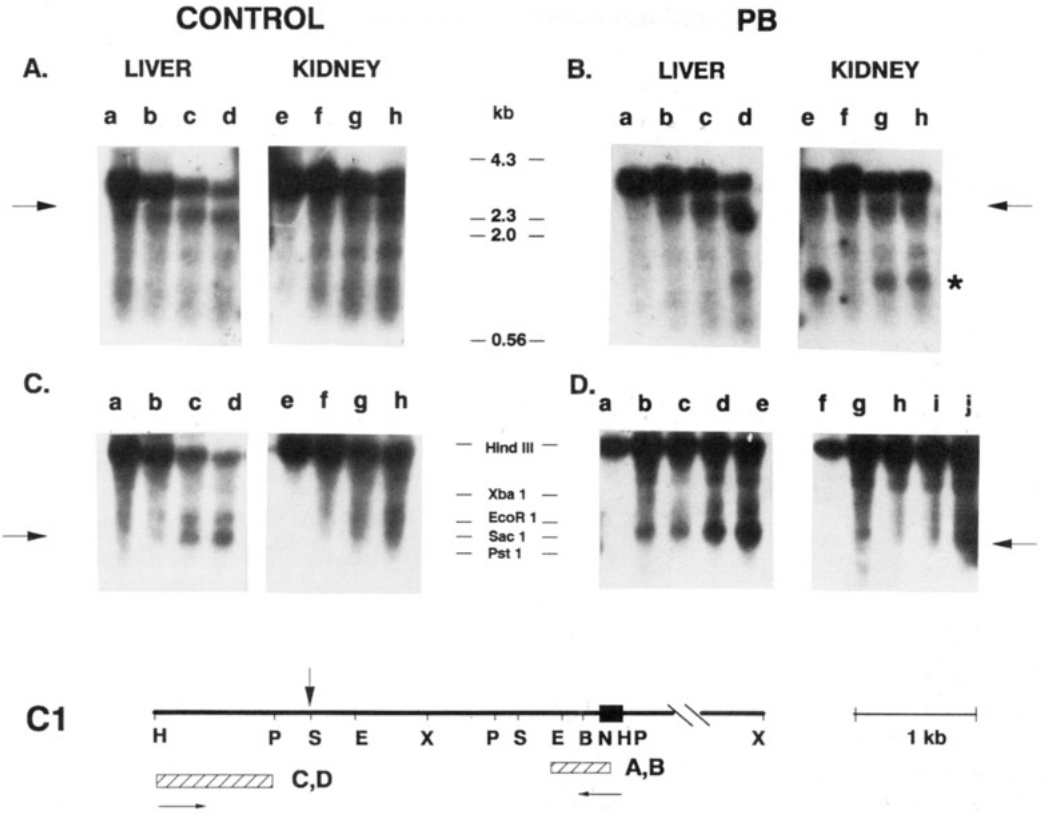

FIGURE 4: Distal DNase I hypersensitive sites in the 5′-flanking region of the P450IIC1 gene. At the bottom, a restriction map of the P450IIC1 5′-flanking region is shown. Thick arrows represent the major distal DNase I hypersensitive site, while thin arrows indicate the direction of the scan. See Figure 1 for restriction site abbreviations. (A, B) Liver and kidney nuclei were isolated from control (A) and phenobarbital-treated (B) rabbits and incubated with 0 (a, e), 4 (b, f), 8 (c, g), or 12 (d, h) μg/mL DNase I at 0 °C for 10 min. The DNA was purified from each of these samples and digested with *Hin*dIII, and 15 μg of DNA for each sample was electrophoresed on a 1.0% agarose gel. The *Eco*RI/*Nco*I fragment shown as a hatched rectangle (A, B) was used as a probe for hybridization. Positions of fragments from *Hin*dIII-digested λ phage DNA used as size markers are shown. The asterisk indicates an artifactual band not consistently observed. (C, D) For moderate resolution mapping, liver and kidney nuclei from control (C) rabbits were isolated and incubated with 0 (a, e), 4 (b, f), 8 (c, g), or 16 (d, h) μg/mL DNase I at 0 °C for 10 min. Nuclei from phenobarbital-treated animals (D) were subjected to digestion with DNase I at 0 (a, f), 0.5 (b, g), 2 (c, h), 4 (d, i), or 8 (e, j) μg/mL. The DNase I treated genomic DNA was purified and restricted with *Hin*dIII. Fifteen micrograms of each sample was run on a 1.5% agarose gel. The *Hin*dIII/*Pst*I fragment located near the major distal DNase I hypersensitive site was selected as a probe [hatched rectangel (C, D)]. Positions are indicated for fragments which were prepared from *Xba*I, *Eco*RI, *Sac*I, and *Pst*I restriction enzyme digestions of the 3.8 kbp *Hin*dIII fragment of the P450IIC1 gene and which were used as internal size markers.

