Aryl hydrocarbon-induced interactions at multiple DNA elements of diverse sequence—a multicomponent mechanism for activation of cytochrome P4501A1 (CYP1A1) gene transcription

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ABSTRACT

In vivo footprinting experiments, augmented with gel shift and transfection analyses suggest that activation of the CYP1A1 gene by aryl hydrocarbons may be a multicomponent process. During the first 30 minutes of exposure to aryl hydrocarbon carcinogens and environmental contaminants, *in vivo* footprints appear at nine distinct sites within a 281 bp region centered 950 bp upstream of the CYP1A1 transcription start site. Six of these sites are unrelated in sequence to the three xenobiotic response elements (XREs) within this region, at which the aryl hydrocarbon (AH) receptor is known to bind. These six display a variety of footprint patterns, are diverse in sequence and range in G-C content from 60 to 75%. This diversity suggests that multiple nuclear factors may be responsible for these six *in vivo* footprints. These observations are consistent with competition gel shift experiments showing that the nuclear factors binding at two of these sites are different from each other, as well as from the AH receptor. Gel shifts also indicate that the sequence-specific factors binding at these sites are expressed constitutively. This is consistent with a model in which *in vivo* footprints are induced at these six sites, not through direct activation or *de novo* synthesis of DNA-binding factors, but through a two phase mechanism in which binding of the nuclear AH receptor complex to XREs facilitates the binding of constitutive factors at these sites. This facilitation could be mediated either through specific protein–protein interactions or through alterations in chromatin structure that make these sites accessible to constitutive nuclear factors. A function for the sequences at which aryl hydrocarbons induce *in vivo* footprints is suggested by transfection experiments showing that one of these sequences cooperates with a weak XRE to confer on a reporter gene responsiveness to aryl hydrocarbons.

INTRODUCTION

Cytochrome P4501A1 catalyzes the mixed function oxidation of many carcinogenic and toxic environmental contaminants, as well as many endogenous compounds. Understanding the regulation of the gene encoding this enzyme has been of particular concern in chemical carcinogenesis, since cytochrome P4501A1 is capable of activating aryl hydrocarbons, such as benzo[*a*]pyrene, to highly carcinogenic forms (1, 2). Since the transcription of the cytochrome P4501A1 gene (CYP1A1) is strongly activated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and by other halogenated aromatic hydrocarbons, CYP1A1 has also been used as a model system for studying the biological actions of this important class of environmental contaminants. Extensive research has established that TCDD and many other planar aryl hydrocarbons exert their biological effects through the aryl hydrocarbon (AH) receptor (3–5), which has recently been shown to be a member of a new class of nuclear receptors, distinct from the steroid and thyroid hormone receptor super-family (6–8). Since CYP1A1 is the most thoroughly studied of the genes regulated by this receptor, it serves as a useful system for studying the molecular mechanisms through which this new class of receptors acts.

Transient transfection experiments with chloramphenicol acetyl transferase (CAT) reporter gene constructs (9, 10) and *in vitro* transcription studies (11) have identified a class of response elements, xenobiotic response elements (XREs), also called dioxin or AH response elements, through which aryl hydrocarbons activate transcription of CYP1A1. *In vitro* footprinting and gel shift experiments have also shown that, subsequent to binding aryl hydrocarbons, the AH receptor is activated to a form that binds specifically to XREs (12–17). Information regarding the *in vivo* DNA–protein interactions important in regulating CYP1A1 transcription has been provided through both low-resolution studies, employing DNase I (18), exonuclease III (19), and restriction enzymes (20), and, recently, through high-resolution *in vivo* footprinting studies (21–24).

