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Liver Cells Contain Constitutive DNase I-Hypersensitive Sites at the Xenobiotic Response Elements 1 and 2 (XRE1 and -2) of the Rat Cytochrome P-450IA1 Gene and a Constitutive, Nuclear XRE-Binding Factor That Is Distinct from the Dioxin Receptor

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Dioxin stimulates transcription from the cytochrome P-450IA1 promoter by interaction with the intracellular dioxin receptor. Upon binding of ligand, the receptor is converted to a form which specifically interacts in vitro with two dioxin-responsive positive control elements located in close proximity to each other about 1 kb upstream of the rat cytochrome P-450IA1 gene transcription start point. In rat liver, the cytochrome P-450IA1 gene is marked at the chromatin level by two DNase I-hypersensitive sites that map to the location of the response elements and exist prior to induction of transcription by the dioxin receptor ligand β-naphthoflavone. In addition, a DNase I-hypersensitive site is detected near the transcription initiation site and is altered in nuclease sensitivity by induction. The presence of the constitutive DNase I-hypersensitive sites at the dioxin response elements correlates with the presence of a constitutive, labile factor which specifically recognizes these elements in vitro. This factor appears to be distinct from the dioxin receptor, which is observed only in nuclear extract from treated cells. In conclusion, these data suggest that a certain protein-DNA architecture may be maintained at the response elements at different stages of gene expression.

Transcription of the cytochrome P-450IA1 gene is dramatically induced in response to the environmental contaminant dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) or related compounds. The induction process is mediated by the intracellular dioxin receptor protein and initiated by binding of the inducing chemical to the receptor, which in its non-ligand-occupied state most probably is located in the cytoplasmic compartment of target cells (for a review, see reference 29). In the absence of ligand, the cytosolic dioxin receptor is recovered as a latent, non-DNA-binding species (30). However, upon exposure to ligand in vivo, the dioxin-receptor complex undergoes an activation process involving a poorly understood structural alteration that enables it to translocate to the nucleus (29). In vitro, the ligand-activated dioxin receptor has been shown to represent a DNA-binding protein (references 5 and 47 and references therein) which specifically recognizes dioxin-responsive positive control elements (xenobiotic response elements [XREs]) found in the promoter and upstream regions of the cytochrome P-450IA1 and glutathione *S*-transferase Ya genes (9, 16, 17, 21, 31, 33). The XREs, in turn, modulate the activity of linked promoters, thereby serving as dioxin-inducible enhancers. Thus, the mechanism of action of dioxin is similar to that of steroid hormones (reviewed in references 2, 19, and 48) in that (i) both categories of receptors directly transduce an extracellular signal to the target transcription units they regulate and (ii) the dioxin and steroid receptors require ligand for function.

A striking property of the dioxin receptor is that the ligand strictly controls the activation of the receptor from a latent species to a DNA-binding form also under cell-free conditions (7, 17, 30). In the case of steroid hormone receptors, the glucocorticoid response element of the rat tyrosine aminotransferase gene is protected against methylation by dimethyl sulfate in hepatoma cells only after hormone administration (3), supporting the hypothesis that the glucocorticoid receptor interacts in vivo with its target sequence in a ligand-dependent manner. Moreover, glucocorticoids induce the formation of a DNase I-hypersensitive site at the glucocorticoid response elements of the rat tyrosine aminotransferase gene in both hepatoma cells and rat liver (4, 25, 32), suggesting that the hormone-activated receptor may alter the DNA or chromatin configuration at these regulatory elements.

To assess the function of dioxin-responsive elements defined by cellular transfection studies (16), we have examined chromatin structural changes associated with activation of the cytochrome P-450IA1 gene in rat liver. Two sequence elements (XRE1 and -2) mediating dioxin receptor-dependent activation of this gene have been defined and are localized about 1 kb (extending from positions −1029 to −997 and from positions −1092 to −1069, respectively) upstream of the transcription initiation site (16). Both the XRE1 and -2 elements are recognized by the ligand-activated form of dioxin receptor in vitro (17, 21). In this study, we

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have observed in untreated rat liver two DNase I-hypersensitive sites which map to the positions of the two XREs, respectively. Interestingly, these sites remained unaltered upon induction of gene expression by the dioxin receptor ligand β-naphthoflavone. The presence of these constitutive DNase I-hypersensitive sites could suggest that a specific protein-DNA architecture is maintained at the XREs, regardless of changes in the transcriptional state of the gene. In agreement with this notion, we have identified and characterized an XRE-specific DNA-binding factor in vitro that is present in nuclear extract from nontreated cells and appears to be distinct from the receptor itself.

MATERIALS AND METHODS

Animals, cell culture, isolation of nuclei, and preparation of extracts. Male Sprague-Dawley rats weighing 200 to 300 g were used in animal studies. In indicated cases, the rats were treated 4 or 20 h prior to sacrifice with one single intraperitoneal injection of β-naphthoflavone (dissolved in corn oil) at a concentration of 80 mg/kg of body weight. The rats were sacrificed by cervical dislocation. For DNase I hypersensitivity studies, liver tissue was homogenized in 15 mM Tris-HCl (pH 7.4)–60 mM KCl–0.35 M sucrose–15 mM NaCl–2 mM EDTA–0.5 mM EGTA–0.15 mM spermine–0.5 mM spermidine–1 mM phenylmethylsulfonyl fluoride, and nuclei were prepared essentially as described previously (15). Nuclear proteins were extracted from rat liver tissue as described previously (18). Rat Rueber hepatoma cells (H4IIE) were grown in monolayer culture in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 8% (vol/vol) heat-inactivated fetal calf serum and benzylpenicillin-streptomycin (GIBCO). The wild-type Hepa 1c1c7 cell line and the mutant line c4 derived from it (20) were grown in minimum essential medium as described previously (6, 46). The mutant c4 line expresses a dioxin receptor phenotype which is deficient in nuclear translocation (nt^-; 28) and DNA-binding activity (5) of ligand-occupied receptor. In indicated cases, cells were treated with 1 nM dioxin or [^{3}H]dioxin (Chemsyn, Lenexa, Kan.) for 1 h. Nuclear extract was prepared from untreated or treated hepatoma cells according to Dignam et al. (10). This protocol was modified in indicated experiments to allow separation of constitutive XRE-binding factor(s) from the receptor. Briefly, isolated nuclei were preextracted with a low salt concentration (final concentration, 0.15 M KCl) prior to a second extraction of nuclear proteins at a final concentration of 0.42 M KCl. Cytosolic extracts from untreated wild-type Hepa 1c1c7 cells were prepared as described previously (46).