DNase I hypersensitive site was mapped near the *Sac*I site (Figure 4, bottom).

DNase I Hypersensitivity in the 5′-Flanking Region of the Constitutive P450IIC3 Gene. Unlike the P450IIC1 and P450IIC2 genes, the amount of P450IIC3 mRNA is not increased by phenobarbital treatment (Leighton & Kemper, 1984; Chan & Kemper, 1990). To examine the pattern of DNase I hypersensitivity in the 5′-flanking region of the P450IIC3 gene, genomic DNA was digested with *Eco*RI and probed with a *Bcl*I/*Eco*RI fragment from the intron 1 region. The *Eco*RI fragment extended from intron 1 to about 3.2 kbp into the 5′-flanking region (Figure 5). One major subfragment was generated by DNase I treatment in liver but not kidney samples regardless of phenobarbital treatment. Using internal markers, this region mapped near the *Hph*I site at about −200 bp. The P450IIC3 gene is very AT-rich in this region (Chan & Kemper, 1990), so it is possible that the DNA is naturally hypersensitive to DNase I. However, digestion of deproteinized P450IIC3 DNA with DNase I did not result in preferential cleavage in this region (data not shown). Furthermore, the absence of DNase I hypersensitivity in the kidney nuclei provides additional support that the hypersensitive site in the liver samples is due to chromatin structure. The appearance of this hypersensitive site correlates well with the expression pattern of the P450IIC3 gene. In contrast to the phenobarbital-inducible genes, there was no evidence for a distal DNase I hypersensitive site up to 3.0 kbp from the RNA initiation site (Figure 5). Analysis of *Xba*I-digested genomic DNA using a *Hin*dIII/*Xba*I fragment as a probe did not reveal any major hypersensitive sites up to 5.0 kbp from the RNA initiation site (data not shown).

DNA Sequences of the Distal Hypersensitive Regions. The original genomic clone for P450IIC2 (Govind et al., 1986) did not extend to the distal hypersensitive region. In order to sequence this region, an additional genomic clone was isolated that contained 5′-flanking sequences, and a 3.4 kbp *Hin*dIII fragment, including the distal hypersensitive region, was subcloned. Significant similarity of the 5′-flanking sequences of the P450IIC1 and P450IIC2 genes extended throughout the region sequenced originally to −700 bp (Govind et al., 1986; Zhao et al., 1990). To determine whether similar sequences are present in the region of the distal hypersensitive sites of these two genes, the DNA sequence around the *Sac*I site of each gene was determined. With the introduction of gaps, the overall similarity of the sequences was 67% over about 500 bp (Figure 6). The sequence extending in the 3′ direction from the *Sac*I site for 165 bp was well conserved with a similarity of near 80%. At the end of this region of high similarity, a stretch of 15 identical bases contained a sequence (underlined) that resembled the consensus binding motif for HNF-1/LF-B1, GTTAATNATTAAC (de Simone & Cortese, 1988). The first eight nucleotides of the 15 bp conserved