The experiments presented here reveal aryl hydrocarbon-inducible footprints at several sequences distinct from XREs, as

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well as establish a function for these sequences and their interactions with regulatory factors. This expands the focus of CYP1A1 transcriptional regulation beyond XREs, and establishes that the inducible *in vivo* footprints, previously reported around -950 bp upstream from the CYP1A1 promoter, are part of a much broader array of aryl hydrocarbon-inducible DNA-protein interactions. Thus, transcriptional regulation of CYP1A1, like that of many other genes (25-27), involves interactions between several, different regulatory factors and sequences. Specifically, we show that, within a 281 bp domain, interactions are coordinately induced at nine sites. Six of these are diverse in sequence and have different footprint patterns, while the remaining three are XREs. Further experiments show that the nuclear proteins recognizing two of these six diverse regions are different from each other and are expressed constitutively. Together, these experiments provide evidence that interaction of the nuclear AH receptor complex with XREs may facilitate binding of constitutive DNA-binding factors at diverse sequence elements upstream of CYP1A1. A role for these secondary interactions in regulation of transcription is suggested by transfection experiments showing that one such element, located 1100 bp upstream of the CYP1A1 promoter, can cooperate with XREs to activate transcription of a reporter gene in response to aryl hydrocarbons.

MATERIALS AND METHODS

In vivo footprinting

Rat hepatocyte-derived LCS7 cells, a clonal derivative of RALA255-qpG (28, 29), were grown to confluence in 150 mm tissue culture dishes as previously described (12). Cells were treated with 5 μ g/ml β -naphthoflavone in dimethylsulfoxide for 30 minutes prior to dimethyl sulfate treatment. This relatively nontoxic polycyclic aromatic compound was used in all experiments in order to reduce risk to the experimenter. At the concentrations and under the conditions used, the effects of this compound were indistinguishable from those of other strong CYP1A1 inducers, such as TCDD. Dimethyl sulfate treatment, DNA extraction, piperidine treatment of DNA, and the ligation-mediated polymerase chain reaction were performed as described (30), except for the following: (1) Cells were incubated in DNA harvest buffer at 37°C overnight. (2) The dimethyl sulfate stop buffer contained 250 mg/ml calf liver tRNA, not 100 mg/ml yeast tRNA. (3) Prior to first strand synthesis, DNA was denatured at 97°C for 5 minutes. (4) Two labeling cycles were performed. Labeled products were resolved on denaturing 6% polyacrylamide gels, and visualized by autoradiography.

Transfections, plasmids and oligonucleotides

Primary cultures of adult rat hepatocytes were prepared and maintained in culture as previously described (31). HepG2 cells were grown in the same medium with 10 percent fetal bovine serum on plates that were first coated with Matrigel (Collaborative Research, Bedford, MA). Transfections were carried out as previously described (32). All plasmids were generated by inserting the oligonucleotides described below in native orientation into the *Bgl*III site immediately upstream of the minimal SV40 promoter driving the CAT gene carried in plasmid pA₁₀CAT (33). All constructs were confirmed by DNA sequence analysis. Oligonucleotide XRE2 included bases -1098 to -1070 upstream from the CYP1A1 transcription start site, while DXE1 spanned bases -1108 to -1085, and DXE2 included bases -1078 to

-1055. DX46 included XRE2 and DXE1, spanning bases -1108 to -1070. X112 extended from -1109 to -998, and included XREs 1 and 2 and DXEs 1, 2, and 3. The Sp1 oligonucleotide sequence was 5'-GATCCAGACCCCGCCCACTA-3' (upper strand). All oligonucleotides included *Bam*HI and *Bgl*III sites at the 5' and 3' ends, respectively.

Mobility shift assay

Nuclear extracts were prepared from LCS7 cells and mobility-shift DNA-binding protein assays were carried out, as described (12). The oligonucleotides used are described in the previous paragraph.

RESULTS

Aryl hydrocarbon-induced DNA-protein interactions

Figures 1 and 2 show that β -naphthoflavone coordinately induced footprints, not only at XREs, as previously reported (21-24), but also at six other sites within a 281 bp region. Whereas the footprints over XREs were all similar, the sequences of, and footprints at, the other six sites were diverse, although somewhat enriched in guanines (60-75% G+C). In this paper, these diverse sequence xenobiotic responsive elements will be referred to as DXEs to distinguish them from XREs, the sites where the AH receptor is known to bind.

