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INTERACTION OF THE REGULATORY DOMAINS OF THE MURINE *Cyp1a1* GENE WITH TWO DNA-BINDING PROTEINS IN ADDITION TO THE *Ah* RECEPTOR AND THE *Ah* RECEPTOR NUCLEAR TRANSLOCATOR (ARNT)

FRANCE CARRIER,* CHING-YI CHANG,† JAUH-LIN DUH,†
DANIEL W. NEBERT† and ALVARO PUGA†‡

*Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and †Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056, U.S.A.

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Abstract—The *a*romatic *h*ydrocarbon (*Ah*) receptor complex is a ligand-activated transcriptional activator consisting of at least two protein components. The ligand-binding component is the AhR protein, a cytosolic receptor encoded by the *Ahr* gene, which, upon ligand binding, translocates to the nucleus in a heterodimeric complex with the ARNT (*Ah* receptor nuclear translocator) component. The complex binds to several discrete DNA domains containing *a*romatic *h*ydrocarbon *r*esponsive *e*lements (AhRE) present in the regulatory region of the murine cytochrome P_1450 *Cyp1a1* gene and of the other genes in the [*Ah*] gene battery. As a consequence of binding, a transcriptional complex is formed that activates the expression of these genes by as yet unidentified mechanisms. We have analyzed DNA–protein interactions in four of these domains, specifically, the AhREs located between −1085 and −482 (sites A, C, E, and D) of the upstream regulatory region of the murine *Cyp1a1* gene. We found that two DNA-binding proteins, present in cytosolic and nuclear extracts of mouse Hepa-1 cells, showed overlapping DNA-binding specificities to those of the *Ah* receptor. One of these proteins had an apparent molecular mass of 35–40 kDa, bound only to AhRE3 (site D), and has been identified tentatively as a member of the C/EBP family of transcription factors. The second protein, purified by DNA-affinity chromatography, had an apparent molecular mass of 95 kDa and bound to a larger DNA motif that included the AhRE sequence, in AhRE3 and AhRE5 (sites D and A), but not in AhRE1 or AhRE2 (sites C and E). This protein was not AhR nor was it ARNT, since it was found in receptorless (Ahr^-) and in nuclear translocation-defective ($Arnt^-$) cells, as well as in cells that had not been exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin), a potent inducer of *Cyp1a1* expression. Evidence from *in vivo* methylation protection indicated that two G residues flanking AhRE3, one of which is required for binding of the 95-kDa protein, may be protected from methylation in uninduced cells and become exposed upon dioxin treatment, suggesting that the 95-kDa protein may be constitutively bound to AhRE3, and be displaced by binding of the *Ah* receptor complex. These results lend support to the concept that the transcriptional regulation of the [*Ah*] battery genes could be modulated by combinatorial interactions of the *Ah* receptor complex with other transcription factors.

Key words: cytochrome P450; transcriptional regulation; *Ah* receptor; *Cyp1a1* gene; gel retardation; *in vivo* footprinting

Polycyclic aromatic hydrocarbons such as TCDD§ (dioxin), 3-methylcholanthrene, and benzo[*a*]pyrene are environmental pollutants that elicit a variety of toxic, teratogenic, and carcinogenic responses in exposed animals [1–7]. Most of these responses are mediated by a cytosolic receptor protein termed the *a*romatic *h*ydrocarbon (*Ah*) receptor. Upon ligand binding, the receptor undergoes a temperature-dependent transformation accompanied by translocation to the nucleus. The nuclear receptor binds to AhREs (also termed XREs and DREs by other authors) in the regulatory region of the murine cytochrome P_1450 *Cyp1a1* gene and possibly of several other genes in the [*Ah*] gene battery. The result of this interaction is the transcriptional activation of the Ah-responsive genes [3, 5, 6, 8]. Possibly as part of the transcriptional activation mechanism, binding induces bending of the DNA at or near the site of the protein–DNA interaction [9].

The gene encoding the ligand-binding receptor has been cloned recently and shown to contain a 95-kDa helix-loop-helix motif, characteristic of many transcriptional activators [10, 11]; however, a transcriptionally functional *Ah* receptor is not a single protein, but a heterodimer that contains, in addition to the ligand-binding moiety, a second helix-loop-helix 84-kDa protein component, known as ARNT, needed for nuclear translocation of the

‡ Corresponding author: Dr. Alvaro Puga, Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, OH 45267-0056. Tel. (513) 558-0916; FAX (513) 558-0925.

§ Abbreviations: AhRE, *a*romatic *h*ydrocarbon *r*esponsive *e*lement, also termed XRE and DRE, for xenobiotic and dioxin responsive element, respectively; ARNT, *Ah* receptor nuclear translocator; C/EBP, CAATT/enhancer binding protein; DTT, dithiothreitol; LMPCR, ligation-mediated polymerase chain reaction; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

receptor [12, 13]. It has been shown recently that the *Ah* receptor and ARNT have little if any DNA-binding activity as monomers, and that a heterodimer of the two proteins is required for DNA binding and for transcriptional activation [14–16]. In addition to the ligand-binding moiety and the nuclear translocator protein, other cytosolic proteins have a transient role in receptor complex activity. One of these is the 90-kDa heat shock protein, which binds the unoccupied receptor and inhibits its DNA-binding activity [17].

Several helix-loop-helix transcriptional activators can readily form protein–protein interactions with other transcription factors, particularly those in the bZIP family, and modify the nature of the transcriptional response [18, 19]. Given the conservation of a TCDD-binding protein with the DNA-binding properties of the *Ah* receptor complex throughout the vertebrates [20–23], it is attractive to speculate that, in addition to its role in detoxification, the *Ah* receptor may have other regulatory functions that are modulated by interactions with other proteins. Our laboratories are interested in the analysis of these other possible functions and of the various protein components that may be part of the regulatory complexes. Here we present evidence to indicate that, in addition to the *Ah* receptor and ARNT, at least two other protein components can interact with the AhRE motifs of the *Cyp1a1* gene.

MATERIALS AND METHODS

Protein extraction and purification

To prepare protein extracts from tissue culture cells, mouse hepatoma Hepa-1 cells [24] were grown to confluence in 150-mm diameter plates in α-MEM supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Extracts were also prepared from three mutant derivatives of Hepa-1 cells, namely the *c*37, *c*2, and *c*4 cell lines. The *c*37 line is Cyp1a1 metabolism-deficient [25, 26]; *c*2 is a "receptorless" mutant that has undetectable levels of *Ah* receptor and *Ahr* mRNA [10, 25, 27, 28], and *c*4 is a mutant defective in nuclear translocation of the *Ah* receptor [12, 25, 28]. Cells from 100 plates were harvested in PBS solution and centrifuged at 1500 *g* for 5 min; then the pellets were resuspended in 50 mL of 10 mM HEPES, pH 7.9. After swelling on ice for 10 min, the cells were pelleted again and the pellets were resuspended in 15 mL of MDH buffer (1.5 mM $MgCl_2$, 2 mM DTT, 25 mM HEPES, pH 7.9). Nuclear and cytosolic proteins were recovered by modifications of methods described by others [29]. The cells were homogenized with 25 strokes of the "A" pestle of a Dounce homogenizer, brought to 0.5% NP-40 and centrifuged at 1700 *g*. The supernatant, containing cytosolic proteins, was precipitated by the addition of ammonium sulfate to 46% (w/v) saturation. The pellet of nuclei was resuspended in 4 mL of HDEK1/10 buffer (25 mM HEPES, pH 7.9, 2 mM DTT, 2 mM EDTA, 0.1 M KCl) and, after the nuclei were in suspension, glycerol and KCl were added to 10% and 0.4 M, respectively. The extract was placed in a rotating wheel for 30 min at room temperature, and then it was centrifuged at 15,000 *g* for 20 min. The pellet was discarded, and the supernatant, containing extracted nuclear proteins, was adjusted to 5–10 mg/mL of protein and kept at −70° until needed. The ammonium sulfate precipitate of cytosolic proteins was collected by centrifugation, and the pellet was dissolved in HDEK1/10 buffer and dialyzed against 100 vol. of the same buffer.