Mapping of DNase I-hypersensitive sites in nuclei. DNase I digestions were performed at 4°C on isolated rat liver nuclei essentially as described previously (14), and protein-free DNA was recovered as described previously (37). Electrophoretic separation of DNA, transfer to nitrocellulose, and hybridization to ^{32}P-labeled probes were as described previously (37). A 1-kb ladder (Bethesda Research Laboratories) was used as molecular weight standards. The various DNA fragments used as probes were isolated by restriction digestion of various subclones of the rat cytochrome P-450IA1 gene (41, 42).

Chromatographic fractionation of nuclear proteins. Hepatoma cell nuclear extract was fractionated by high-performance gel permeation chromatography on a prepacked Superose 12 (Pharmacia) column (10 by 300 mm) or by anion-exchange chromatography on a prepacked Mono Q HR 5/5 column (Pharmacia) as described previously (21, 46). The anion-exchange column was equilibrated in 20 mM phosphate (pH 7.2)–1 mM EDTA–2 mM 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride and eluted at a flow rate of 0.5 ml/min with linear salt gradients as indicated.

In vitro DNA-binding assays. DNA-binding activities were characterized in rat liver and hepatoma cell nuclear extracts by a gel mobility shift assay as described previously (21, 30). DNA-binding reaction mixtures were assembled in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9)–10% (vol/vol) glycerol–0.5 mM dithiothreitol–3 mM $MgCl_2$–60 mM KCl–4 mM spermidine in a final volume ranging between 20 to 50 μl. In these experiments, double-stranded 36-bp-long oligonucleotides spanning the wild-type rat cytochrome P-450IA1 XRE1 or XRE2 (16) sequence motif were used as specific, radiolabeled probes. Oligonucleotides containing point mutations in the XRE1 motif (7) were also synthesized and used in either direct binding or DNA-binding competition experiments. For sequences of the wild-type and mutant oligonucleotides, see Fig. 5A. Finally, a 38-bp oligonucleotide spanning an octamer motif from the BCL1 immunoglobulin heavy-chain promoter (34) was used as an XRE-unrelated sequence in the oligonucleotide competition experiments.

Safety precautions. The experimental use of dioxin required special handling procedures as outlined previously (reference 5 and references therein). Contaminated materials were disposed of by high-temperature incineration.

RESULTS

A DNase I-hypersensitive site within the 5' flank of the cytochrome P-450IA1 gene coincides with the location of XRE1 and XRE2. Expression of the cytochrome P-450IA1 gene is highly inducible by dioxin receptor ligands in rat liver (reviewed in reference 29). To examine the chromatin structure of the 5' flank of the cytochrome P-450IA1 gene in this tissue, nuclei were isolated from uninduced rat liver and digested to various extents with DNase I. DNA was purified, digested with restriction endonuclease, fractionated by gel electrophoresis, and analyzed by blot hybridization. In one such experiment (Fig. 1), the genomic DNA was digested with *Pst*I to generate a fragment which contained about 2.2 kb of DNA sequences upstream of the cytochrome P-450IA1 cap site, the first exon, and the major part of the first intron (shown schematically in Fig. 1A).

Digested DNA was indirectly end labeled with a sequence (designated probe 394 in Fig. 1A) spanning the 5' end of the genomic *Pst*I fragment. The prominent band of about 4.4 kb (Fig. 1B) corresponds to the *Pst*I fragment not cleaved by DNase I. Digestion of control rat liver nuclei with increasing concentrations of DNase I (Fig. 1B, lanes 1 to 9) generated a band of about 1.1 ± 0.1 kb which corresponded to a hypersensitive site (HS1) within the 5' flank of the gene at about 1 kb relative to the transcription start site. Thus, this site appears to overlap with the dioxin-responsive elements XRE1 and XRE2, which also constitute dioxin receptor-binding sequences mapped in vitro (17, 21). Taken together, the XRE1 and XRE2 elements span sequences from −997 to −1092 relative to the cap site (16). At high concentrations of DNase I (lanes 8 and 9), a nuclease-induced band of about 2.1 ± 0.1 kb was detected, indicative of a second hypersensitive site (HS2) near the cap site of the promoter. DNase I digestion of protein-free high-molecular-weight DNA from rat liver showed that the hypersensitive sites mapped in isolated nuclei had no counterpart in protein-free DNA (data

not shown), indicating that the mapped hypersensitivity is a consequence of chromatin structure.

Induction of cytochrome P-450IA1 transcription leads to no change in chromatin structure at the XREs. We have previously shown that transcription from the rat liver cytochrome P-450IA1 promoter is induced by the dioxin receptor ligand β-naphthoflavone (40). Following injection of a single dose of β-naphthoflavone (80 mg/kg), maximal transcription levels are observed at 4 h, as assessed by run-on elongation analyses with isolated rat liver nuclei (40). At 4 h of treatment, gene activation by β-naphthoflavone was not paralleled by any change in sensitivity to DNase I at the HS1 site coinciding with the XREs (Fig. 1B; compare lanes 1 to 9 with lanes 10 to 18). By contrast, the DNase I-hypersensitive site near the point of transcription initiation (HS2) showed a marked increase in sensitivity to nuclease digestion in nuclei from β-naphthoflavone-treated liver (lanes 10 to 18) compared with control nuclei (lanes 1 to 9).

In a second set of experiments, rats were treated for 20 h with β-naphthoflavone. During this period of time, β-naphthoflavone-induced cytochrome P-450IA1 gene transcription decreases to control levels (40). Blot hybridization analysis with fragment 394 of DNase I and *Pst*I-digested DNA from rat liver nuclei after 20 h of in vivo treatment also demonstrated the hypersensitive site at the positions of XRE1 and XRE2. In fact, DNA blot analysis of the HS1 site at high resolution showed that it could be resolved into two bands (HS1A and HS1B) with sizes corresponding to hypersensitive sites at about −1 kb ± 50 bp and −1.1 kb ± 50 bp, respectively, relative to the transcription start site (Fig. 1C). Thus, these two sites map to the locations of XRE1 (at −1 kb) and XRE2 (at −1.1 kb; 16), respectively. As in Fig. 1B, the HS1A and HS1B sites were detected in liver nuclei from untreated as well as β-naphthoflavone-induced rats (Fig. 1C; compare lanes 1 to 9 with lanes 10 to 18).