As is shown in Figure 1A and summarized in Figure 3A, the guanines in region DXE1 (labeled D1 in Figure 1A) underwent striking alterations in dimethyl sulfate reactivity following exposure of cells to β -naphthoflavone. The 3'-most guanine within this domain became hypersensitive, while seven of the other eight guanines became protected from dimethyl sulfate following exposure to β -naphthoflavone. DXE2 and DXE3, located between XRE1 and XRE2, were also altered in reactivity to dimethyl sulfate following exposure to β -naphthoflavone. Two bases were protected on the upper strand of DXE2 (labeled D2A in Figure 2A-C) while a single guanine was protected on the lower (labeled D2B in Figure 1A). Figures 2A and 2B show that marked hypersensitivity was induced in a single guanine located within DXE3, which lies between DXE2 and XRE2. The guanines marked as protected or hypersensitive in Figures 1 through 3 are those that consistently, in three or more experiments, displayed β -naphthoflavone-induced alterations in reactivity.

The compound β -naphthoflavone also induced an *in vivo* footprint at DXE4. In this region, labeled D4 in Figure 2A, the reactivity of four guanines to dimethyl sulfate decreased, while a fifth guanine became hypersensitive. There was some variation in the intensity of inducible footprints among the DXEs, as well as among XREs. The footprint at DXE4 was the weakest of these. Although ours is the first report of an inducible *in vivo* footprint at DXE4, it has been previously reported that, in the murine cell line Hepa 1, this sequence is a functional enhancer element (34). Thus transfection data support the functional significance of the sequences corresponding to this, quite modest, *in vivo* footprint. Since this footprint is the weakest of those reported here, this implies that the even more distinct footprints observed at other DXEs are also likely to be of functional significance. The sequence of DXE4 corresponds to the consensus sequence of the transcription factor Sp1 (35). However, the hypersensitive base, which is part of the DXE4 footprint, distinguishes it from that reported for Sp1, which contains no hypersensitive guanines (35).

Figure 1B demonstrates that exposure to β -naphthoflavone alters the reactivity of two guanines on the same strand of DXE5,

Figure 1. Inducible *in vivo* interactions detected at XREs and DXEs — lower strand. Sequence corresponds to lower strand in Figure 3C. X1–X5 indicate XREs, D1–D6 indicate DXEs. Letters above each panel indicate lanes displaying ladders from free DNA (F), untreated cells (C) and β-naphthoflavone-treated cells (B). Duplicate lanes represent analyses of DNA isolated from separate plates of LCS7 cells that were treated in parallel with inducers and carried in parallel through the footprinting procedure. Only changes that were observed in duplicate in several experiments were considered significant. (A) Primers were P2A, P2B, and P2C, whose locations are specified in Figure 3C. (B), (C) and (D) Primers were P4A, P4B, and P4C.

which is located between DXE4 and XRE3. One guanine within this region becomes hypersensitive, while a second becomes protected. Although there are other guanines on both strands of DXE5, these are not altered in reactivity by β-naphthoflavone.

Although the appearance of footprints at XREs and at DXEs is coordinate, there appears to be no consistent positional relationship between these two classes of sequences, except that most DXEs are within 30 bp of a functional XRE. However, DXE6 breaks even this rule; the closest functional XRE (XRE3)

is located 80 bp upstream. As shown in Figures 1B, 1C and 1D and summarized in Figure 3A, the pattern of dimethyl sulfate reactivity at DXE6 is similar to that observed at DXE3, although the DXE6 core sequence differs from that of DXE3 by the inversion of an A-T base pair. At both of these sites, β-naphthoflavone causes a single guanine to become strongly hypersensitive.

Even though the alterations in dimethyl sulfate reactivity at DXE3 and DXE6 involve only single bases, the hypersensitivity

Figure 2. Inducible *in vivo* interactions detected at XREs and DXEs — upper strand. Sequence corresponds to upper strand in Figure 3C. Nomenclature as shown in Figure 1. Primers were P1A, P1D and P1E.

