Aryl Hydrocarbon (Ah) Receptor DNA-binding Activity

SEQUENCE SPECIFICITY AND Zn\(^{2+}\) REQUIREMENT*

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The aryl hydrocarbon (Ah) receptor, also called the xenobiotic or TCDD receptor, mediates transcriptional activation of the cytochrome P-450c (CYP1A1) gene by interacting with Ah or xenobiotic response elements. This paper presents evidence that a metal ion, probably Zn\(^{2+}\), is an essential cofactor for the Ah receptor. This paper also maps in detail the interactions between the Ah response element XRE1 and the Ah receptor from the rat hepatocyte-derived cell line LC57. Interactions were mapped by three methods, 1) methylation interference footprinting, 2) mobility shift competition experiments, using a series of synthetic oligonucleotides with systematic alterations in the Ah response element core sequence, and 3) orthophenanthroline/Cu\(^{2+}\) footprinting. These findings suggest the following consensus sequence for DNA recognition by the Ah receptor: CNA/TNA/TCACCGTA/T/T. The chelators 1,10-phenanthrol ine and oxalic acid inhibited the sequence-specific DNA-binding activity of the Ah receptor in a concentration dependent manner, suggesting that the DNA-binding activity of the receptor requires divalent metal ions. Inhibition was due to metal-chelation, since: 1) inhibition was almost completely prevented by the presence of Zn\(^{2+}\), or other divalent metal ions having high affinity for the chelators used, while metal ions with low affinity did not protect; 2) the DNA-binding activity of the receptor could be restored by dialysis to remove 1,10-phenanthrol ine, but only in the presence of Zn\(^{2+}\), while dialysis in the absence of metal ions reversed inhibition by the nonchelating isomer 4,7-phenanthrol ine. The involvement of a divalent cation in receptor function, possibly bound via sulfhydryls, was also suggested by the finding that Cd\(^{2+}\) and Co\(^{2+}\) inhibited DNA-binding activity. Once bound to the XRE1 DNA sequence, the receptor could not be inhibited by 1,10-phenanthrol ine, suggesting that the essential metal ion must become inaccessible to chelation when the receptor binds DNA. The Zn\(^{2+}\) requirement of the Ah receptor is similar to that of the estrogen and the glucocorticoid receptors and is consistent with the hypothesis that the Ah receptor is a member of the steroid and thyroid hormone receptor superfamily.

The polycyclic aromatic hydrocarbon inducible cytochrome

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P-450IA1 subfamily of the cytochromes P-450 is capable of both detoxifying polycyclic aromatic compounds and converting some compounds to highly active carcinogens and mutants. Treatment with polycyclic compounds, such as TCDD, 3-methylcholanthrene, and β-naphthoflavone, causes P-450IA1 mRNA levels to increase 100-fold both in hepatocyte cultures and in liver (Fagan et al., 1986; Kawajiri et al., 1984; Pasco et al., 1988). Both transcriptional and post-transcriptional mechanisms have been shown to be important in the regulation of the CYP1A1 gene of rat (P-450c; Pasco et al., 1988), mouse (P-450; Kimura et al., 1986), and human (P-450; Thierry et al., 1987). DNA-mediated gene transfer studies have identified multiple regulatory elements, both positive and negative, associated with the CYP1A1 gene. One of these is responsive to polycyclic compounds and is termed the Ah response element, TCDD response element, or xenobiotic response element (Fujisawa-Sehara et al., 1986, 1987; Sogawa et al., 1986; Jones et al., 1985, 1986a, 1986b; González and Nebert, 1985; Neuhold et al., 1986).

The proposed model for the action of TCDD and other polycyclic compounds is remarkably similar to the current model of steroid hormone action (Ringold, 1985; Yamamoto, 1986), in which a hydrophobic ligand binds with high affinity to a soluble receptor protein and the resulting ligand-receptor complex acts as a modulator of transcription for several genes (Poland and Knutson, 1982; Poland and Kimbrough, 1984).

The Ah receptor, also known as the TCDD receptor and xenobiotic receptor, when complexed with its ligand can undergo temperature-dependent transformation to become a DNA-binding protein (Gasiewicz and Bauman, 1987; Hannah et al., 1986). The sequence-specific interactions of the murine Ah receptor with the Ah response element of the CYP1A1 gene have been previously demonstrated by the mobility shift DNA-binding assay (Denison et al., 1988a, 1988b; Fujisawa-Sehara et al., 1988, 1989). In this paper we map in detail, by three independent methods, the interactions of the rat Ah receptor with the Ah response element of the rat CYP1A1 (P-450c) gene. This new, detailed information makes it possible to construct a much more precise model of the interactions between the Ah receptor and its cognate sequence.