Consistent with the observations in Fig. 1B, β-naphthoflavone treatment dramatically altered the sensitivity to DNase digestion of the HS2 site, which was detected as a band of 2.1 kb ± 0.1 kb, positioning this site in close proximity to the cap site (Fig. 1C). Interestingly, the HS2 site showed a sensitivity to nuclease digestion after 20 h of

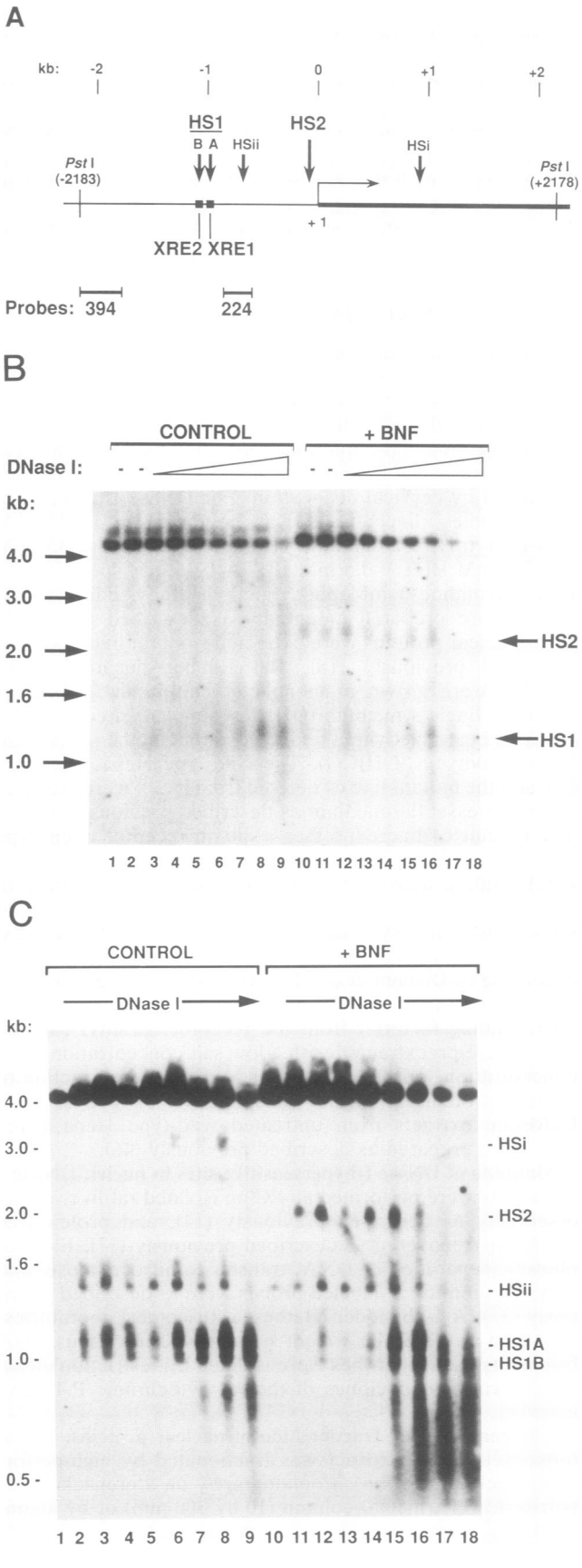

FIG. 1. Mapping of DNase I-hypersensitive sites in the 5′ flanking region of the cytochrome P-450IA1 gene in untreated and β-naphthoflavone-treated rat liver. (A) Schematic representation of the promoter region of the rat cytochrome P-450IA1 gene. The thick line represents the transcribed region; arrows indicate the DNase I-hypersensitive sites designated; filled boxes represent the dioxin-responsive elements XRE1 and XRE2; the bent arrow indicates the start site of transcription. The fragments used as radiolabeled probes for DNA blot hybridization experiments are indicated below the map. Probes 394 and 224 represent a 394-bp *Pst*I-*Pvu*II (−2183 to −1789) and a 224-bp *Bam*HI-*Kpn*I (−847 to −623) fragment, respectively, of the rat cytochrome P-450IA1 gene. (B) Hybridization analysis. Nuclei from uninduced (control) and β-naphthoflavone-induced (BNF) rat livers (4 h of treatment) were isolated and treated in the absence (lanes 1, 2, 10, and 11) or presence (lanes 3 to 9 and 12 to 18) of increasing concentrations of DNase I (0.03 to 2.1 U/μl). After purification, the DNA was digested with *Pst*I, and 35 μg of DNA per lane was separated on a 1% agarose gel. The DNA was blotted to a filter and hybridized to the 394-bp probe. (C) Mapping of DNase I-hypersensitive sites at higher resolution. Digested DNA was isolated from untreated rat liver or liver treated for 20 h with β-naphthoflavone and size separated on a 1.5% agarose gel. See legend to panel B for details. The parallel mobilities of DNA molecular weight markers are shown at the left.