To prepare protein extracts from bacterial cells expressing C/EBPα from an inducible *lac* promoter [30, 31] (a gift of Peter F. Johnson), the cells were grown to mid-log phase in LB medium supplemented with 50 μg/mL of ampicillin and harvested 3 hr after induction with 1 mM isopropylthio-β-galactoside (IPTG). Bacterial pellets were resuspended in HDEK1/10 with 10% glycerol and lysed by sonic disruption. Nucleic acid fragments were removed by passing the extract through DEAE-cellulose in the same buffer. The flow-through fraction was collected and kept at −70° until needed.

DNA-affinity chromatography

A support for DNA-affinity chromatography was prepared essentially as described by others [32, 33]. Two columns were prepared, one containing a double-stranded 29-mer synthetic oligonucleotide with the sequence of the AhRE3 element (−1006) 5′-TCCAGGCTCTTCTCACGC-AACTCCGGGGC-3′ (−978) (the underline denotes the central *Ah* receptor recognition core), and the other with the sequence 5′-TCCAGGCTCTTCT-GAGGGAAGTCCGGGGC-3′, containing four transversions (underlined) in the central recognition core. The two complementary strands of each oligonucleotide were synthesized separately in an Applied Biosystems synthesizer, and they were phosphorylated with T4 polynucleotide kinase, annealed to the corresponding complementary strand, and catenated. Two milligrams of each oligonucleotide were used to prepare DNA affinity columns. The DNA was mixed with 2 mL of CNBr-activated Sepharose 4B, and the mixture was brought to a final volume of 6 mL with 10 mM potassium phosphate buffer, pH 8.0 (KPB). The coupling reaction was allowed to proceed with gentle agitation for 16 hr at room temperature. The mixture was then spun down, the buffer was removed, and the remaining reactive groups were blocked by incubation in 0.2 M glycine, pH 8.0, for 2 hr at room temperature. The DNA matrix was washed successively with 10 mL of 10 mM KPB, 1 M KPB, 1 M KCl, twice with water, and twice with binding buffer B (20 mM Tris–HCl, pH 7.8, 60 mM KCl, 1 mM $MgCl_2$, 1 mM DTT). Approximately 80% of the DNA was coupled to the Sepharose, as determined from the recovery of trace amounts of radioactively labeled oligonucleotide mixed in with the bulk of the DNA at the beginning of the procedure. For binding, proteins in binding buffer B were recirculated over the columns at a rate of 0.2 mL/min for 24 hr and washed with the same buffer; bound proteins were eluted by increasing the molarity of KCl in the buffer.

DNA-binding activity assays

For electrophoretic mobility shift assays, 10–15 μg of crude nuclear extract or 100–200 ng of affinity-purified protein were incubated in a total volume of 30 μL with 10,000 dpm of double-stranded probe in a buffer containing 20 mM HEPES, pH 7.8, 240 mM KCl, 1 mM EDTA, 1 mM DTT, 1 μg poly(dI·dC)-poly(dI·dC), and 10% glycerol. The *Ah* receptor in nuclear and in cytosolic extracts from untreated cells was activated to a DNA-binding form by TCDD and high salt treatments [34]. Binding of the probe was allowed to take place for 20 min at room temperature, and the samples were then loaded in non-denaturing 4% polyacrylamide gels that were pre-run for 45 min at 10 V/cm in 0.5X Tris-borate buffer. Electrophoresis proceeded for 2 hr and, after it was completed, the gel was dehydrated in 10% methanol, 10% acetic acid for 20 min, dried and exposed to an X-ray film. The AhRE probes used for binding are specified in the legends. The C/EBPα probe was the cognate recognition motif 5′-GATCAATTGGG-CAATTCAGGCCTGATTGCCCAATT-3′ [35]. When using antisera, the extracts were preincubated with a 1:100 dilution of the antiserum for 30 min on ice prior to the DNA binding reaction. The anti-C/EBPα antiserum was a gift of Alan Friedman; the control anti-CTDBP serum that we used was raised against rat liver proteins bound to a calf-thymus DNA affinity column and was a gift of Rita Hannah.

For southwestern blots, proteins were separated by electrophoresis in denaturing 6% or 7.5% polyacrylamide–SDS gels and were electrophoretically transferred to Nytran membranes (TM, Schleicher & Schuell). After transfer, membranes were washed in buffer B, and non-specific binding sites were blocked by incubation for 1 hr at room temperature in 5% non-fat milk in buffer B. Binding of the DNA probes took place for 2 hr at room temperature in 0.25% non-fat milk in high-ionic strength buffer B, containing 240 mM KCl and 2×10^6 dpm of probe. After binding, the membranes were washed three times in a buffer of the same composition, blotted and exposed to X-ray film. The probes used for DNA-binding assays were prepared by labeling each strand separately at the 5′ end with T4 polynucleotide kinase in the presence of [γ-^{32}P]-ATP and then allowing the strands to reanneal. For southwestern blots, the labeled probes were also catenated by ligation with T4 DNA ligase.

In vivo *methylation protection analyses*

Hepa-1 cells were grown to 80–90% confluence and treated with 10 nM TCDD for the lengths of time indicated or left untreated. Methylation was carried out for 3 min by adding 0.2% dimethyl sulfate in PBS to the cell monolayers. The cells were washed with PBS and lysed on the dish by addition of a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% SDS and 200 μg/mL of proteinase K. After incubation for 2 hr at 37°, nucleic acids were extracted with phenol:chloroform (1:1) and precipitated with ethanol. RNA was removed by RNase treatment and, to decrease viscosity, DNA was digested with *Eco*RI. Following a second phenol:chloroform extraction and ethanol precipitation, methylated DNA was cleaved with 1 M piperidine at 90° for 30 min.

LMPCR was carried out essentially as described by others [36, 37], using 5 μg of DNA for each sample and a set of three nested oligonucleotides for each DNA strand. For first strand synthesis, the coding strand primer was 5′-AACCTGCTCCA-TCCTCTGGG-3′ and the non-coding strand primer was 5′-GTTCCCCTCCCCCAGCTA-3′. For PCR, the coding strand primer was 5′-CATC-CTCTGGGGGCAGAGGT-3′, and the non-coding strand primer was 5′-CCCAGCTAGCGTGAC-AGC-3′. These two primers were used for PCR in conjunction with the linker primer 5′-GCGGTG-ACCCGGGAGATCTGAATTC-3′. For the coding strand, the end-labeling primer was 5′-GG-CAGAGGTCGGGCTGCC-3′, and for the non-coding strand it was 5′-GTGACAGCACTGGG-ACCCG-3′. DNA sampleswere denatured at 95° for 5 min, and first strand synthesis was with 1 U of Vent™ DNA polymerase (New England Biolabs) for 30 min at 54° followed by 10 min at 70°. Unidirectional linkers were ligated for 16 hr at 16° using 12 U of DNA ligase per sample. Following phenol extraction and ethanol precipitation of the ligation reactions, PCR was carried out for 20 cycles of 1 min at 95°, 2 min at 60° and 3 min at 72°. Conditions for 2 cycles of extension of the end-labeling primers were 3.5 min at 95°, 2 min at 60° and 10 min at 76°. For end labeling, primers were labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Approximately 5–8 pmol of end-labeled primer was added to each reaction. After extraction with phenol:chloroform and ethanol precipitation, samples were analyzed in 6% acrylamide sequencing gels, and the DNA fragments were visualized by autoradiography. Experiments were repeated with two different sets of methylated DNA samples, with similar results.