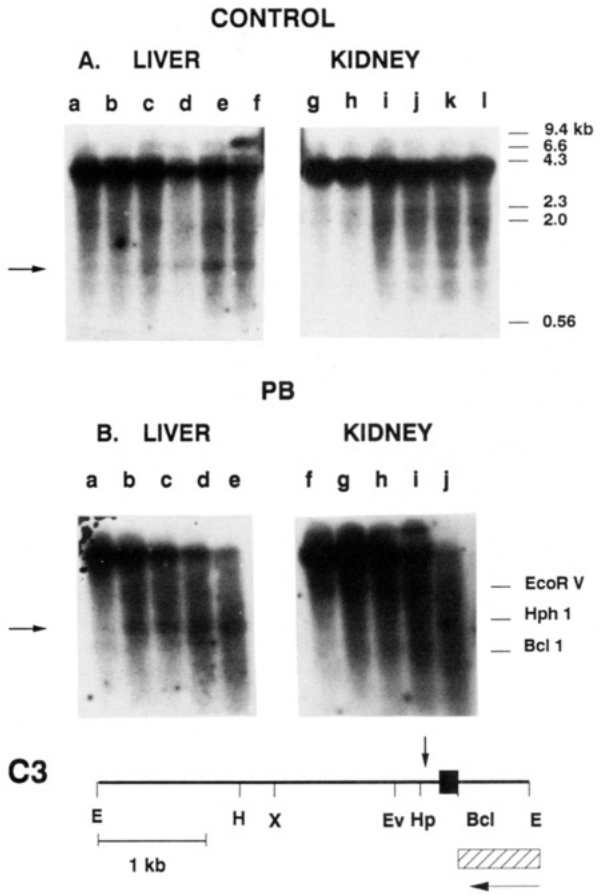

FIGURE 5: DNase I hypersensitive sites in the 5′-flanking region of the P450IIC3 gene. Liver and kidney nuclei from a control rabbit (A) were digested with 0 (a, g), 4 (b, h), 6 (c, i), 8 (d, j), 12 (e, k), or 16 (f, l) μg/mL DNase I. Nuclei from a phenobarbital-treated rabbit (B) were incubated with 0 (a, f), 4 (b, g), 8 (c, h), 12 (d, i), or 16 (e, j) μg/mL DNase I at 0 °C for 10 min. Ten micrograms of genomic DNA was restricted with *Eco*RI and separated by electrophoresis on a 1.8% agarose gel. The *Bcl*I/*Eco*RI fragment from the intron 1 was used as a probe (hatched rectangle). The positions are indicated of DNA fragments generated by digestion with *Eco*RV, *Hph*I, and *Bcl*I of the *Eco*RV/*Eco*RI P450IIC3 fragments which were used as internal size markers. Thick arrows mark the positions of the major DNase I hypersensitive site. The restriction map of the 5′-flanking region of the P450IIC3 gene is shown at the bottom. See Figure 1 for restriction site abbreviations.

sequence are identical to the first half of the consensus sequence. A second highly conserved region containing 24 of 26 identical nucleotides beginning at nucleotide 150 for P450IIC2 (Figure 6) also contains a sequence that is similar to the HNF-1 sequence. In addition, this region contains a 10 bp imperfect direct repeat (dashed arrow, Figure 6). The region in the P450IIC2 gene from nucleotide 523 to nucleotide 579 was 95% similar to the 3′ regions of long interspersed repetitive DNA of the L1 family present in rabbit globin genes (Demers et al., 1989). These 3′ regions are present in 66 000 copies per haploid genome, so that this repetitive DNA marks the end of potential gene-specific transcriptional regulatory sequences in the distal hypersensitive site.