We also report in this paper the effect of several divalent cations and of the chelating agents 1,10-phenanthroline and oxalic acid on the DNA-binding activity of the Ah receptor. Our data show that Cd\(^{2+}\), Co\(^{2+}\), 1,10-phenanthroline, and oxalic acid all inhibit binding of the Ah receptor to the Ah response element XRE1 and that Zn\(^{2+}\) can prevent inhibition by 1,10-phenanthroline and can reverse this inhibition when

1 See Nebert et al. (1989) for current P-450 nomenclature.
2 The abbreviations used are: P-450, cytochrome P-450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CDTA, 1,2-cyclohexanediaminetetraacetic acid.
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present during removal of 1,10-phenanthroline by dialysis. Similarly, metal ions chelated by oxalic acid prevent the inhibitory effect of this agent. These results suggest that a metal ion, most likely Zn\(^{2+}\), is an essential cofactor for the DNA-binding activity of the Ah receptor. Since the "zinc finger" motif is a common feature of the DNA binding domains of all known members of the steroid and thyroid hormone receptor superfamily, and since Zn\(^{2+}\) is an essential cofactor for the glucocorticoid receptor (Freedman et al., 1988) and for the estrogen receptor (Sabbah et al., 1987), the findings reported here provide additional support for the earlier hypothesis that the Ah receptor is a member of this receptor superfamily.

The work of the bulk presented in this paper has been carried out in the rat hepatocyte-derived cell line LCS7 (Chou, 1983; Chou and Schlegel-Haueter, 1981), in which it has been demonstrated that TCDD and other polycyclic compounds induce the transcription of the P-450c gene and probably influence post-transcriptional events as well (Teifeld et al., 1989). All previously reported research concerning cis-acting transcriptional regulatory sequences associated with the P-450c gene and concerning putative regulatory proteins that interact with these sequences, has been carried out using a single cell line, the murine hepatoma-derived cell line Hepa1 (Hankinson, 1979) and its derivatives, primarily because of the availability of Ah receptor regulatory variants of this line (Hankinson, 1979; Legraverend et al., 1982; Miller et al., 1983). Even researchers who have been investigating the function of the rat P-450c regulatory sequences have used this mouse derived cell line (Fujisawa-Sehara et al., 1986, 1987, 1988).

We have chosen to use the LCS7 cells in order to examine the regulation of the rat P-450c gene in a cell line derived from that same species and to evaluate the regulation of this gene in a second cell culture system.

**Experimental Procedures**

**Materials**—MgCl\(_2\), MnCl\(_2\), CoCl\(_2\), CaCl\(_2\), CdCl\(_2\), and oxalic acid were obtained from Fisher, ZnCl\(_2\) from J. T. Baker Chemical Co., and 1,10-phenanthroline and 4,7-phenanthroline from GFS Chemicals (Columbus, OH).

**Electrophoresis DNA-binding Assay**—Nuclear extracts were incubated with approximately 5000 cpm (0.02 ng) of an end-labeled, methylated 40-base pair oligonucleotide, whose sequence was 5'-CCATCGATTGATGCGGATACGGGTCCGTCATGCTGTAACGCTTATA-3', which was generated by transfection of line RALA255-cmG (Chou, 1983), which was generated by transfection of the plasmid pSV40 virus, which is temperature-sensitive for maintenance of expression. To demonstrate the reproducibility of the method.