RESULTS

Detection of AhRE3-binding proteins in cultured wild-type and mutant mouse hepatoma cells

When analyzed by gel mobility shift, evidence for the presence of a TCDD-dependent *Ah* receptor–DNA complex is found only in protein extracts from wild-type cells. Protein extracts from mutant mouse hepatoma cell lines that lack a functional *Ah* receptor or are deficient in the nuclear translocator protein ARNT lack this complex [12, 27, 38, 39]. To determine whether there were other proteins with similar binding specificities to the *Ah* receptor and ARNT, we examined extracts from these mutant cells for the presence of a protein that would bind specifically to the cognate AhRE3 probe. Nuclear extracts from TCDD-treated and untreated wild-type and the mutant cell lines *c*37, *c*2, and *c*4 were separated by electrophoresis and analyzed in southwestern blots. At least three distinct proteins, present in all cell extracts, were found to bind AhRE3 with a high degree of specificity, particularly a protein of 95–100 kDa, and two others at 35–40 kDa and 72 kDa. These proteins were localized to the nucleus even in the absence of TCDD treatment, and, in the case of the 95-kDa protein,

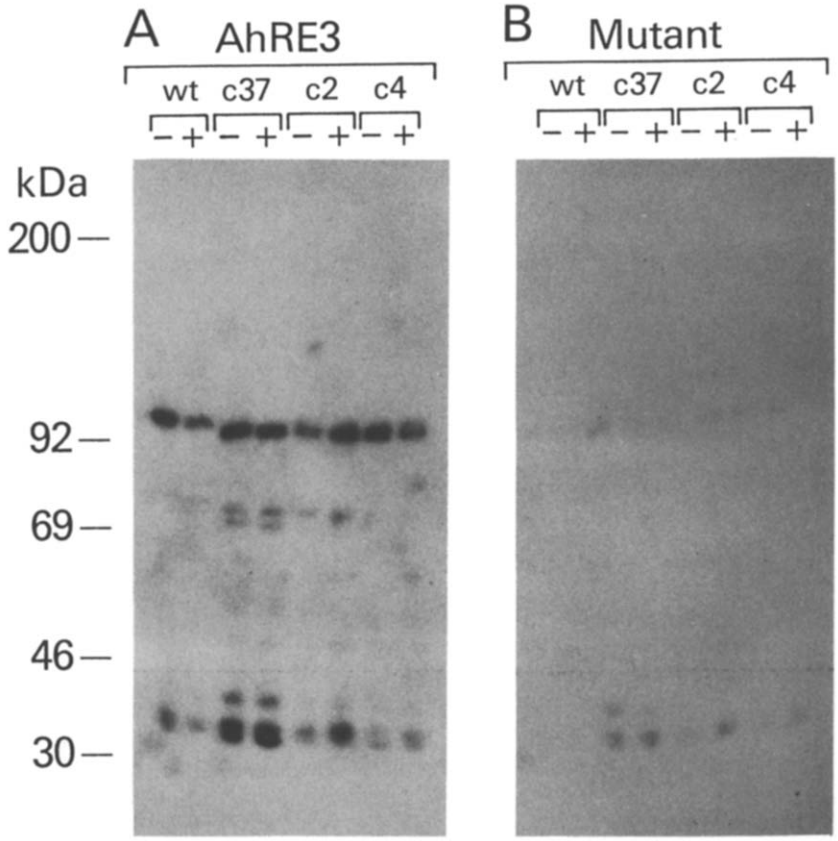

Fig. 1. Southwestern blot analysis of wild-type and mutant Hepa-1 cells. Nuclear extracts were prepared from TCDD-treated (+) and untreated wild-type (wt), *c37*, *c2*, and *c4* cells (−), and 25 μg of protein was separated by electrophoresis in duplicate denaturing 6% polyacrylamide–SDS gels. Proteins from each gel were transferred to Nytran and probed for DNA-binding with the AhRE3 probe (panel A) or the mutant probe (panel B).

it appeared to be as abundant in mutant as in wild-type cells (Fig. 1A). This protein had the binding specificity of the DNA-binding *Ah* receptor complex, since a mutant oligonucleotide, containing four transversions in the central recognition core, showed little affinity for the 95-kDa protein (Fig. 1B). The length of time required to get comparable X-ray images was 20–50 times shorter with the AhRE3 probe than with the mutant probe, suggesting that the mutant probe had a considerably lower affinity for the specific AhRE motif (data not shown). The binding specificity of the AhRE3 probe was greatly enhanced at the higher ionic strength; when binding and washings were done at lower K^+ concentrations, the AhRE3 probe bound to many other proteins, and the mutant probe also bound strongly to the 95-kDa band (data not shown). Similar findings have been reported for the binding of the glucocorticoid receptor to its cognate site [40].

Two of the mutant cell lines used in these experiments have been shown to lack expression of the *Ah* receptor complex components responsible for DNA binding. The *c4* cell line is ARNT-deficient [12, 25, 28], whereas the *c2* cell line has negligible *Ah* receptor activity [10, 25, 27, 28]. Therefore, it is unlikely that the 95-kDa protein detected in these experiments is ARNT or AhR. Rather, our results would indicate that this protein is an uncharacterized DNA-binding factor that recognizes the same binding motif as the *Ah* receptor complex.

Likelihood that the 35–40-kDa protein is a member of the C/EBP family

It has been shown recently that C/EBPα, a bZIP transcription factor involved in the regulation of many liver genes [31, 41, 42], binds to the glutathione *S*-transferase Ya gene AhRE motif and regulates expression of this gene, possibly in cooperative interaction with the *Ah* receptor [43]. For this reason, we conjectured that the 35–40-kDa protein detected in southwestern blots might be C/EBPα. To test this hypothesis, we examined the binding of oligonucleotides containing different AhRE motifs to C/EBPα protein made in *Escherichia coli* by a bacterial expression plasmid. Of the four AhREs tested, only one of them, AhRE3, was efficiently bound by this protein (Fig. 2A). Preincubation of the bacterial extract with anti-C/EBPα antiserum, but not with an antiserum against rat liver proteins eluted from a calf thymus DNA affinity column, abolished the AhRE3-binding activity in the bacterial extract (Fig. 2B), confirming that the binding activity