Discussion

As summarized in Figure 7, our results demonstrate that major DNase I hypersensitive sites are present in the P450IIC genes both proximal, −200 bp, and distal, −2.0 to −2.2 kbp, to the RNA initiation site. In agreement with that observed in other genes, the presence of the proximal site correlated well with the expression state of the genes (Stadler et al., 1980; McGhee et al., 1981). This site was present (1) in the P450IIC1 gene only in liver samples from phenobarbital-treated rabbits, (2) in the P450IIC2 gene in treated and untreated samples from both kidney and liver, and (3) in the P450IIC3 gene in liver samples from either treated or untreated rabbits. The presence of this proximal site parallels the expression of these genes based on the concentrations of mRNA present (Leighton & Kemper, 1984). The appearance of a DNase I hypersensitive site near the promoter region in the P450IIC1 gene after phenobarbital treatment may result from a direct effect of phenobarbital on liver nuclear factors binding in this region. Altered binding of a nuclear factor in the proximal region of the rat P450IIB1/2 gene has been reported after phenobarbital treatment (Rangarajan & Padmanaban, 1989). In preliminary studies, we have detected at least two nuclear proteins that bind in this region, but have not detected differences in their binding after phenobarbital treatment (J. Zhao, D. Chen, J. Kim, and B. Kim, unpublished results). Alternatively, phenobarbital may exert a direct effect on other regulatory regions, which indirectly alters the chromatin structure at the proximal site.

The DNA sequences in the region of the distal hypersensitive sites are about 67% similar between P450IIC1 and P450IIC2. This similarity is equivalent to that observed for the sequences from −1 to −700 bp. This indicates that the gene duplication that resulted in these two genes extended at least 2.5 kbp into the 5′-flanking region. Within this region, there are highly conserved regions suggesting a functional role for these sequences. Rabbit liver nuclear proteins bind to a 100 bp DNA fragment containing an HNF-1-like site (nucleotides 402–414 for P450IIC2 in Figure 6). The binding to this sequence as well as similar sequences in the proximal regions of P450IIC1 and P450IIC2 can be competitively displaced by an oligonucleotide containing a proximal region HNF-1-like sequence in the P450IIC4 gene (data not shown). This raises the interesting possibility that binding of HNF-1-like proteins to both the distal and proximal regions may be important for regulation of the genes.

The functional significance of the distal DNase I hypersensitive site is unclear. A distal site was present in the phenobarbital-inducible genes, P450IIC1 and P450IIC2, but was not detected in the constitutive P450IIC3 gene, suggesting a possible role of this region in phenobarbital induction. However, the presence of this site was not dependent on phenobarbital treatment since it was present in untreated animals. Subtle changes in chromatin structure or protein binding within the DNase I hypersensitive site would not have been detected by the indirect Southern technique. There are numerous studies in other genes showing a lack of change in DNase I hypersensitive sites between expressed and nonexpressed states after hormonal treatment (Durrin & Gorski, 1985; Becker et al., 1984), during differentiation (Weintraub et al., 1981; Forrester et al., 1986), and in different cell types (Peterlin et al., 1987). These results are similar to those described for other P450 genes. No changes were observed in discrete DNase I hypersensitive regions in the mouse (Durrin & Whitlock, 1989) and rat (Foldes & Bresnick, 1989) P450IA1 gene and the rat P450m gene (Einck et al., 1985) after treatment with inducers, in contrast to an earlier report of a change in the location of DNase I hypersensitive site in the 5′-flanking region of the rat P450IA1 gene (Einck et al., 1986). Changes in hypersensitivity at distal regulatory regions, therefore, do not always correlate with gene expression.