**Preparation of Linear Extracts**—Nuclear extracts, containing the Ah receptor, were prepared according to Whitlock and Galeazzi (1984), with minor changes. Briefly, confluent LC57 cells were harvested from tissue culture roller bottles by scraping into phosphate-buffered saline (without Ca\(^{2+}\) or Mg\(^{2+}\)) and centrifuging at 2000 rpm for 5 min in a Beckman JS7 rotor. The cell supernatant was resuspended in 5 volumes of 10 mM HEPES, pH 7.5. After swelling 10 min on ice, the cells were collected by centrifugation as before and resuspended in 5 volumes of 3 mM MgCl\(_2\), 1 mM dithiothreitol, 25 mM HEPES, pH 7.5. Cells were again collected by centrifugation and resuspended in 1 volume of the same buffer. Cells were broken with glass beads in a Dounce homogenizer using a tight pestle. The cell homogenate was immediately centrifuged for 30 s in an Eppendorf microcentrifuge and the crude nuclear pellet resuspended in 2 volumes of 0.1 M KCl, 25 mM HEPES, pH 7.5, and 1 mM dithiothreitol. Nuclei were lysed by adding 2 M KCl to a final concentration of 0.4 M at 4°C for 30 min. The lysates were adjusted to 20% glycerol and centrifuged at 45,000 rpm for 30 min in a Beckman Ti-70 rotor. The transparent supernatant was stored at -80°C in small aliquots. All procedures were performed at 4°C.

**Methylation Interference Assay**—Methylation interference assays were performed as described previously (Gilman et al., 1986) with a few modifications. End-labeled oligonucleotides were treated with dimethyl sulfate as described (Hendrickson and Schleif, 1985). Partially methylated oligonucleotides were incubated with nuclear extracts as described above for mobility shift assays, except that the reaction was scaled up to 100 ml and contained approximately 300,000 cpm of DNA/reaction. After electrophoresis, DNA was electrotransferred from the gel to Whatman DE81 paper overnight at 12 volts. The DE81 paper was exposed to x-ray film for 1 h at -70°C to locate the complexed and free DNA bands, which were excised, and the DNA was eluted by incubation in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM NaCl for several hours at 37°C. Carrier tRNA (10μg) was added to the eluates, which were extracted twice with phenol/chloroform/isooamyl alcohol (25:24:1, v/v) and once with chloroform/isooamyl alcohol (24:1, v/v). DNA was precipitated twice with EtOH, resuspended in 80% (v/v) formamide, 50 mM Tris borate, pH 8.3, and 1 mM EDTA, denatured at 100°C for 2 min and applied to a standard 10% polyacrylamide sequencing gel.

**Footprinting**—A preparative scale mobility shift reaction was carried out and electrophoresed as described above. The gel was then treated with ortho-phenanthroline/Cu\(^{2+}\) reagent, which cleaves the DNA while still in the gel (Kuwabara and Sigman, 1987). DNA was electrotransferred to DB81 paper, isolated, and run on standard sequencing gels, as described above.

**Results**

**DNA Binding Activity of the Ah Receptor from Rat Hepatocyte-derived LC57 Cells**—The Ah receptor from the murine hepatoma cell line Hepa1 has been previously shown to bind sequence specifically to Ah response elements (Denison et al., 1988a, 1988b; Fujisawa-Sehara et al., 1988; Hanpood et al., 1989). Fig. 1 shows that a similar DNA-binding activity is present in the nuclei of LC57 cells, a rat hepatocyte-derived cell line. LC57 cells were treated for the specified times with the polycyclic compound β-naphthoflavone and nuclear extracts prepared by the same procedure used to prepare extracts containing the Ah receptor from Hepa1 cells (Denison et al., 1988a, 1988b).

**Conclusions**

The mobility shift, or gel electrophoresis, DNA-binding assay (Fried and Crothers, 1981; Garner and Rezvain, 1981; Strauss and Vashavsky, 1984) was used to detect nuclear proteins that bind sequence specifically to the Ah response element XRE1. A 32P-labeled double stranded, 40-base pair oligonucleotide, whose sequence was
5'-CTCCAGGCTTCACGGAACCTCGGGGCAC-3' (coding strand), was used as probe in these experiments. This oligonucleotide corresponded in sequence to the region from -997 to -1028 from the transcription start site of the P-450c gene, with additional bases at each end to create BamHI- or BglII-ligatable ends. This sequence spans the core region of XRE1, bases -1007 to -1021, one of the Ah response elements of the P-450c gene (Fujisawa-Sehara et al., 1987). This domain is identical in sequence to one of the Ah response elements of the murine P-450IA1 gene and has been used to characterize the DNA-binding properties of the murine Ah receptor from Hepa1 cells (Denison et al., 1988a, 1988b; Hapgood et al., 1989).