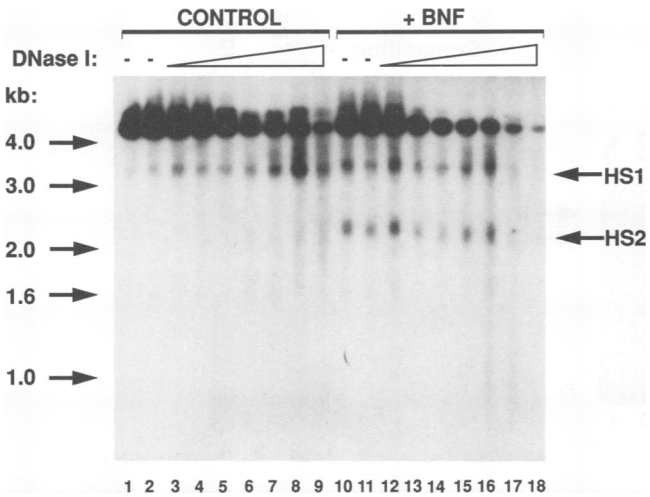

FIG. 2. DNase I-hypersensitive sites in rat cytochrome P-450IA1 chromatin. The autoradiograph shows hypersensitive sites mapped in nuclear chromatin of untreated rat liver (control; lanes 1 to 9) or rat liver treated for 4 h with β-naphthoflavone (BNF; lanes 10 to 18), using probe 224. See legend to Fig. 1B for details. The HS1 and HS2 sites showed different degrees of sensitivity to endogenous nucleases in different preparations of nuclei (compare, for instance, lanes 1 and 2 with lanes 10 and 11).

treatment similar to that observed after 4 h of treatment (Compare Fig. 1B and C). Thus, the dioxin-induced change in chromatin structure in the vicinity of the transcription start site was not reversed after 20 h of treatment, although the cytochrome P-450IA1 gene is transcribed at a low rate at this time point. In addition to the two major DNase I-hypersensitive sites HS1 and HS2, this analysis visualized a ~3.1-kb band corresponding to a hypersensitive site (HSi) at about +1 kb in the first intron of the gene. Upon β-naphthoflavone induction, the sensitivity of this site to nuclease digestion was decreased, in agreement with an earlier report (13). Moreover, a second constitutive hypersensitive site (HSii) was detected at approximately 0.75 kb relative to the transcription start site, and a rather broad region of β-naphthoflavone-stimulated DNase I hypersensitivity was indicated at more than 1.4 kb upstream of the transcription start site (Fig. 1C; compare lanes 1 to 9 with lanes 10 to 18).

The chromatin structure of the 4.4-kb *Pst*I fragment from the cytochrome P-450IA1 gene was further characterized by using an internal probe (designated 224 in Fig. 1A) that hybridizes to sequences in the 3′ vicinity of the XREs. This analysis did not reveal any DNase-I hypersensitive sites in addition to two major bands of 3.1 ± 0.1 kb and 2.1 ± 0.1 kb, respectively (Fig. 2). The 2.1-kb band showed increased sensitivity to nuclease digestion following β-naphthoflavone stimulation and corresponded to the previously identified HS2 site located in close proximity to the cap site, whereas the 3.1-kb band was constitutively present (Fig. 2; compare lanes 1 to 9 with lanes 10 to 18). Given the background that the intronic DNase I-hypersensitive site at +1 kb disappeared upon β-naphthoflavone induction (Fig. 1C), the constitutive 3.1-kb band most probably corresponds to the HS1 site at about −1 kb relative to the start site. Thus, this analysis confirms the earlier assignment of the HS1 and HS2 sites to the positions of the XREs and the transcription start site, respectively.

The constitutive DNase I-hypersensitive site at the XREs correlates with the presence of a labile, constitutive XRE-specific factor. The constitutive accessibility of the XREs in cytochrome P-450IA1 chromatin may facilitate interaction with nuclear proteins also in noninduced cells, or, conversely, such a factor(s) may play a role in the formation of the hypersensitive site. This hypothesis prompted us to examine whether proteins other than the dioxin receptor could specifically recognize XRE target sequences in vitro. To identify such factors, nuclear extracts were prepared from untreated rat liver and H4IIE rat hepatoma cells. DNA-binding activities were monitored by a gel mobility shift assay, using as specific probe an oligonucleotide which spanned the XRE1 sequence from positions −1026 to −999 relative to the cytochrome P-450IA1 gene transcription start site. In nuclear extract obtained from noninduced rat liver, an XRE-binding activity was detected (Fig. 3A). This DNA-binding activity formed a single complex (indicated by an arrow) which appeared to be the result of specific protein-DNA interaction, since an excess of unlabeled XRE1 oligonucleotide competed more efficiently for complex formation than did an excess of an unrelated oligonucleotide containing the octamer element of immunoglobulin promoters (Fig. 3A). A similar XRE-specific DNA-binding activity was also observed in nuclear extract from noninduced H4IIE rat hepatoma cells (data not shown).

The dioxin receptor has been extensively characterized in Hepa 1c1c7 mouse hepatoma cells (reviewed in references 27 and 46), in which transcription of the cytochrome P-450IA1 promoter is highly inducible (24). To characterize the putative constitutive XRE-binding factor(s) and to relate it to the dioxin receptor, nuclear extracts were prepared from dioxin-treated or untreated hepatoma cells. We observed that a labile XRE-binding factor could be detected in nuclear extract from untreated cells. In gel mobility shift assays, this factor generated a complex (Fig. 3B, lane 1; designated C1) exhibiting a faster relative mobility on nondenaturing polyacrylamide gels than did the bona fide dioxin receptor-XRE complex (designated R), which was detected only in nuclear extract from treated cells (compare lane 1 with lanes 2 to 4).

We confirmed that the different XRE-binding activities resulted from sequence-specific protein-DNA interaction by competition with unlabeled DNA fragments. Thus, formation of both the C1 complex and the receptor-dependent complex was greatly reduced by the presence of a 40-fold molar excesses of the unlabeled XRE1 oligonucleotide (Fig. 3B, lanes 1 to 3), whereas little or no competition for binding was observed by coincubation with a 40-fold molar excess of an unrelated fragment containing the octamer motif of an immunoglobulin heavy-chain promoter (lane 4). Interestingly, the XRE-specific C1 complex was also detected in nuclear extract of nt^- mutant hepatoma cells (Fig. 3C), which show no detectable levels of receptor-dependent XRE-binding activity following dioxin induction (17, 21).