The cooperative interaction between two distantly separated regions is important for the regulation of many eukaryotic genes. Distal and proximal cis-acting regions have been

```
C2          TGCTGATGAACATTTAGACTATCGTGTATCCTGGTTGCTGTGAATAATGCTGAGTTATTGTGGTTATTTCTAACTAAAATAAAGAGAC   88
C1 AAACTCACCAGGTGATGATGAACATTAGGCTATTTCTGTACCCAGATGACGGTGCACTATGCTGGATAGTTGCAGCTATTTCTAAATGCAGTAAGGAAAA  100

C2 ATCTAGTTTTGTAAAGACTACAGATATGCAACATTTATAATTGGTCATGTTTGTAAGGATTCTGATAATTTACACAGAATTTTCACATCTATCATATCAA  188
C1 GTCAGTTTTTCTCAAGAACTCAGATACACAAAATT--GAATTGTTCATCTCTAAAAATATGCTGATAATTTAAAGAGAATTTTCACACCTATAATGTTTC  198
                                                          ---------> --------->

C2 TTTGGATTCCCTCAATTACCTTTGC----------------------------CTGTCCCTTGGCCCAGT-----GAAGCAGCTCCCAAAGGAGGCA  252
C1 TTTAGAATCTCTCATCTACCTTTGCTTAAGATGAGATGATTCCCATTTTAGAAATTCTGCCCCTTGCTCAAATAATTGGTTGTGGTTCCTGCAAGAGGCA  298

C2 GAGCTCATGTCAAATTCCTTGTCCCATGAGTCATGCCACTTTCCTACCAAGCAGACAGTCAATTCAGCA-GGTTTACCGATATGAACTTACAGTGACATT  351
C1 GAGCTCAGGTTGAATTTCTTATCGCCTGAGTCCTGCTACCTTCCTAACAAGTAGACAATCAGTTCAACAGGGTTCACCAATATTTACTTACTGT-CTTCA  397

C2 CAGTATTGACTTTTAAGTAAGCATGAACTGAGTCAATAATTGAGGCACAGGTTAATGAAAATGAGGGTCAGATAAGGTCTGTTTCATAACCTATAAAATT  451
C1 CAGCAATGACTTGCAAATAAACATGAAGTTAGTCAATTATTGGAGCACAAGTTAATGAAAATGAGAATTGGAAAAAATTGGCTCTGTA------GAATGC  491

C2 ATGCAAGTAAAATTTTGATTTTAGGACTCTACAAGAATTGTATACCTTATTTGGATAAAACAAAATAAAAGGATCACTGGGAGTAGTGAGATGGCATTGG  551
C1 TTGCAAGTCCAATTTTGCTTCT-TGACTGGGCTCTGCAGGCATGCAAGCTTTCCCTATAGTGAGTGG                                    557

C2 TACATGCCACCTCAATGGGATTGAATTC                                                                            574
```

FIGURE 6: Nucleotide sequences of the distal DNase I hypersensitive sites of the P450IIC1 and P450IIC2 genes. The nucleotide sequence between two *Eco*RI sites in P450IIC2 and between a *Pst*I and *Eco*RI site in P450IIC1 (see Figure 7) was determined. The 3′ terminal of the sequences shown corresponds to about −1900 and −1800 for P450IIC1 and P450IIC2, respectively. The two sequences were aligned with the GAP program of the GCG University of Wisconsin package. Asterisks indicate identical nucleotides. The position of the conserved *Sac*I site is underlined, and sequences that resemble HNF-1-like binding sites are double-underlined. The broken arrows indicate a repeated sequence.

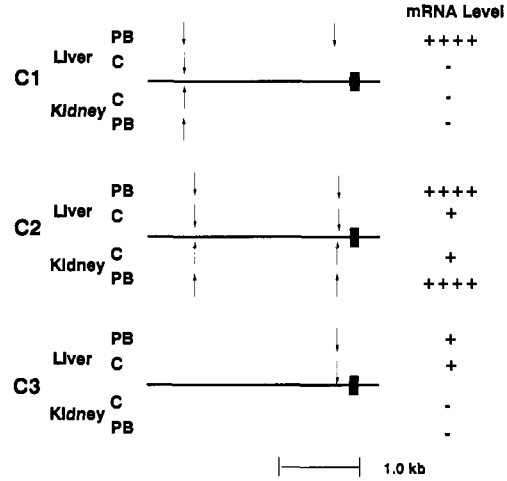

FIGURE 7: Summary of the major DNase I hypersensitive sites of the P450IIC subfamily genes. The horizontal lines indicate the 5′-flanking and intron 1 regions of the IIC genes. The solid boxes represent exon 1. The major distal and proximal DNase I hypersensitive sites are shown as vertical arrows. Differential induction and tissue-specific expression of the P450IIC genes are also illustrated by mRNA levels of liver and kidney tissues from control and phenobarbital-treated rabbits determined by dot blot and Northern blot experiments (Leighton & Kemper, 1984; Zhao et al., 1987). The level of mRNA is proportional to the number of (+)'s.