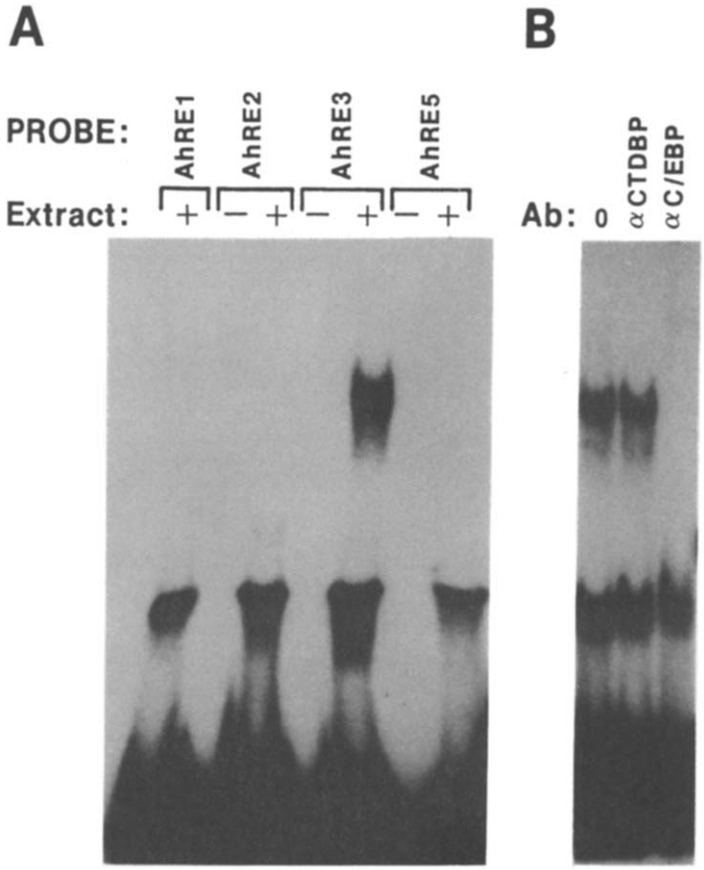

Fig. 2. Electrophoretic mobility shift analysis of bacterially made C/EBPα binding to AhRE motifs. (A) Approximately 5 μg of an extract of *E. coli* cells engineered to express C/EBPα was analyzed for binding of each of the four AhRE probes whose nucleotide sequences are shown in Table 1. Probes were assayed in the presence (+) as well as in the absence (−) of extract. (B) Prior to addition of probe, the bacterial extract was preincubated with no further additions (0); a 1:100 dilution of anti-calf thymus DNA binding proteins from rat liver (*αCTDBP*); or anti-C/EBPα (*αC/EBP*) under the conditions described in Materials and Methods.

Table 1. DNA sequence of oligonucleotides containing AhRE motifs used in electrophoretic mobility shift studies

Motif	Site	Sequences
AhRE1	Site C	-919 TGGAGCAGCTTACG**CACGC**TAGCCTCAG-892
AhRE2	Site E	-1085 TTCCCCTCCCCCAGCTA**GCGTG**ACAGCACTGGG-1053
AhRE3	Site D	-1006 TCCAGGCTCTTCT**CACGC**AACTCCGGG-980
AhRE5	Site A	-503 TGCGCTTCT**CACGC**GAGCTTGG-482

AhRE motifs defined in Ref. 44 have also been given the indicated site denominations [45]. Only one strand of the double-stranded molecule is presented. The AhRE motif, CACGC, or its complement, GCGTG, is shown in bold characters.

in the bacterial extract was indeed that of C/EBPα, and not that of an irrelevant bacterial protein.

Examination of the oligonucleotide sequence of the four AhREs tested (Table 1) suggested that one possible reason for the unique behavior of AhRE3 was the presence of a CAACT motif, resembling the C/EBP cognate motif CAATT [35]. To explore this possibility, we used gel retardation analyses to measure the ability of cognate, wild-type, and mutant AhRE3 oligonucleotides to compete for binding to bacterial C/EBPα. Binding of the cognate C/EBPα probe, containing the motif GGCAATT, was efficiently competed by itself as well as by wild-type AhRE3, containing the motif CACGCAAC, but not by a mutant AhRE3 that contains the motif CACGCACA (Fig. 3, probe B). The differences in the amount of oligonucleotides needed for complete competition suggest that the C/EBPα protein binds to its cognate site with a higher affinity than to AhRE3. Binding of wild-type AhRE3 could be

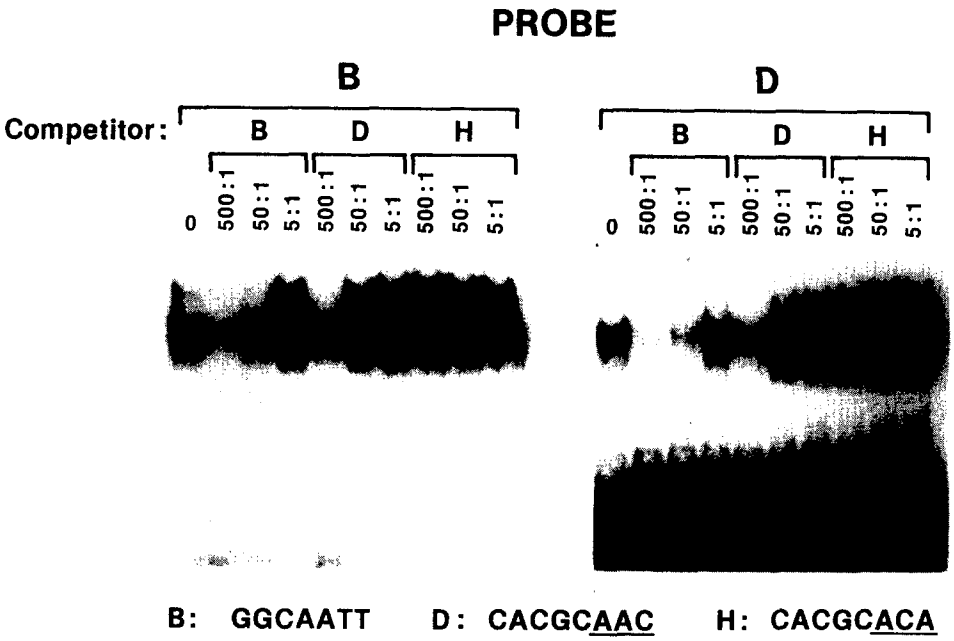

Fig. 3. Competition analysis of C/EBPα binding specificity to AhRE3. Approximately 4 μg of the same bacterial extract used in Fig. 2 was allowed to interact with various amounts of unlabeled oligonucleotides bearing the cognate recognition sequence of C/EBPα (B), AhRE3 (D), or a mutant AhRE3 (H) for 5 min under conditions favoring DNA–protein binding. Thereafter, radioactively labeled probes were added, incubation was continued for 20 min, and complexes were analyzed by gel retardation. The molar ratios of unlabeled to labeled oligonucleotides are indicated at the top of each lane. The sequences shown denote only the central binding motif of each oligonucleotide; the complete sequences are presented in Materials and Methods and in Table 1.

efficiently competed by itself as well as by the cognate C/EBPα oligonucleotide, but not by the mutated AhRE3 (Fig. 3, probe D). These results show that C/EBPα specifically recognizes AhRE3, the motif present in the probe used for southwestern analysis, and suggest that the 35–40-kDa protein detected in Fig. 1A is likely to be a member of the C/EBP family of transcription factors.

Partial purification of the 95-kDa DNA-binding component

We used DNA-affinity chromatography to obtain a partially purified preparation of the 95-kDa Hepa-1 protein. We made use of the different binding characteristics of the two oligonucleotides tested in southwestern blots to prepare two columns, one containing the wild-type AhRE3 sequence and the other containing the mutant AhRE3 sequence. Nuclear and cytosolic extracts from TCDD-treated and untreated cells were bound to each of these columns in low-ionic strength buffer and eluted by increasing the ionic strength of the buffer. The presence of a TCDD-dependent, *Ah* receptor-dependent DNA–protein complex was clearly detectable as a slow-moving band in gel mobility shift analysis of crude nuclear extracts from TCDD-treated but not from untreated cells (Fig. 4A, lanes 1–2). The identity of this band and of several of the other complexes has been well characterized by this and many other laboratories [39, 46–52]. Chromatography of the extracts through a column containing the mutant oligonucleotide yielded no DNA-binding protein of sufficient affinity to be retained by the column. All the extracts, regardless of source and treatment, gave the same negative result. As an example, mobility shift analyses of eluted fractions from TCDD-treated nuclear extracts are shown in Fig. 4A, lanes 3–5. In contrast, chromatography through a column containing the cognate AhRE3 sequence resulted in the enrichment of a DNA-binding protein that, when assayed by gel mobility shift, presented two unexpected characteristics. First, its DNA-binding activity per unit protein was very high in nuclear extracts from TCDD-treated cells, but it was almost as high in nuclear extracts from untreated cells (Fig. 4A, compare lanes 6–8 with lanes 9–11). The activity was also present, although less abundantly, in treated and untreated cytosolic extracts (Fig. 4A, lanes 12–15). Second, the mobility of this protein when complexed with AhRE3 DNA was quite different from the mobility of the *Ah* receptor-containing band, appearing to migrate at the same rate as the fastest-moving complex of those found in crude extracts. Notwithstanding, the DNA-binding protein purified by affinity chromatography was the same 95-kDa protein detected in crude extracts, since, when we analyzed these proteins in southwestern blots, the size of the affinity-purified protein was 95 kDa, identical to the size of the protein detected in crude extracts (Fig. 4B).