The constitutive nuclear XRE-specific factor is distinct from the dioxin receptor. To biochemically characterize the different XRE-binding factors, Hepa 1c1c7 hepatoma cells were treated with [^{3}H]dioxin, and a standard (10) nuclear extract was prepared. Consequently, material was generated that contained both the constitutive XRE-binding activity and ligand-activated dioxin receptor, as assessed by gel mobility shift analysis (Fig. 4B, input lane). We next fractionated this extract by fast protein liquid-anion exchange chromatography on Mono Q columns. As has been reported previously (46), specifically bound [^{3}H]dioxin was recovered in two distinct peaks centered around Mono Q fractions 19

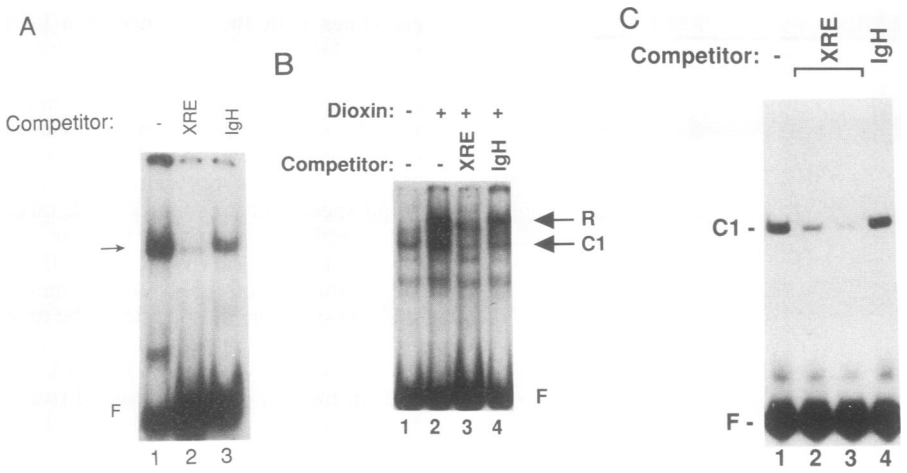

FIG. 3. Detection of an XRE-binding factor in nuclear extract from untreated rat liver and hepatoma cells. XRE-binding activities in crude or fractioned nuclear extracts were monitored by a gel mobility shift assay, using as a specific probe a radiolabeled oligonucleotide spanning the XRE1 sequence motif. In certain DNA-binding reactions, the labeled XRE1 fragment was incubated with nuclear extract in the presence of an excess of either unlabeled XRE1 oligonucleotide (XRE) or the unrelated immunoglobulin octamer sequence element (IgH), as indicated above the lanes. (A) XRE-binding activity present in nuclear extract from untreated rat liver. Lanes: 1, no competitor; 2 and 3, 50-fold molar excess of competitor. The arrow indicates the XRE-specific complex. (B) XRE-binding activities in a high-salt nuclear extract from untreated (lane 1) or dioxin-induced (lanes 2 to 4) hepatoma cells in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of a 40-fold molar excess of the indicated competitor fragment. (C) XRE-binding activities in a high-salt nuclear extract from dioxin-induced mutant nt$^-$ hepatoma cells. Lanes: 1, no competitor; 2, 100-fold molar excess of competitor; 3 and 4, 200-fold molar excess of competitor. Constitutive (C1) and receptor-induced (R) complexes are indicated by arrows. F, free (unbound) probe.

(eluting at 27 mS/cm, corresponding to ~0.2 M KCl) and 23 (eluting at 40 mS/cm, corresponding to ~0.35 M KCl), respectively (Fig. 4A). The receptor-dependent XRE-binding activity was recovered in Mono Q fraction 19 of the columns (Fig. 4B), whereas fraction 23 has been shown to harbor a non-DNA-binding form of ligand-occupied dioxin receptor (46). The factor(s) generating the constitutive C1 complex could be separated from both forms of receptor by Mono Q anion-exchange chromatography. Thus, C1-forming activity was detected predominantly in Mono Q fraction 26, which was eluted at a higher (~0.45 M KCl) salt concentration than both the DNA and non-DNA-binding forms of dioxin receptor. In addition, a second constitutive XRE-specific complex exhibiting a slightly faster relative mobility than the C1 complex was eluted at a low salt concentration (centered around 0.15 M KCl) in Mono Q fractions 16 and 17 (Fig. 4B). As discussed below, a possible degradation product of the C1-forming factor could be responsible for generating this complex. In support of this notion, the C1 complex appeared to be unstable during the Mono Q fractionation procedure, generating higher levels of the lower band (representing the possible degradation product) relative to the C1 complex than were observed in the input material. Gel permeation chromatography of crude nuclear extract from induced Hepa 1c1c7 cells showed elution of the C1-forming factor in a region of the column corresponding to a Stokes radius of 4.5 nm (data not shown), in contrast to the dioxin receptor which, under identical conditions, elutes as a very asymmetric protein with a Stokes radius of 6.9 nm (21). Moreover, the C1-forming factor sedimented in the 2.8S region of sucrose gradients (data not shown), giving a calculated molecular size of about 53 kDa. In conclusion, three lines of evidence argue that the C1-forming factor is distinct from the dioxin receptor: (i) in contrast to the dioxin receptor, the factor is detected in nuclear extract from untreated wild-type cells and thus exhibits a constitutive

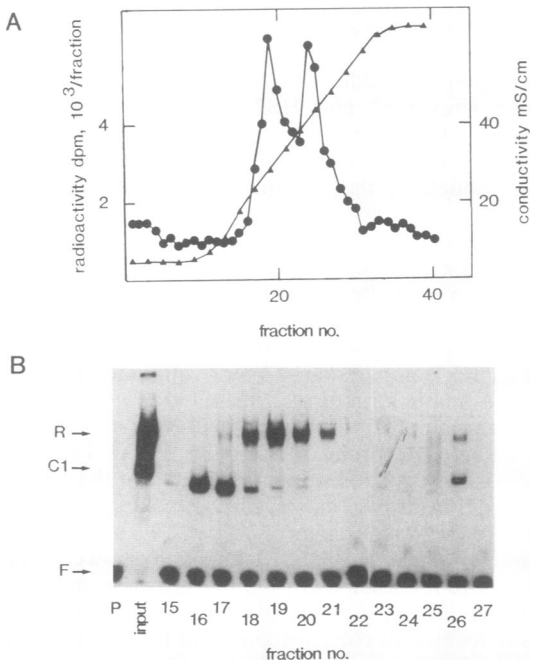

FIG. 4. Chromatographic separation of constitutive XRE-binding factors from the dioxin receptor. Hepa 1c1c7 cells were treated with [^{3}H]dioxin, and nuclear extract was prepared as described by Dignam et al. (10). Two milliliters (corresponding to about 6 mg of protein) were applied to a Mono Q anion-exchange chromatography column. Retained material was eluted with a 0 to 0.6 M KCl gradient. (A) Profile of the eluted [^{3}H]dioxin-binding activity. (B) Gel mobility shift analysis of XRE1-binding activity. R, dioxin receptor-dependent XRE1 complex; C1, constitutive protein-DNA complex; F, free (unbound) ^{32}P-labeled XRE1 probe.