Fig. 1 shows that, following treatment with β-naphthoflavone, XRE1-binding activity rapidly appeared in the nuclei of LCS7 cells, with kinetics similar to those observed for the appearance of the Ah receptor in the nuclei of Hepa1 cells (Denison et al., 1988a). Fig. 1 also shows that the rapid appearance of XRE1-binding activity in the nuclei of LCS7 cells was not blocked by inhibition of protein synthesis, as has been reported for the Ah receptor from Hepa1 cells (Denison et al., 1988a). These and other findings concerning the nuclear XRE1-binding activity isolated from LCS7 cells suggests that this activity corresponds to the Ah receptor observed in nuclear extracts from Hepa1 cells. The findings supporting this conclusion include the DNA footprinting data presented below.

Mobility Shift Competition Experiments—A series of mobility shift competition studies were performed, first to determine if the DNA-protein complexes detected in the experiments shown in Fig. 1 were sequence-specific, and second to precisely map these interactions.

The experiment presented in Fig. 2 tested the ability of a series of "mutant" double-stranded oligonucleotides to compete with 32P-labeled XRE1-40 for binding to the Ah receptor present in nuclear extracts from LCS7 cells. As shown in Fig. 3, each mutant oligonucleotide differed by 3 bases from the sequence of XRE1-15, a 15-base pair oligonucleotide containing the core domain of XRE1. Fig. 2 shows that the bands corresponding to the Ah receptor diminished when unlabeled XRE1-40 (lanes 2–4) or XRE1-15 (lanes 5–7) was included in DNA binding reactions at molar concentrations 5-, 25-, and 100-fold molar excess, respectively) of competitor; XRE1-40 (lanes 2–4), XRE1-15-base pair core region (lanes 5–7), XRE1-related oligonucleotides, M1 (lanes 8–10), M2 (lanes 11–13), M3 (lanes 14–16), M4 (lanes 17–19), M5 (lanes 20–22). The positions of the specific protein-DNA complexes (B) and free probe (F) are indicated.

FIG. 1. Ah receptor from rat LCS7 cells. A, LCS7 cells were treated with β-naphthoflavone (5 μg/ml) for 0–120 min, as specified. Cells were harvested and nuclear extracts prepared as described under "Experimental Procedures." Specific binding to XRE1-40 was measured by the mobility shift assay. B, before harvesting, cells were treated for 30 min with β-naphthoflavone (B) and/or 60 min with 10 μg/ml cycloheximide (X). B indicates specific DNA-protein complexes, and F indicates free XRE1-40 cDNA.

FIG. 2. Specific binding of the Ah receptor to the Ah response element XRE1 of the P-450c gene. Nuclear extracts from β-naphthoflavone-treated LCS7 cells were incubated with XRE1-40 (0.04 ng, 10,000 cpm), and the reactions were resolved by electrophoresis. Lane 7 contained no competitor DNA; each subsequent set of three lanes contained 0.1, 0.5, or 2.0 ng (5-, 25-, and 100-fold molar excess, respectively) of competitor; XRE1-40 (lanes 2–4), XRE1-15-base pair core region (lanes 5–7), XRE1-related oligonucleotides, M1 (lanes 8–10), M2 (lanes 11–13), M3 (lanes 14–16), M4 (lanes 17–19), M5 (lanes 20–22). The positions of the specific protein-DNA complexes (B) and free probe (F) are indicated.

FIG. 3. Sequence of XRE1-related oligonucleotides used in competition studies. The 15-base pair XRE1 core sequence is specified. Only those bases which differ from the wild-type (WT) XRE1 sequence are indicated for the mutant oligonucleotides.

decreased only slightly in reactions carried out in the presence of oligonucleotide M1 (lanes 8–10). Similar concentrations of oligonucleotide M2 competed weakly, while those of M5 competed almost as effectively as XRE1-15 itself for binding of 32P-labeled XRE1-40 by the Ah receptor.