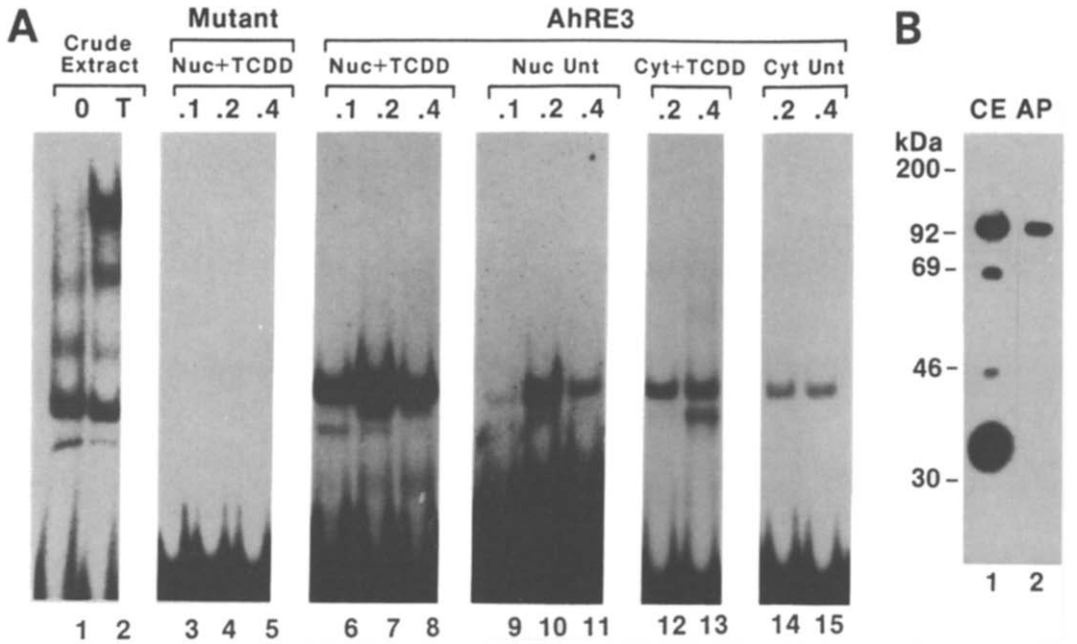

Fig. 4. Analysis of DNA-binding proteins in affinity-purified extracts. (A) Proteins from nuclear and cytosolic extracts were adsorbed to an AhRE3-affinity column and bound proteins were eluted by increasing the molarity of KCl (lanes 6–15), and analyzed by gel mobility shift using an AhRE3 probe. The KCl molarity, indicated by the decimals above lanes 3–15, was increased up to 1 M, but only negligible binding activities were detected above 0.5 M and are not shown in the figure. A nuclear extract from TCDD-treated cells was adsorbed to a mutant-affinity column and similarly analyzed (lanes 3–5). A mobility shift assay of unfractionated nuclear extracts from TCDD-treated (T) and untreated (0) cells is included for comparison (lanes 1 and 2). (B) Proteins from the unfractionated crude nuclear extract of TCDD-treated cells (CE) and from the affinity-purified (AP) material obtained from the same extract were analyzed in southwestern blots using AhRE3 as the binding probe.

The results from these experiments suggest that the 95 kDa is unlikely to be one of the known components of the *Ah* receptor complex, because the complex of the affinity-purified protein with DNA shows a much faster mobility rate than the DNA–*Ah* receptor complex, and, furthermore, because the same 95-kDa protein can be found in cells lacking AhR as well as ARNT (Fig. 1A).

Analysis of the binding sites of the 95-kDa protein

To evaluate the binding specificity of the 95-kDa protein and to compare it with that of the *Ah* receptor complex/ARNT, we examined crude nuclear extracts and affinity-purified protein for their ability to bind to all four AhREs. Using gel retardation assays, the presence of the characteristic *Ah* receptor complex could be detected in nuclear extracts from TCDD-treated wild-type cells, but not in extracts from untreated cells; the complex could be found regardless of which of the four AhRE probes was used (Fig. 5A). In contrast, the band at the mobility of the 95-kDa complex, present in crude extracts, was formed only when AhRE3 and AhRE5 were used as probes, and not when the probes were derived from AhRE1 and AhRE2 (Fig. 5A). In addition, unlike the *Ah* receptor complex, this band was present in extracts from both TCDD-treated and untreated cells. These observations were confirmed when the affinity-purified extract was used as the source of binding protein. The 95-kDa protein had nearly undetectable binding activity to AhRE1, but it showed strong binding to AhRE5 (Fig. 5B), similar to the binding to AhRE3 (compare Fig. 5B with Fig. 4A).

These results suggested that the fast-moving complex observed in crude extracts and the affinity-purified 95-kDa protein were the same, and that the binding specificity of this protein was very similar to the specificity of the *Ah* receptor complex, although subtle sequence differences allowed it to interact with AhRE3 and AhRE5, but not with AhRE1 or AhRE2. Examination of the sequences involved (Table 1) pointed at the motif TTCT (or its complement, AGAA), immediately upstream of the CACGC receptor-binding motif, as a likely determinant for the binding differences.

We used competition analyses to confirm this conclusion. Six double-stranded oligonucleotides harboring consecutive 2-base pair mutations in the AhRE3 motif were used to compete binding of the wild-type probe in electrophoretic mobility shift assays. As shown before [51], oligonucleotides M3 and M4, bearing mutations affecting bases in the central GCGTG (or its complement, CACGC) motif, failed to compete the binding of the wild-type probe to the *Ah* receptor complex, which, on the