XRE-binding activity; (ii) the factor is also detected in nuclear extract from mutant hepatoma cells which are dioxin unresponsive and deficient in nuclear translocation and DNA-binding activity of ligand-occupied dioxin receptor; and (iii) the factor does not copurify with any of the dioxin receptor forms observed in nuclear extract from dioxin-induced cells.

DNA-binding specificities of XRE-binding factors. In the dioxin-responsive XRE1 sequence of the cytochrome P-450IA1 gene, the hexanucleotide motif 5′-TCACGC-3′ is critical for dioxin receptor interaction in vitro (7, 31) and, in turn, is conserved in the rat XRE2 element (16). In gel mobility shift experiments using as specific probes oligonucleotides of identical length (sequences shown in Fig. 5A) carrying either the XRE1 or XRE2 motif, neither element was recognized by the cryptic form of cytosolic Hepa 1c1c7 dioxin receptor (Fig. 5B, lanes 1 and 3). Upon ligand-dependent in vitro activation (7, 30), however, the DNA-binding activity of the cytosolic receptor was unmasked, demonstrating that both the in vitro (Fig. 5B, lanes 2 and 4)- and in vivo (17, 21)-activated forms of dioxin receptor bind to both the XRE1 and XRE2 elements. The relative affinity of the ligand-activated dioxin receptor is about four- to fivefold lower for the XRE2 sequence than for the XRE1 motif (21). Thus, sequences flanking the critical hexamer motif may stabilize receptor-DNA interaction.

We observed that the constitutive C1-generating factor could be preferentially extracted from isolated nuclei of untreated or dioxin-treated cells at a low salt concentration (~0.15 M KCl), in contrast to the in vivo-activated receptor, which requires salt concentrations of 0.42 M KCl for efficient extraction from treated nuclei. Following incubation with the constitutive factor(s) present in the low-salt nuclear extract from untreated Hepa 1c1c7 cells, C1 was generated by probes containing either the XRE1 (Fig. 5B, lanes 5 and 7) or XRE2 (lanes 6 and 8) motif.

To further characterize the DNA-binding specificity of the constitutive factor responsible for C1 formation, we performed oligonucleotide competition experiments in gel mobility shift assays using the low-salt nuclear extract from untreated hepatoma cells. To this end, the ability of the XRE1 wild-type sequence to compete for binding was compared with that of mutant oligonucleotides that carry one single or four transversions in the core hexanucleotide motif. The sequences of the two mutant oligonucleotides are presented in Fig. 5A along with the sequences of the XRE1 and XRE2 wild-type probes.

Formation of C1 with the XRE1 probe was strongly inhibited in the presence of a 200-fold molar excess of competitor fragments containing either the unlabeled XRE1 sequence (Fig. 5C, compare lanes 2 and 3) or the mutant motif (XM1) containing only a single transversion in the conserved hexamer motif (compare lanes 2 and 5). In contrast, no significant competition for formation of C1 was detected at an identical molar excess of the oligonucleotide (XM4) containing a cluster of four point mutations in the hexamer motif (compare lanes 2 and 4). This mutant oligonucleotide appeared to be inert in competition experiments to a similar extent as unrelated oligonucleotides spanning either the octamer sequence motif from immunoglobulin promoters or a glucocorticoid response element (compare lanes 2, 4, 6, and 7).

In the low-salt nuclear extract, a faint band migrating with a relative mobility slightly greater than that of the major C1 band was detected (Fig. 5C). In addition, the factor responsible for formation of this band indicated a DNA-binding specificity similar to that of the C1-forming factor. This band varied in intensity in different protein preparations and thus may represent a discrete degradation product of the C1-forming factor rather than an additional distinct XRE-binding factor.

In control experiments, the cytosolic dioxin receptor was activated to a DNA-binding form by dioxin treatment in vitro (7), resulting in recognition of the wild-type XRE1 sequence (Fig. 5D, lane 1). Competition experiments demonstrated that formation of the receptor-DNA complex (indicated by an arrow) was abrogated only in the presence of a 25- to 50-fold molar excess of the wild-type XRE competitor fragment (Fig. 5D, lanes 2 to 4). Receptor-DNA interaction was not significantly disturbed in the presence of identical molar excesses of either the XM4 or XM1 mutant competitor fragment (lanes 5 to 8). Taken together, these results indicate similar but not identical DNA-binding specificities for the dioxin receptor and the constitutive C1-forming factor. Whereas a single point mutation of the fifth base in the conserved hexamer motif is sufficient to dramatically impair interaction of the dioxin receptor with the XRE1 target sequence, such a mutation did not significantly inhibit C1 formation (7; Fig. 5D). Finally, the oligonucleotide competition experiments indicated that the C1-forming factor and the receptor may exhibit different relative affinities for the XRE1 target sequence. Thus, about 5- to 10-fold-higher concentrations of competitor oligonucleotide were required to abrogate XRE complex formation with the C1-forming constitutive factor(s) compared with the concentrations required to inhibit dioxin receptor-XRE interaction. A tentative quantitation by gel mobility shift analysis of both factors indicated that they exhibit approximately the same relative abundance in high-salt nuclear extract of treated hepatoma cells.