induced at these sites is quite strong and is highly reproducible. It is well known that the hypersensitivity of guanines to dimethyl sulfate is indicative of DNA–protein interactions, just as protection is. Whereas protection is usually due to DNA–protein interactions that prevent dimethyl sulfate from gaining access to a particular guanine, hypersensitivity is due to DNA–protein interactions that increase the reactivity of a guanine to dimethyl sulfate, usually through increasing its accessibility to the footprinting reagent. The absence of broader footprints at DXE3 and DXE6 is probably due to the fact that methylation by dimethyl sulfate provides a less-than-complete picture of the interactions of proteins with a particular DNA domain. This is because dimethyl sulfate (a) reacts only with guanines, (b) is sensitive

only to interactions of proteins within the major groove of the DNA molecule, and (c) is excluded only by very tight DNA–protein interactions. Thus, a protein could bind weakly in the major groove of the DNA, or bind primarily in the minor groove and/or to the phosphate backbone of even a highly guanine-rich domain and yet not protect those guanines from methylation by dimethyl sulfate. In fact, such asymmetric binding might deform the DNA molecule, making one or more guanines more reactive and thereby generating hypersensitivity at those sites. The possibility that a protein could bind to DXE3 or DXE6 without protecting guanines from methylation by dimethyl sulfate is also increased by the fact that the guanines within these regions are concentrated on only one strand. We conclude that the strong

Figure 3. Summary of footprinted regions. Open triangles indicate bases protected from methylation. Filled triangles indicate hypermethylated bases. (A) Diverse sequence xenobiotic responsive elements. (B) Xenobiotic response elements. Sequences were aligned relative to common features. Only bases that were altered in reactivity in three separate experiments are indicated here. Data for the guanine on the upper strand of XRE3 are not presented in Figure 2. (C) Sequence overview. Sequences of interest are boxed. Primers are indicated by arrows.

and highly reproducible hypersensitivity observed at single bases within DXE3 and DXE6 constitutes reliable evidence for β -naphthoflavone-induced changes in DNA-protein interactions in these regions. However, it will be necessary to use other *in vivo* footprinting methods to characterize these interactions more thoroughly. It is worth noting here that an asymmetric distribution of guanines between strands is characteristic of all of the DXEs examined, except for DXE2. This probably contributes to the fact that all of these regions, except DXE2, display footprints on only one strand.

Figure 3A, which compares the six DXEs discussed above, reveals the diversity of the footprints at these sites: only two of these regions, DXE3 and DXE6, are similar both in sequence and in footprint pattern. The diversity of the footprints at DXEs

admits the possibility that the coordinately induced interactions at these sites may be due to multiple DNA-binding factors. The interactions at DXEs are, however, sequence-specific, since global alterations in dimethyl sulfate reactivity are not apparent in Figures 1 and 2 in either untreated or β -naphthoflavone-treated cells.

As shown in Figures 1 and 2 and summarized in Figure 3B, β -naphthoflavone induced *in vivo* footprints at three of the five XRE core motifs located in the region examined. Interactions were not detected, in either untreated or induced cells, over the other two putative XRE cores present in this region (designated XRE4 and XRE5 in Figures 1A and 1B). Inducible interactions at XREs 1, 2 and 3 are consistent with the observations in mouse (21–24). XRE4 is not present in murine CYP1A1, while the

Figure 4. A. Transcriptional regulatory function of DXE1. Primary cultures of adult rat hepatocytes were plated at 0.7×10^6 cells/60 mm dish and co-transfected (32) 48 hours later with the specified plasmids and with the β -galactosidase expression vector CH110 (37). Forty hours after transfection, cells were treated with β -naphthoflavone ($5 \mu\text{g/ml}$) for 24 hours or left untreated. Cells were then harvested and CAT (38) and β -galactosidase (37) activities determined. All plasmids were generated by inserting the oligonucleotides described in Materials and Methods in native orientation into the *Bgl*III site immediately upstream of the SV40 early region promoter-CAT fusion gene carried in the plasmid pA₁₀CAT (33). X112 is the native sequence containing DXE1, XRE2, DXE2, and XRE1 (5' to 3' order). DX46 is the native sequence containing DXE1 and XRE2 (5' to 3' order). CAT activities are reported as percent of chloramphenicol acetylated, normalized to β -galactosidase activity. **B.** XRE2 and DXE1 cooperate to mediate transcriptional activation by aryl hydrocarbons in HepG2 cells. The human hepatoma cell line HepG2 was plated at 0.5×10^6 cells/60 mm dish and co-transfected 24 hours later with the specified plasmids and with the β -galactosidase expression vector CH110. Starting 24 hours after transfection, cells were exposed to β -naphthoflavone ($5 \mu\text{g/ml}$) for 24 hours or left untreated. Cells were then harvested, and CAT and β -galactosidase activities determined. All plasmids were generated by inserting the oligonucleotides described in Materials and Methods in native orientation into the *Bgl*III site immediately upstream of the SV40 early region promoter-CAT fusion gene carried in the plasmid pA₁₀CAT. X112 is the native sequence containing DXE1, XRE2, DXE2, DXE3 and XRE1 (5' to 3' order). DX46 is the native sequence containing DXE1 and XRE2 (5' to 3' order). CAT activities are reported as percent of chloramphenicol acetylated/ β -galactosidase activity, normalized by setting the CAT activity of pA₁₀CAT to 1.0 for all experiments. N was ranged from 8 to 16 for all points. Error bars represent one standard deviation.