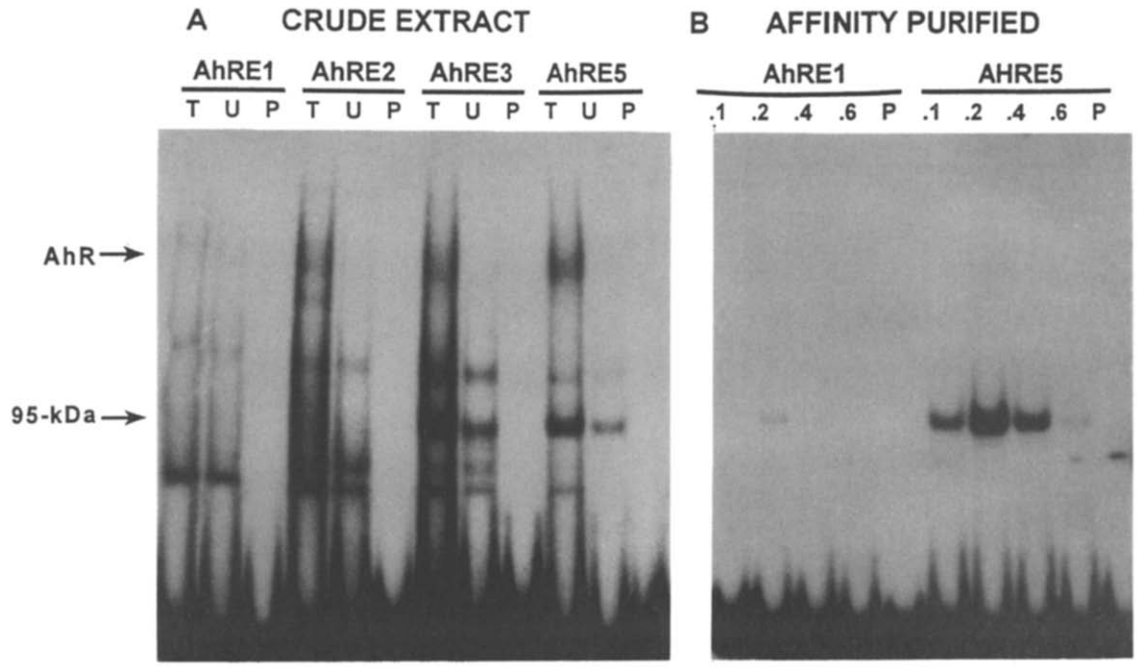

Fig. 5. Binding of the 95-kDa protein to only AhRE3 and AhRE5. (A) Gel mobility shift analysis of the four probes indicated (AhRE1, AhRE2, AhRE3, and AhRE5) was carried out with nuclear proteins from a crude extract of TCDD-treated (T) or untreated (U) cells. (P) denotes the probe alone, run in the absence of extract. (B) Equal volumes of the fractions eluted from an AhRE3 affinity column with 0.1, 0.2, 0.4, and 0.6 M KCl were tested for binding of AhRE1 and AhRE5 probes. The *arrows* point at the positions of the DNA:*Ah* receptor and the DNA:95-kDa protein complexes.

other hand, was competed efficiently by the other four mutants tested (Fig. 6A). The binding specificity of the affinity-purified protein appeared to be subtly different. As for the *Ah* receptor complex, mutants M3 and M4 failed to compete wild-type probe binding, but in addition, mutants M1 and M2, bearing mutations in the AGAA sequence next to the AhRE motif, also failed to compete the wild-type probe (Fig. 6B). These results indicate that the binding target for the 95-kDa affinity-purified protein is larger than the AhRE3 motif, encompassing the complete CGTGAGAA sequence defined by mutants M1, M2, M3, and M4 (Fig. 6C), and also found in AhRE5. Judging from the relative band intensities in the autoradiograms of Fig. 6B, it appears that M1 and M4 are better competitors than M2 and M3, suggesting that the central TGAG motif is of more consequence for efficient binding.

Localization of the 95-kDa protein to the cell nucleus even in the absence of TCDD induction (Fig. 4) suggested the possibility that this protein could be constitutively bound to DNA. To determine whether a protein was bound to AhRE3 in uninduced cells, we used *in vivo* methylation protection and analyzed the genomic DNA by LMPCR. The results of these experiments indicated that TCDD treatment caused changes in the methylation patterns of the four G residues in the sequence GCGTGAG of the AhRE3 antisense strand. As already shown by others [45, 53], between 30 and 90 min after TCDD treatment, the two internal G residues, available for methylation in untreated cells, became protected as a result of *Ah* receptor complex binding. In contrast, the two outer G residues, protected from methylation in untreated cells, became more sensitive to methylation within the first 30 min of TCDD treatment (Fig. 7). In the sense strand, the single G residue in the sequence became protected by TCDD treatment, in agreement with previous reports [45, 53] (data not shown).

The results presented in this section are consistent with the idea that a constitutive protein, unrelated to the *Ah* receptor, may occupy the AhRE3 motif and possibly other AhREs in uninduced cells. After dioxin treatment, displacement of this protein would be required to allow binding of the *Ah* receptor complex.

DISCUSSION

In this paper, we present evidence to indicate that proteins other than the *Ah* receptor and ARNT can bind to the AhRE regulatory elements in the upstream sequences of the *Cyp1a1* gene. One of these proteins has a molecular mass of 35–40 kDa, and is likely to be a member of the C/EBP family of transcription factors, since bacterially made C/EBPα can specifically recognize and bind to AhRE3. C/EBPα binding depends on the presence of the triplet AAC 3′ to the central CACGC AhRE motif, and it is abolished by its double transversion to ACA. None of the three other AhREs tested,

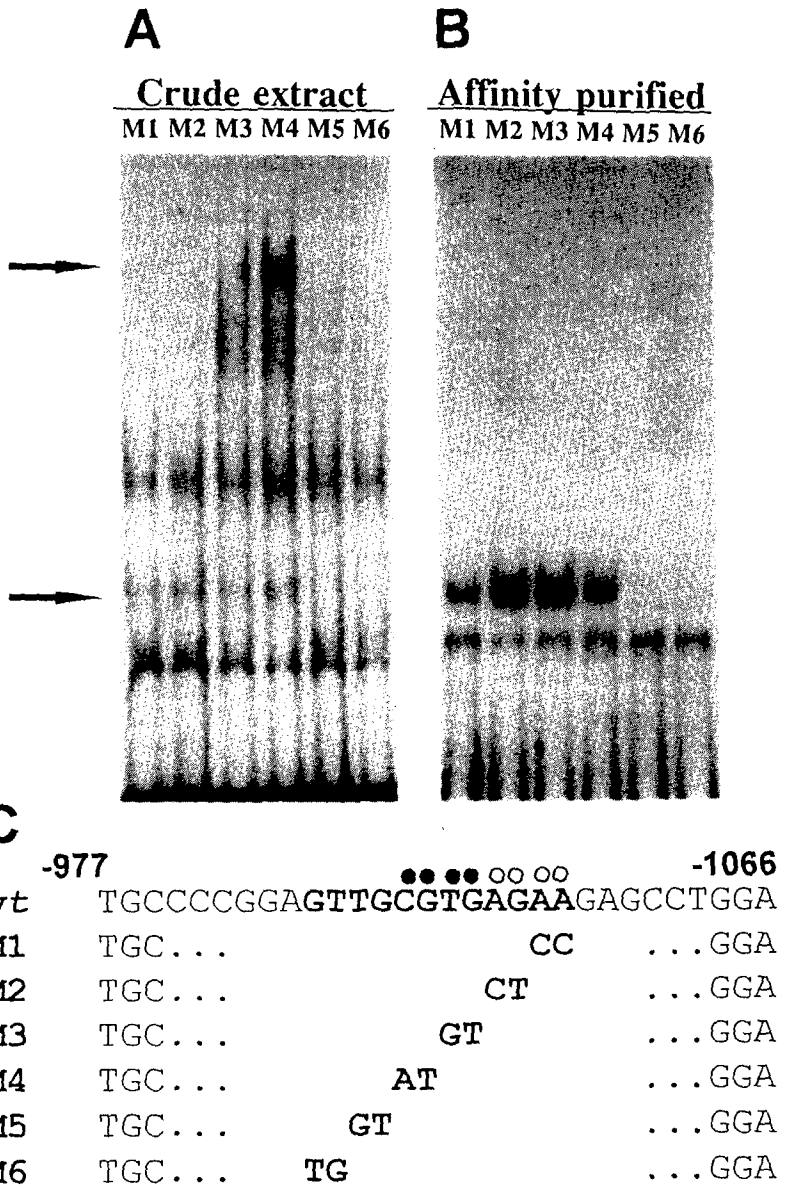

Fig. 6. Competition gel mobility shift assays of crude extract and affinity-purified 95-kDa protein. Crude nuclear extract (A) or affinity-purified 95-kDa protein (B) was allowed to interact with a 200-fold molar excess of each of the six unlabeled oligonucleotides shown in (C) (only the antisense strand shown). After 5 min, ^{32}P-labeled wild-type (wt) AhRE3 probe was added, and incubation was continued for an additional 5 min. Complexes were separated in non-denaturing polyacrylamide gels. The *top arrow* indicates the band containing the *Ah* receptor complex; the *bottom arrow* points at the band containing the 95-kDa protein. The *solid circles* denote bases required for binding of both the *Ah* receptor complex and the 95-kDa protein; the *open circles* denote bases required solely for binding of the 95-kDa protein.