DISCUSSION

The chromatin structure at the dioxin response elements of the rat cytochrome P-450IA1 gene is unaltered in the process of gene activation. In this report, we show that two DNase I-hypersensitive sites (collectively termed HS1) in close proximity to each other coincided with the cytochrome P-450IA1 dioxin response elements XRE1 and XRE2, which both are located about 1 kb upstream of the transcription initiation site. Importantly, this hypersensitive site was detected in nuclei from both untreated and induced rat liver, indicating a constitutively open chromatin structure at this segment of the gene. Previously, the failure to detect any discrete DNase I-hypersensitive sites in the 5′ flank of the cytochrome P-450IA1 gene in hepatoma cells has been reported (12). The reason for this discrepancy in results remains unclear. By contrast to the constitutive DNase I-hypersensitive site observed at the XREs in this study, glucocorticoids or progestins induce DNase I-hypersensitive sites at the hormone response elements in chromatin flanking, for instance, mouse mammary tumor virus (50), the rat tyrosine aminotransferase gene (4, 25, 32), and the chicken lysozyme (14, 22) genes. In fact, the observed hormone-induced chromatin changes in the tyrosine aminotransferase (4) and lysozyme (14) genes facilitated the identification and definition of functional hormone response elements (22, 25). More recently, it has been shown that both glucocorticoid and progesterone receptors induce formation of the DNase I-hypersensitive site at the glucocorticoid response elements in tyrosine aminotransferase chromatin in an indistinguishable manner (43), suggesting that the two receptors modulate

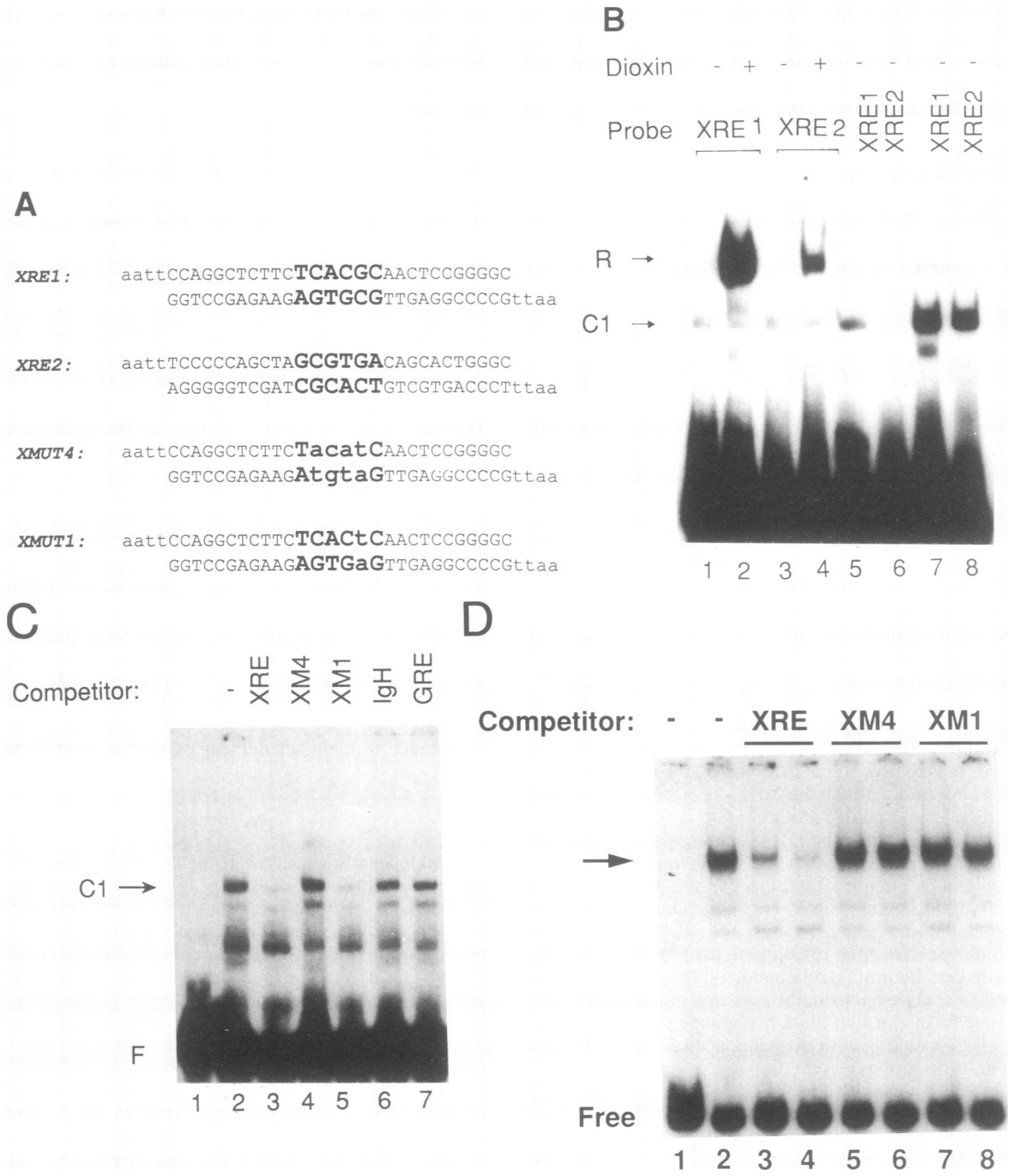

FIG. 5. DNA-binding specificities of constitutive and inducible XRE-binding factors. (A) Nucleotide sequences of wild-type and point-mutated oligonucleotides used in gel mobility shift DNA-binding experiments. Oligonucleotides XRE1 and XRE2 span the XRE1 and XRE2 elements, respectively, of the rat cytochrome P-450IA1 gene. Bold letters indicate the conserved core hexanucleotide motif. Oligonucleotides XMUT1 (XM1) and XMUT4 (XM4) carry point mutations in the XRE1 sequence motif. Lowercase letters indicate point-mutated nucleotides or linker sequences. (B) Evidence that the constitutive XRE-binding factor recognizes both XRE1 and XRE2 sequences. Latent (lanes 1 and 3) and in vitro-activated cytosolic Hepa 1c1c7 dioxin receptor (lanes 2 and 4) and 2 (lanes 5 and 6) and (lanes 7 and 8) 4 μl of low-salt nuclear extract containing the constitutive XRE-binding factor from Hepa 1c1c7 cells were incubated with labeled XRE1 or XRE2 probe as indicated. The receptor was activated by incubation of cytosol (4 mg of protein per ml) from untreated, wild-type Hepa 1c1c7 cells with 10 nM dioxin at 25°C for 3 h. (C) DNA-binding specificity of the C1-forming factor. The labeled XRE1 probe was incubated with a fixed amount of low-salt nuclear extract from nontreated Hepa 1c1c7 cells in the absence (lane 2) or presence of a 200-fold molar excess of unlabeled competitor DNA as indicated above the lanes. IgH, immunoglobulin octamer sequence element; GRE, glucocorticoid response element. Lane 1 shows the mobility of free probe (F) in the absence of any added protein. (D) Binding studies with in vitro-activated dioxin receptor. The receptor was in vitro activated as described above. Gel mobility shift assays were performed with labeled XRE1 probe in the absence (lane 2) or presence of either a 20-fold (lanes 3, 5, and 7) or 40-fold molar (lanes 4, 6, and 8) excess of the indicated unlabeled competitor fragments. Lane 1 shows a control DNA-binding reaction in the absence of any added protein. The receptor-dependent XRE complex is indicated by an arrow. Free, unbound ^{32}P-labeled probe.