Figure 5. Different constitutive nuclear factors bind to DXE1 and DXE2. (A) Nuclear extracts were from untreated (C) or β -naphthoflavone-treated ($5 \mu\text{g/ml}$, B) cells. The ^{32}P -labeled probes (0.50 ng , $2.0 \times 10^4 \text{ cpm/10 ml}$ reaction) were XRE2 (lanes 1–4), XRE1 (lanes 5–8) and DXE1 (lanes 9–12). Reactions were run in duplicate to establish reproducibility. (B) Nuclear extracts were from untreated (lane 1) and β -naphthoflavone-treated (lanes 2–14) cells and the probe was ^{32}P -labeled DXE2 (0.14 ng , $1.5 \times 10^4 \text{ cpm/10 } \mu\text{l}$ reaction). Lanes 1 and 2, no competitor. Each subsequent set of three lanes contained 5-fold, 25-fold, and 100-fold molar excess of the following competitors: DXE1 (lanes 3–5), DX46 (lanes 6–8), DXE2 (lanes 9–11), and Sp1 (lanes 12–14). The arrows indicate the DXE2 specific complexes. (C) Nuclear extracts were from untreated (lane 1), and β -naphthoflavone-treated (lanes 2–20) cells and the probe was DXE1 (0.16 ng , $1.5 \times 10^4 \text{ cpm/10 } \mu\text{l}$ reaction). Lanes 1 and 2 contained no competitor. Competitor was present at 2-, 5-, 25-, or 100-fold molar excess for DXE1, DXE2, and DX46, and 5-, 25-, and 100-fold excess for Sp1 and XRE2. Competitors are specified above lanes. The arrow indicates the position of the DXE1-specific protein–DNA complex.

murine and rat sequences at XRE5 are sufficiently divergent to account for the fact that *in vivo* interactions are detected at the former but not at the latter. The detection of footprints over only a subset of XRE core motifs is consistent with previously reported studies (14, 36), showing that XRE function requires sequences in addition to the core motif.

DXE1 is a functional regulatory element

Although *in vivo* footprinting data for DXE1, similar to those presented in Figures 1 and 2, have been previously reported (21, 24), a gene regulatory function for this sequence has not been previously described. Figure 4 shows that DXE1 acts as a transcriptional regulatory element that can cooperate with XRE2 to render the chloramphenicol acetyl transferase (CAT) reporter gene responsive to aryl hydrocarbons.

Initial experiments using all known methods for DNA-mediated gene transfer showed that the rat hepatocyte-derived cell line LCS7, could be transfected with only very low efficiency. This made it impractical to evaluate the function of DXEs in this cell line. Instead, we chose to carry out such experiments in primary cultures of adult rat hepatocytes, the cell type from which LCS7 cells were originally derived, and in HepG2 cells, a human hepatoma cell line. Figure 4A presents results for isolated hepatocytes, showing that CAT expression could not be induced by aryl hydrocarbons in cells transfected with constructs in which either a single copy of the weak response element XRE2, or a single copy of DXE1, was placed upstream of the CAT reporter gene. However, CAT expression could be induced in cells transfected with a construct, DX46, containing both XRE2 and DXE1. CAT expression could be induced even more strongly in cells transfected with X112, which contains XRE1, DXE2 and DXE3, in addition to DXE1 and XRE2.