AhRE1 (CACGCTAG), AhRE2 (CACGCTAG), and AhRE5 (CACGCGAG), formed complexes with C/EBPα, because the complete CACGCAAC motif is found only in AhRE3. A motif identical to AhRE3, present in the glutathione *S*-transferase Ya gene, has been shown recently to bind C/EBPα and to confer C/EBPα-dependent transcriptional activation to a reporter gene [43]. We would expect similar results for reporter genes bearing the AhRE3 of *Cyp1a1*, but not other AhREs.

Nuclear and cytosolic extracts from uninduced and TCDD-induced cells were found to contain a second protein, with a molecular mass of 95 kDa, that bound to AhRE3 and AhRE5, but not to AhRE1 and AhRE2. Its binding specificity indicates that this protein is not AhR or ARNT, and this conclusion is confirmed by its presence in wild-type and Cyp1a1-deficient cells, as well as in receptorless and nuclear translocation-negative cells. Most likely, this protein is one of several that have been observed

Fig. 7. *In vivo* protein–DNA interactions at AhRE3. Hepa-1 cells were uninduced (0) or treated with 10 nM TCDD for 30 min (30) or 90 min (90). Genomic DNA was isolated and analyzed by LMPCR. The lane designated *C* contained a control of cloned genomic DNA methylated *in vitro*. *Filled* and *open symbols* denote G residues that exhibited decreased or increased methylation, respectively, after exposure to TCDD. The sequence shown to the right is the antisense strand of the AhRE3 (complement of the sequence shown in Table 1). The lane containing the control DNA was exposed for 2 hr; exposure of the other lanes was for 48 hr.

to interact with the AhREs by other investigators [52, 54–56]. Its binding motif overlaps the GCGTG sequence (or its complement, CACGC) in the AhRE; however, competition analyses indicate that the complete binding site is formed by the sequence 5′-CGTGAGAA-3′, which includes the next four 3′ nucleotides from AhRE3. To date, no transcription factors have been identified with this sequence specificity.

In vivo footprinting analyses revealed changes in the methylation patterns of the four G residues in the antisense strand of the AhRE3 sequence 5′-TTGCGTGAGAA-3′. The first and last G residues are protected in uninduced cells and become exposed after TCDD treatment, whereas the reverse is the case for the two middle Gs, which are exposed in uninduced cells and become protected after exposure to TCDD. Protection of these two G residues after exposure to TCDD has been observed previously [45, 53] and has been interpreted as evidence for *Ah* receptor complex binding, a conclusion with which we fully agree. Exposure of the first G in uninduced cells and its protection after TCDD treatment have also been observed before, but it has been ascribed to an effect of DNA bending or to some other unknown reason [45, 53] rather than to occupancy of the site in uninduced cells. In the absence of further evidence to the effect, however, it seems more warranted to conclude that protection of this G residue is also due to protein binding. We believe that changes in methylation protection of this and also of the fourth G residue in the sequence suggest that, in uninduced cells, the site is occupied by a protein that must be displaced for transcriptional activation by the *Ah* receptor complex. At present, we cannot determine whether this putative protein is the 95-kDa protein, detected in southwestern blots, that we have purified by affinity chromatography. In this regard, it is interesting to note that another laboratory has identified a constitutive binding factor that recognizes the AhRE motifs and that may be associated with transcriptional repression of the *Cyp1a1* gene in certain cell types [56]. It is attractive to speculate that the putative repressor identified in those studies is the same as the 95-kDa protein that we have detected, and that binding of this protein to AhRE sequences intervenes in the negative regulation of the *Cyp1a1* gene. Other negative regulators of human *CYP1A1* gene expression have been found that do not interact with AhRE motifs [57].

Of all the AhRE motifs tested, only AhRE3 appears to interact with C/EBPα, and only AhRE3 and AhRE5 bind the 95-kDa protein. Interaction of the *Ah* receptor complex with these two other factors is likely to modulate the regulation of *Cyp1a1* expression at the level of these two AhREs. Our data support the concept that the transcriptional regulation of the *Ah* battery genes is modulated by combinatorial interactions of the *Ah* receptor complex with other transcription factors. It is unlikely, however, that we have identified all the proteins that can interact with the regulatory sequences in these genes. More likely, many other DNA–protein and protein–protein interactions of interest remain to be recognized.

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REFERENCES

1. Poland A, Knutson J and Glover E, Studies on the mechanism of action of halogenated aromatic hydrocarbons. *Clin Physiol Biochem* **3**: 147–154, 1985.
2. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**: 517–554, 1982.
3. Whitlock JP Jr, Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol* **30**: 251–277, 1991.
4. Nebert DW and Gonzalez FJ, P450 genes. Structure, evolution and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
5. Nebert DW, The *Ah* locus: Genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol* **20**: 153–174, 1989.
6. Landers JP and Bunce NJ, The *Ah* receptor and the mechanism of dioxin toxicity. *Biochem J* **276**: 273–287, 1991.
7. Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–288, 1988.
8. Greenlee WF and Neal RA, The *Ah* receptor: A