These findings indicate that the band labeled B in Figs. 1 and 2 represents a sequence-specific DNA-protein complex, since the ability of oligonucleotides to compete for binding with 32P-labeled XRE1-40 was sequence-dependent. These results also indicate which bases within the XRE1 core sequence are most important for formation of this complex. For instance, in oligonucleotide M1, the triplet TCT, spanning base pairs -1020 to -1018 in XRE1, had been replaced with AGA. The greatly attenuated ability of this oligonucleotide to compete with XRE1-40 for binding to form DNA-binding complexes indicated that at least one of these 3 base pairs was important in binding the Ah receptor. Similarly, the inability of oligonucleotides M3 and M4 to compete indicated that at least 1 base pair in the triplet spanning positions -1011 to -1009 and at least 1 base pair in the triplet spanning positions -1008 to -1006 must have been important in the interaction of the Ah receptor with XRE1-40. Since oligonucleotides M2 and M5 competed to some extent, we conclude that the positions at which these oligonucleotides differ from XRE1-15 must have been less important for binding of the Ah receptor. It should be pointed out that all of the mutant oligonucleotides which competed with XRE1-40 for binding of the Ah receptor did so less strongly than the wild-type XRE1 core sequence. It is possible that alteration of three consecutive bases anywhere in the core sequence reduces binding somewhat, either by causing local alterations in DNA
conformation or by disrupting secondary DNA-protein contacts.

**Methylation Interference Footprinting**—To further characterize the interactions of the Ah receptor with XRE1, the methylation interference footprinting technique was used (Hendrickson and Schleif, 1985). This procedure identifies guanine residues that, when methylated at the N-7 position in the major groove, prevent or weaken binding of a factor. End-labeled XRE1-40 was partially methylated with dimethyl sulfate (Hendrickson and Schleif, 1985) and used as probe in a preparative scale mobility shift reaction. Free and bound DNAs were separated by electrophoresis, isolated from the acrylamide gel, cleaved with piperidine, and run on a standard 10% sequencing gel. When XRE1-40, 5'-end-labeled on the coding strand, was used as a probe, one band corresponding to the guanine at position -1011 was dramatically reduced in intensity in complexes formed with the Ah receptor (Fig. 4), indicating that this guanine was important for the interaction of the receptor with XRE1. When the contacts on the non-coding strand were mapped, it was found that methylation of the guanines located at -1010, -1012, and -1014 suppressed binding of the Ah receptor. Thus, the guanines at position -1011 on the coding strand and at positions -1010, -1012, and -1014 on the noncoding strand were involved in formation of complexes with the Ah receptor.

**ortho-Phenanthroline/Copper Footprinting**—Footprinting by the ortho-phenanthroline/Cu²⁺ footprinting technique (Kuwabara and Sigman, 1987) was used as a third technique to map the interactions of the Ah receptor with XRE1. In this procedure, DNA-protein complexes were resolved from free DNA by electrophoresis and then subjected to cleavage by ortho-phenanthroline/Cu²⁺ reagent while still in the gel. This takes advantage of the increased stability of DNA-protein complexes when they enter the gel matrix. After cleavage, the DNA was eluted from the gel and run on a standard sequencing gel. As shown in Fig. 5, the Ah receptor protected and therefore interacted with the same general region of XRE1-40 shown by competition mobility shift and methylation interference mapping to be important for Ah receptor binding. The boundaries of the protected domain on the coding strand were at bases -1004 to -1005 and -1018 and on the noncoding strand at bases -1006 and -1022.

**Inhibition of the DNA Binding Activity of the Ah Receptor by 1,10-Phenanthroline**—We have investigated the possible role of divalent metal ions in the DNA-binding function of the Ah receptor from LCS7 cells. The experiment presented in Fig. 6 investigated the effect of the chelator 1,10-phenanthroline on the formation of complexes between ³²P-labeled XRE1-40 and the Ah receptor present in nuclear extracts from β-naphthoflavone-treated LCS7 cells. Incubation of nuclear extract with 1,10-phenanthroline prior to the addition of ³²P-labeled XRE1-40 inhibited formation of Ah receptor-XRE1 complexes in a concentration-dependent manner (Fig. 6a, compare lanes 1 and 2 with lanes 3-7). Half-maximal inhibition was observed at 1 mM 1,10-phenanthroline and maximum with 3.0 mM. Note that treatment with 1,10-phenanthroline did not decrease the intensity of other bands, some of which have been shown to be due to sequence-specific interactions of other factors with XRE1. This indicates that the effect of 1,10-phenanthroline on the Ah receptor was

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**Fig. 4. Methylation interference analysis.** Methylation interference footprinting was performed as described under "Experimental Procedures" using XRE1-40, ³²P-labeled on either strand, as indicated, and nuclear extracts from β-naphthoflavone-treated LCS7 cells. F represents the free probe, showing cleavage at all guanines, and B represents DNA-protein complexes, as in Figs. 1 and 2. The nucleotide sequence corresponding to the region of interest is presented at the side of the figure. The guanines at which methylation blocks binding are indicated by arrows on the sequence.