Much more extensive analysis in the HepG2 cell line confirmed results obtained with isolated hepatocytes, showing with high statistical significance that DXE1 and XRE2 can cooperate to confer responsiveness to aryl hydrocarbons. Figure 4B establishes that in HepG2 cells DXE1 alone does not confer responsiveness to β -naphthoflavone, although it is clearly a basal enhancer element. Likewise, XRE2 alone does not confer responsiveness to β -naphthoflavone; it consistently yields basal CAT expression even lower than that of pA₁₀CAT. In contrast, DX46, which contains both DXE1 and XRE2, is responsive to β -naphthoflavone. We have carried out paired t-tests establishing that, although the fold induction with the DX46 construct by β -naphthoflavone is only approximately 2, the difference between the levels of expression in control and β -naphthoflavone-treated cells is highly significant ($p = 0.001$). This compares favorably with significance for responsiveness of X112 ($p = 0.007$). The lower p value for X112 is probably due to a smaller n (10 versus 16) and larger variability in the extent of induction. The small, but highly reproducible, extent of induction is due primarily to the use of a minimal promoter in the constructs tested. We chose to use this promoter because it displayed more clearly the interactions between DXE1 and XRE2. A reporter gene driven by a more complex set of sequences would have afforded a more robust response to β -naphthoflavone, but the effects of those promoter sequences might have obscured potential interactions between DXE1 and XRE2. These results strongly confirm our observations in isolated hepatocytes, that DXE1 can cooperate with XRE2 to confer responsiveness to aryl hydrocarbons on the CAT reporter gene. In conjunction with those findings, the data from HepG2 cells establish the generality of the cooperative

functions of DXE1 and XRE2, making it quite likely that these elements function similarly in the hepatocyte-derived LCS7 cell line used in other experiments in this paper.

Different constitutive factors bind to DXE1 and DXE2

Although the *in vivo* footprint at DXE1 had been reported (21, 24), *in vitro* experiments characterizing the interaction of nuclear factors with this sequence have not been described previously. Figure 5 presents experiments characterizing, *in vitro*, the nuclear factors that bind specifically to DXE1 and DXE2. In light of the experiments presented in Figures 1 and 2, showing that β -naphthoflavone induces interactions at both of these sites *in vivo*, the results presented in Figure 5 are of significance for understanding the mechanism by which aryl hydrocarbons induce CYP1A1 transcription.

Figures 5A and 5C present *in vitro* analyses of DNA-binding activity showing that the nuclear factors specific for DXE1 and DXE2 are expressed constitutively and cannot be induced further by β -naphthoflavone. The experiment presented in Figure 5A shows that nuclear extracts from untreated and β -naphthoflavone-treated cells contain equal levels of DNA-binding activity specific for ³²P-labeled, double-stranded DXE1 oligonucleotide (lanes 9 and 10 versus lanes 11 and 12). Figure 5C shows that the nuclear factor which specifically recognizes DXE2 is also present constitutively (lane 1 versus lane 2). As a control, the experiment presented in Figure 5A also measured the DNA-binding activity of the nuclear AH receptor, assessed using ³²P-labeled XRE1 and XRE2 oligonucleotides. XRE-binding activity was highly inducible; it could not be detected in extracts from untreated cells (lanes 1, 2, 5, and 6), but was present in substantial amounts in extracts from β -naphthoflavone-treated cells (lanes 3, 4, 7, and 8), as previously reported (13–15). Thus, in contrast to the inducible footprints at XREs, which are probably due to β -naphthoflavone activation of the AH receptor, the inducible *in vivo* footprints observed in Figures 1 and 2 at DXE1 and DXE2, are probably not due to the activation of cryptic DXE-binding factors, nor are they likely to be due to factors newly synthesized in response to β -naphthoflavone, since DXE1- and DXE2-specific factors are present constitutively.