biochemical and biological perspective. In: *The Receptors* (Ed. Conn PM), Vol. 2, pp. 89–129. Academic Press, New York, 1985.
9. Elferink CJ and Whitlock JP Jr, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-inducible, Ah receptor-mediated bending of enhancer DNA. *J Biol Chem* **265**: 5718–5721, 1990.
10. Burbach KM, Poland A and Bradfield CA, Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci USA* **89**: 8185–8189, 1992.
11. Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Gotoh O, Funae Y and Fujii-Kuriyama Y, cDNA cloning and structure of mouse putative Ah receptor. *Biochem Biophys Res Commun* **184**: 246–253, 1992.
12. Hoffman EC, Reyes H, Chu F-F, Sander F, Conley LH, Brooks BA and Hankinson O, Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**: 954–958, 1991.
13. Legraverend C, Hannah RR, Eisen HJ, Owens IS, Nebert DW and Hankinson O, Regulatory gene product of the *Ah* locus. Characterization of receptor mutants among mouse hepatoma clones. *J Biol Chem* **257**: 6402–6407, 1982.
14. Whitelaw M, Pongratz I, Wilhelmsson A, Gustafsson J-Å and Poellinger L, Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. *Mol Cell Biol* **13**: 2504–2514, 1993.
15. Hankinson O, Research on the aryl hydrocarbon (dioxin) receptor is primed to take off. *Arch Biochem Biophys* **300**: 1–5, 1993.
16. Reyes H, Reisz-Porszasz S and Hankinson O, Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256**: 1193–1195, 1992.
17. Wilhelmsson A, Cuthill S, Denis M, Wikström A-C, Gustafsson J-Å and Poellinger L, The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. *EMBO J* **9**: 69–76, 1990.
18. Li L, Chambard J-C, Karin M and Olson EN, Fos and Jun repress transcriptional activation by myogenin and MyoD: The amino terminus of Jun can mediate repression. *Genes Dev* **6**: 676–689, 1992.
19. Bengal E, Ransone L, Scharfmann R, Dwarki VJ, Tapscott SJ, Weintraub H and Verma IM, Functional antagonism between c-Jun and MyoD proteins: A direct physical association. *Cell* **68**: 507–519, 1992.
20. Mason ME and Okey AB, Cytosolic and nuclear binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor in extrahepatic tissues of rats and mice. *Eur J Biochem* **123**: 209–215, 1982.
21. Bigelow SW, Zijlstra JA, Vogel EW and Nebert DW, Measurements of the cytosolic Ah receptor among four strains of *Drosophila melanogaster*. *Arch Toxicol* **56**: 219–225, 1985.
22. Poland A, Glover E and Bradfield CA, Characterization of polyclonal antibodies to the *Ah* receptor prepared by immunization with a synthetic peptide hapten. *Mol Pharmacol* **39**: 20–26, 1991.
23. Swanson HI and Perdew GH, Detection of the Ah receptor in rainbow trout: Use of 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin in cell culture. *Toxicol Lett* **58**: 85–95, 1991.
24. Bernard HP, Darlington GJ and Ruddle FH, Expression of liver phenotypes in cultured mouse hepatoma cells: Synthesis and secretion of serum albumin. *Dev Biol* **35**: 83–96, 1973.
25. Hankinson O, Dominant and recessive aryl hydrocarbon hydroxylase-deficient mutants of mouse hepatoma line Hepa-1 and assignment of recessive mutants to three complementation groups. *Somat Cell Genet* **9**: 497–514, 1983.
26. Kimura S, Smith HH, Hankinson O and Nebert DW, Analysis of two benzo[a]pyrene-resistant mutants of the mouse hepatoma Hepa-1 $P_1$450 via cDNA expression in yeast. *EMBO J* **6**: 1929–1933, 1987.
27. Puga A, RayChaudhuri B and Nebert DW, Transcriptional derepression of the murine *Cyp1a-1* gene by mevinolin. *FASEB J* **6**: 777–785, 1992.
28. Hankinson O, Andersen RD, Birren B, Sander F, Negishi M and Nebert DW, Mutations affecting the regulation of transcription of the cytochrome P_1-450 gene in the mouse Hepa-1 cell line. *J Biol Chem* **260**: 1790–1795, 1985.
29. Dignam JD, Lebovitz RM and Roeder RG, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**: 1475–1489, 1983.
30. Landschulz WH, Johnson PF, Adashi EY, Graves BJ and McKnight SL, Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev* **2**: 786–800, 1988.
31. Johnson PF, Landschulz WH, Graves BJ and McKnight SL, Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev* **1**: 133–146, 1987.
32. Kadonaga JT and Tjian R, Affinity purification of sequence-specific DNA binding proteins. *Proc Natl Acad Sci USA* **83**: 5889–5893, 1986.
33. Rosenfeld PJ and Kelly TJ, Purification of nuclear factor 1 by DNA recognition site affinity chromatography. *J Biol Chem* **261**: 1398–1408, 1986.
34. Nemoto T, Mason GG, Wilhelmsson A, Cuthill S, Hapgood J, Gustafsson JA and Poellinger L, Activation of the dioxin and glucocorticoid receptors to a DNA binding state under cell-free conditions. *J Biol Chem* **265**: 2269–2277, 1990.
35. Vinson CR, LaMarco KL, Johnson PF, Landschulz WH and McKnight SL, *In situ* detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev* **2**: 801–806, 1988.
36. Pfeifer GP, Steigerwald SD, Mueller PR, Wold B and Riggs AD, Genomic sequencing and methylation analysis by ligation mediated PCR. *Science* **246**: 810–813, 1989.
37. Mueller PR and Wold B, *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR [published erratum appears in *Science* **248**: 802, 1990]. *Science* **246**: 780–786, 1989.
38. Denison MS, Fisher JM and Whitlock JP Jr, Inducible, receptor-dependent protein–DNA interactions at a dioxin-responsive transcriptional enhancer. *Proc Natl Acad Sci USA* **85**: 2528–2532, 1988.
39. Fujisawa-Sehara A, Yamane M and Fujii-Kuriyama Y, A DNA-binding factor specific for xenobiotic-responsive elements of P-450c gene exists as a cryptic form in cytoplasm: Its possible translocation to nucleus. *Proc Natl Acad Sci USA* **85**: 5859–5863, 1988.
40. Härd T, Dahlman K, Carlstedt-Duke JMB, Gustafsson JA and Rigler R, Cooperativity and specificity in the interactions between DNA and the glucocorticoid receptor DNA-binding domain. *Biochemistry* **29**: 5358–5364, 1990.
41. Landschulz WH, Johnson PF and McKnight SL, The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* **243**: 1681–1688, 1989.
42. Landschulz WH, Johnson PF and McKnight SL, The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* **240**: 1759–1764, 1988.
43. Pimental RA, Liang B, Yee GK, Wilhelmsson A, Poellinger L and Paulson KE, Dioxin receptor and C/EBP regulate the function of the glutathione *S*-transferase Ya gene xenobiotic response element. *Mol Cell Biol* **13**: 4365–4373, 1993.

44. Nebert DW and Jones JE, Regulation of the mammalian cytochrome P_1450 (*CYP1A1*) gene. *Int J Biochem* **21**: 243–252, 1989.
45. Wu L and Whitlock JP Jr, Mechanism of dioxin action: Receptor–enhancer interactions in intact cells. *Nucleic Acids Res* **21**: 119–125, 1993.
46. Jones PBC, Durrin LK, Galeazzi DR and Whitlock JP Jr, Control of cytochrome P_1-450 gene expression: Analysis of a dioxin-responsive enhancer system. *Proc Natl Acad Sci USA* **83**: 2802–2806, 1986.
47. Fujisawa-Sehara A, Sogawa K, Nishii C and Fujii-Kuriyama Y, Regulatory DNA elements localized remotely upstream from the drug-metabolizing cytochrome P-450c gene. *Nucleic Acids Res* **14**: 1465–1477, 1986.
48. Fujisawa-Sehara A, Sogawa K, Yamane M and Fujii-Kuriyama Y, Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: A similarity to glucocorticoid regulatory elements. *Nucleic Acids Res* **15**: 4179–4191, 1987.
49. Denison MS, Fisher JM and Whitlock JP Jr, The DNA recognition site for the dioxin–Ah receptor complex. *J Biol Chem* **263**: 17221–17224, 1988.
50. Hapgood J, Cuthill S, Denis M, Poellinger L and Gustafsson JA, Specific protein–DNA interactions at a xenobiotic-responsive element: Copurification of dioxin receptor and DNA-binding activity. *Proc Natl Acad Sci USA* **86**: 60–64, 1989.
51. Neuhold LA, Shirayoshi Y, Ozato K, Jones JE and Nebert DW, Regulation of mouse *Cyp1a-1* gene expression by dioxin: Requirement of two *cis*-acting elements during induction. *Mol Cell Biol* **9**: 2378–2386, 1989.
52. Saatcioglu F, Perry DJ, Pasco S and Fagan JB, Multiple DNA-binding factors interact with overlapping specificities at the aryl hydrocarbon response element of the cytochrome P450IA1 gene. *Mol Cell Biol* **10**: 6408–6416, 1990.
53. Wu L and Whitlock JP Jr, Mechanism of dioxin action: Ah receptor-mediated increase in promoter accessibility *in vivo*. *Proc Natl Acad Sci USA* **89**: 4811–4815, 1992.
54. Gasiewicz TA, Elferink CJ and Henry EC, Characterization of multiple forms of the Ah receptor: Recognition of a dioxin-responsive enhancer involves heteromer formation. *Biochemistry* **30**: 2909–2916, 1991.
55. Elferink CJ, Gasiewicz TA and Whitlock JP Jr, Protein–DNA interactions at a dioxin-responsive enhancer. Evidence that the transformed Ah receptor is heteromeric. *J Biol Chem* **265**: 20708–20712, 1990.
56. Gradin K, Wilhelmsson A, Poellinger L and Berghard A, Nonresponsiveness of normal human fibroblasts to dioxin correlates with the presence of a constitutive xenobiotic response element-binding factor. *J Biol Chem* **268**: 4061–4068, 1993.
57. Boucher PD, Ruch RJ and Hines RN, Specific nuclear protein binding to a negative regulatory element on the human CYP1A1 gene. *J Biol Chem* **268**: 17384–17391, 1993.