identified in the rat and mouse P450IA1 gene. A dioxin or polycyclic aromatic hydrocarbon response element that has enhancer properties is present about −1.0 kbp from the RNA initiation site [reviewed in Nebert and Jones (1989) and Whitlock et al. (1989)]. A second cis-acting element required for expression of both the rat and mouse genes is present in the proximal promoter region (Neuhold et al., 1989; Yanagida et al., 1990; Jones & Whitlock, 1990). Other eukaryotic genes provide additional examples. Distal hypersensitive sites, −2.5 and −5.4 kbp from the RNA initiation site, have been shown to contain cis-acting elements responsible for full glucocorticoid induction of the rat tyrosine aminotransferase gene in the liver (Jantzen et al., 1987; Grange et al., 1989). Studies consistent with interaction between two separately located regulatory elements have been reported for the rat tryptophan oxygenase (Danesh et al., 1987), the rat phosphoenolpyruvate carboxykinase (Petersen et al., 1988), and the human β-like globin (Tuan et al., 1989) genes. The presence of both distal and proximal hypersensitive sites in the P450IIC genes is consistent with the hypothesis that a similar mechanism is operating in these genes, which must now be tested directly in functional expression assays.

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Low-Temperature Crystallographic Analyses of the Binding of Hoechst 33258 to the Double-Helical DNA Dodecamer C-G-C-G-A-A-T-T-C-G-C-G†

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ABSTRACT: The crystal structure of the complex of Hoechst 33258 and the DNA dodecamer C-G-C-G-A-A-T-T-C-G-C-G has been solved from X-ray data collected at three different low temperatures (0, −25, and −100 °C). Such temperatures have permitted collection of higher resolution data (2.0, 1.9, and 2.0 Å, respectively) than with previous X-ray studies of the same complex. In all three cases, the drug is located in the narrow central A-A-T-T region of the minor groove. Data analyses at −25 and −100 °C (each with a 1:1 drug/DNA ratio in the crystallizing solution) suggest a unique orientation for the drug. In contrast, two orientations of the drug were found equally possible at 0 °C with a 2:1 drug/DNA ratio in solution. Dihedral angles between the rings of Hoechst 33258 appear to change in a temperature-dependent manner. The drug/DNA complex is stabilized by single or bifurcated hydrogen bonds between the two N–H hydrogen-bond donors in the benzimidazole rings of Hoechst and adenine N3 and thymine O2 acceptors in the minor groove. A general preference for AT regions is conferred by electrostatic potential and by narrowing of the walls of the groove. Local point-by-point AT specificity follows from close van der Waals contacts between ring hydrogen atoms in Hoechst 33258 and the C2 hydrogens of adenines. Replacement of one benzimidazole ring by purine in a longer chain analogue of Hoechst 33258 could make that particular site GC tolerant in the manner observed at imidazole substitution for pyrrole in lexitropsins.

Noncovalent DNA minor groove binding drugs are a subject of interest because they interact with the DNA in a sequence-selective manner (Zimmer & Wähnert, 1986). The subject of this analysis, Hoechst 33258 (Figure 1), shares many structural and DNA-binding characteristics with drugs such as netropsin (Kopka et al., 1985), distamycin (Coll et al., 1987), berenil (Brown et al., 1990), and DAPI (Larsen et al., 1989): (1) They all are crescent-shaped assemblies of flat aromatic rings or amide groups, connected by more or less

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