The competition mobility shift experiment presented in Figure 5B shows that the nuclear factor that binds DXE1 is highly sequence specific. A large molar excess of a double stranded oligonucleotide homologous to DXE1 competed strongly for binding with the ³²P-labeled DXE1 probe (lanes 15–17), while equivalent molar excesses of double-stranded oligonucleotides homologous to XRE1, XRE2, Sp1 and DXE2 did not compete (lanes 3–14). Figure 5C presents similar data for DXE2, showing that a double stranded oligonucleotide homologous to DXE2 competed for binding with a ³²P-labeled DXE2 probe, while DXE1, XRE1, XRE2 and Sp1 oligonucleotides did not.

These experiments indicate that the DNA–protein interactions at DXE1 and DXE2 are sequence specific and involve different DNA-binding factors. In addition, the inability of the Sp1 oligonucleotide to compete implies that the factors recognizing DXE1 and DXE2 are not Sp1. We have also found that polyclonal antiserum to human Sp1, known to supershift Sp1–DNA complexes in the mobility shift assay for DNA–protein interactions (41), did not alter the mobility of DNA–protein complexes formed with ³²P-labeled, double stranded DXE1, under the conditions used in Figure 5 (data not shown). This indicates that the DXE1-specific factor present in nuclear extracts from LCS7 cells is not immunologically related to Sp1. This is

an important point, since the sequence footprinted at DXE1 differs by only one base from the Sp1 consensus sequence (cytosine replaced with adenine). Other investigators have also reported mammalian nuclear factors distinct from Sp1 that interact with guanine-rich domains, including the Sp1 consensus sequence, itself (40–43).

DISCUSSION

The central observations reported in this paper are that exposure to aryl hydrocarbons coordinately induces DNA–protein interactions at a large number of sites and that the patterns of DNA–protein interaction detected at these sites are diverse, as are the sequences themselves. These observations emerge from *in vivo* footprint experiments, presented in Figures 1 and 2, that examined the DNA–protein interactions occurring across a large, continuous sequence domain centered around –950 bp upstream of the CYP1A1 start site.

The obvious question that arises from these observations concerns the relevance of these footprints to the mechanism by which aryl hydrocarbons activate transcription of CYP1A1. The transfection experiments, presented in Figure 4, provide insight into this question. These experiments establish that DXE1 is a functional regulatory element that cooperates with XRE2 to confer responsiveness to aryl hydrocarbons. Figure 2 is the first report of an inducible *in vivo* footprint at DXE4, however, Fisher, *et al.* (34), have previously reported that this element can cooperate with the murine equivalent of XRE3 to confer responsiveness to aryl hydrocarbons. Thus two of the six DXEs at which we have detected *in vivo* footprints are similar in function. Work is currently in progress to determine directly the functions of the other four DXEs. However, it can be concluded, based on data for DXE1 and DXE4, that the action of aryl hydrocarbons on CYP1A1 transcription cannot be understood fully in terms of the isolated DNA–protein interactions previously reported (21–24). A broader array of coordinately inducible DNA–protein interactions must be involved. It is well known that enhancers are complex domains consisting of recognition sequences for multiple transcription regulatory factors (25–27). The results presented here establish that this is the case for CYP1A1; several other sequence elements, in addition to the XREs which have been the primary focus of previous work, must play a significant role in CYP1A1 transcriptional regulation.

The *in vitro* experiments examining the interactions of nuclear factors with DXEs provide other insights into the mechanism by which aryl hydrocarbons activate CYP1A1 transcription. Previously, no information had been published on the nuclear factors specific for DXEs. The *in vitro* DNA-binding experiments presented in Figure 5 provide such information for two DXEs. They indicate, first, that different nuclear factors are responsible for the coordinately-induced footprints found at DXE1 and DXE2, and, second, that, the proteins that recognize DXE1 and DXE2 are present constitutively in the nucleus, and do not need to be activated by aryl hydrocarbons in order to bind to DNA. Recent work in this laboratory suggest that these observations can be generalized to all DXE-specific factors (Zhang *et al.*, in preparation and Kim *et al.* preliminary results). Thus current evidence strongly indicates that multiple DNA–protein interactions, at sites of diverse sequence, and involving several different sequence-specific nuclear factors, are important in the regulation of CYP1A1 gene transcription.