chromatin structure by very similar or identical modes of action. Furthermore, in vivo footprinting experiments have demonstrated that the glucocorticoid response element of the tyrosine aminotransferase gene is occupied by a factor (presumably the receptor) only in hormone-induced cells (3). In agreement with these observations, the ligand-induced conversion of the glucocorticoid, estrogen, and progesterone receptors from cryptic to DNA-binding forms has been reconstituted in vitro (1, 8, 26, 34a).

It has been reported that dioxin treatment induces an exonuclease III stop site in mouse cytochrome P-450IA1 chromatin (11). However, this site does not correspond to any functional dioxin-responsive and dioxin receptor-binding sequences in the mouse cytochrome P-450IA1 gene (31), nor does it correspond to any of the mapped DNase I-hypersensitive sites in the rat gene. Thus, in the dioxin receptor system, it is not known how treatment with ligand affects the DNA-binding activity of the receptor in vivo. Under in vitro conditions, however, the XRE-binding activity of the dioxin receptor is under strict control of the ligand (7, 30). Interestingly, the binding affinity of a ligand for the dioxin receptor appears to determine the amount of active form of receptor that is sequestered out of the latent complex (7). Taken together, these in vitro data strongly indicate that the ligand may play several important roles in receptor-mediated signal transduction, including (i) the possible unmasking of a nuclear translocation signal and (ii) triggering release of inhibition of the XRE-binding activity.

Possible functional implications of the constitutive DNase I-hypersensitive site at the XREs. At present, we can only speculate how the chromatin structure at the XREs 1 kb upstream of the cytochrome P-450IA1 transcription start site is maintained in a constitutively accessible configuration. As discussed above, dioxin receptor-dependent XRE-binding activity is not detected in nuclear extracts from untreated cells (9, 17, 21), possibly because of a cytoplasmic intracellular localization and a non-DNA-binding configuration of the receptor in the ligand unoccupied state. It is therefore conceivable that the constitutive C1-forming factor has access to and specifically interacts with XRE target sequences in the uninduced cell.

Recently two constitutive XRE1-binding factors designated XF-1 and XF-2 have been characterized in rat and mouse hepatoma cells (36). In the present study, we have detected only the C1 factor in nuclear extracts from rat liver and a number of hepatoma cells. However, it is formally possible that the larger XF-1 factor is related to or identical to the C1 factor, and given the in vitro lability of the C1 factor, the smaller XF-2 factor may represent a degradation product of XF-1. In any case, it is difficult to assess how the different factors are related to one another since it is not known whether the XF proteins recognize only the XRE1 sequence motif or whether they carry specificity for both XRE1 and XRE2, which are rather dissimilar in the sequences flanking the conserved 5′-TCACGC-3′ core hexamer (16; Fig. 5A).

The functional significance of the interaction between the C1 factor and the XRE element is still unclear. However, both constitutive DNase I-hypersensitive sites and protein-DNA interactions which mediate signal transduction have been described in cyclic AMP- and serum-responsive enhancer elements of the tyrosine aminotransferase and c-*fos* genes, respectively (reviewed in reference 45). In the highly inducible c-*fos* promoter, a complex functional interplay between regulatory factors has been characterized at the serum response element involving at least two DNA-binding factors: a 67-kDa protein (35, 44) and a 62-kDa protein (39). Moreover, it has been demonstrated that the serum response element is protected against methylation by dimethyl sulfate also in nuclei from untreated cells (23), suggesting that the element is constitutively occupied by some factor(s). In fact, the 67-kDa protein has recently been shown to mediate repression of c-*fos* transcription by occupying the serum response element and generating the low levels of expression observed prior to growth factor induction (38). For efficient induction, the 67- and 62-kDa proteins appear to have to interact with one another and the response element (39). Given this background, it remains to be determined whether the presence of the constitutively open chromatin configuration at the XREs and the two different XRE-binding factors implies a similarly complex interplay between these factors in determining the dioxin-induced transcriptional response. For instance, it is formally possible that the constitutive factor will be found to be a component of the possibly heteromeric ~200-kDa form of activated, nuclear dioxin receptor (21). Conversely, it is conceivable that the receptor and the constitutive factor function in a mutually antagonistic manner involving a polypeptide exchange at the XRE of a constitutive repressor versus an inducible activator (i.e., the receptor).

Induced changes in chromatin structure at the cytochrome P-450IA1 transcription start site. Interestingly, the sensitivity of the HS2 site to nuclease digestion in rat cytochrome P-450IA1 chromatin was altered upon induction. We have tentatively mapped this site to between −180 to +20 relative to the transcription start site. Two independent studies have recently demonstrated that, in addition to the dioxin-responsive enhancer elements 1 kb upstream of the transcription start site, more proximal regulatory sequences are required for maximal dioxin inducibility of both the mouse and rat cytochrome P-450IA1 promoters (31, 49). Whereas this region has been defined rather broadly in the mouse promoter to span sequences from positions −245 to −50 relative to the cap site (31), the regulatory sequences have been mapped to an element which extends from positions −53 to −44 and is located 12 bp upstream of the TATA box of the rat promoter (49). Moreover, a constitutive nuclear factor(s) which specifically interacts with this element has been identified (49). It is not clear how this proximal factor(s) acts in combination with the distal, inducible dioxin receptor to enhance transcription from the promoter in response to dioxin treatment. However, it is interesting that the chromatin structure in the proximal region of the promoter becomes more accessible upon induction, suggesting that the interaction between the proximal regulatory element and its related DNA-binding factor(s) may be facilitated. Clearly, in vivo mapping of the chromatin fine structure of the promoter and in vitro reconstitution of the positioning of the nucleosomes are required to further study this issue.

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