Another question that naturally arises concerns the relationship between DNA–protein interactions at XREs and those at DXEs. Based on the finding that exposure to aryl hydrocarbons activates the XRE-binding activity of the AH receptor, while the DNA-binding activity of DXE-specific factors seems to be constitutive, we conclude that the binding of the nuclear AH receptor complex to XREs may facilitate binding of constitutive factors at DXEs. In this two-phase process the response of the AH receptor to aryl hydrocarbons would be primary, while that of DXE-binding proteins would be secondary to that of the receptor.

There are two possible mechanisms by which the nuclear AH receptor might facilitate binding of DXE-specific factors to their cognate sites: (1) The liganded receptor, either while free or while bound to XREs, might interact with DXE-specific factors to facilitate their binding to DXEs. (2) Binding of the receptor to XREs might alter chromatin structure and thereby expose previously inaccessible DXEs for interaction with constitutive nuclear factors. These could, in turn, cooperate with the XRE-bound AH receptor complex to activate CYP1A1 transcription, as is implied by transfection data presented here.

A role for chromatin alteration in regulation of CYP1A1 transcription has been previously proposed (23, 24). This mechanism is not only consistent with several other reports (18, 20), but also accounts most parsimoniously for our results. In particular, the *in vitro* DNA-binding experiments presented in Figure 5 speak strongly against the former mechanism, showing that the nuclear factors binding to DXE1 and DXE2 are active constitutively and do not require activation, either by aryl hydrocarbons or through protein–protein interactions. In addition, the fact that inducible *in vivo* footprints occur at multiple sites that are diverse in sequence, and that these footprints are due to multiple nuclear proteins with different DNA sequence specificities, also favor the chromatin alteration model. The other model would require the existence of multiple nuclear factors with different DNA-binding specificities, all of which would be activated either directly by aryl hydrocarbons or through interactions with a single aryl hydrocarbon-inducible factor, such as the AH receptor. Since the *in vitro* DNA-binding experiments presented in Figure 5 yielded no evidence for the former possibility and since the latter possibility is improbable, the chromatin alteration mechanism seems more likely. However, more direct evidence will be required in order to definitively distinguish between these alternatives.

The ability of the AH receptor to facilitate the binding of other factors to DXEs cannot be the only gene regulatory action of the AH receptor. It is known that transiently transfected CAT reporter gene constructs lack nucleosomes and other features of chromatin organization (44), yet many laboratories have shown, as we have in Figure 4, that aryl hydrocarbons activate CAT expression from such constructs in an AH receptor-dependent fashion. This indicates that the AH receptor must have a transcriptional activation function independent of, and in addition to, the ability to facilitate the binding of DXE-specific factors. An analogous situation has been reported for the glucocorticoid receptor. It can clearly function to alter chromatin organization in a manner that facilitates the interaction of other nuclear factors with their cognate sites (44–46), yet it also activates transcription of reporter genes in transient transfection assays where the template is free of chromatin-like organization (44).

Interestingly, the *in vivo* footprinting data presented in Figures 1 and 2 revealed no reproducible differences in patterns of

dimethyl sulfate reactivity between free DNA, methylated *in vitro*, and DNA from control cells, methylated *in vivo*. This indicates that the region between nucleotides -1298 and -821 upstream of the CYP1A1 gene is quite accessible to dimethyl sulfate, even in cells not treated with aryl hydrocarbons. However, this does not imply that this region is protein-free, nor does it imply that it is free of chromatin-like organization. Many DNA-protein interactions are known not to protect from dimethyl sulfate and are, therefore, invisible to the footprinting method used here. These include interactions involving nucleosomes (47,48), as well as many DNA-protein interactions involving primarily the phosphate backbone or the minor groove of the DNA molecule (49). High resolution footprinting with DNase I and other reagents will be required to investigate more thoroughly both the chromatin organization and the possibility of other constitutive DNA-protein interactions in this region. Detection of such interactions might be expected, since constitutive XRE-binding factors have been detected *in vitro* (50) and since low resolution mapping experiments with DNase I (18) have suggested such interactions.

ABBREVIATIONS

CYP1A1, cytochrome P4501A1 gene. See Nelson *et al.* (51) for current nomenclature; XRE, xenobiotic response element; DXE, diverse sequence xenobiotic responsive element; AH, aryl hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CAT, chloramphenicol acetyl transferase

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