**Fig. 5. ortho-Phenanthroline/Cu⁺ chemical footprinting analysis.** ortho-Phenanthroline/Cu⁺ footprinting was performed as described under "Experimental Procedures" using as probe XRE1-40, end-labeled on the coding and noncoding strands as indicated. B and F, DNA-protein complexes and free DNA, respectively; G, guanine sequencing ladder. Heavy and light bars, those bases within the DNA sequence that were protected completely or partially from ortho-phenanthroline/Cu⁺ cleavage, respectively. Light exposures indicated that radioactivity in excess of that in other lanes had been loaded in lanes B in both panels. This was taken into account when the boundaries of the footprints were defined.

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specific. This inhibition was almost completely prevented if Zn\(^{2+}\) was added to the nuclear extract before addition of 1,10-phenanthroline (Fig. 6A, lane 8). Mg\(^{2+}\) protected much less effectively (less than 20\%), whereas Ca\(^{2+}\) and Mn\(^{2+}\) were ineffective (Fig. 6A, lanes 9–11). When 4,7-phenanthroline, a phenantroline isomer that largely lacks the ability to chelate divalent cations, was tested, inhibition was observed (Fig. 6B, lanes 1–5), but an excess of Zn\(^{2+}\) or other divalent cations could not prevent this inhibition, suggesting inhibition by another mechanism (Fig. 6B, compare lanes 5 and 6).

Inhibition of the DNA-binding Activity of the Ah Receptor by Oxalic Acid—EDTA and CDTA, both of which are effective chelators of several divalent cations, including Zn\(^{2+}\), had no significant effect on the DNA-binding activity of the Ah receptor, even at concentrations as high as 20 mM (data not shown). However, another chelator, oxalic acid, was found to inhibit the DNA-binding activity of the receptor. Fig. 7A shows that oxalic acid inhibited receptor activity in a concentration-dependent manner (compare lanes 1 and 2 with lanes 3–8). Half-maximal inhibition was achieved at 1.0 mM oxalic acid and complete inhibition at 2.25 mM. Zn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\), all of which are known to be chelated with similar affinities by oxalic acid, all prevented receptor inhibition by about 50\% (Fig. 7B, compare lanes 3 and 4 with lanes 5–8). These results support the findings with 1,10-phenanthroline and suggest that the Ah receptor requires a metal ion for DNA-binding activity.

Inactivation of the Ah Receptor by 1,10-Phenanthroline Is Reversible—Although adding Zn\(^{2+}\) to nuclear extracts before the addition of 1,10-phenanthroline prevented inhibition of the DNA-binding activity of the TCDD receptor, once lost, this activity could not be restored by adding Zn\(^{2+}\) in excess of 1,10-phenanthroline to mobility shift reactions (data not shown). The same result was obtained when the experiment was carried out under an argon atmosphere in the presence of dithiothreitol (data not shown). This suggested that the oxidation of sulfhydryl residues, which may be essential to receptor-DNA interactions, was not responsible for the inhibitory action of 1,10-phenanthroline. Therefore, we tried restoring receptor activity by removing 1,10-phenanthroline. When extracts treated with 1,10-phenanthroline were dialyzed in the absence of Zn\(^{2+}\), inhibition persisted (compare lanes 3 and 4 with lanes 9 and 10, Fig. 8). However, dialysis of 1,10-phenanthroline-treated extracts in the presence of Zn\(^{2+}\) restored the DNA-binding activity of the receptor more than 50\% (Fig. 8, compare lanes 3 and 4 with lanes 11 and 12). These observations suggest that the mechanism of inhibition by 1,10-phenanthroline involves the reversible removal of a metal ion(s), probably Zn\(^{2+}\), from the Ah receptor through the formation of a metal-chelator complex. The findings presented in Fig. 8 also indicate that the mechanism by which 4,7-phenanthroline partially inhibits the DNA-binding activity of the receptor must be different from that of 1,10-phenanthroline, since removal of 4,7-phenanthroline by dialysis in the absence of Zn\(^{2+}\) restored receptor activity more than 90\% (Fig. 8, compare lanes 5 and 6 with lanes 13 and 14). This was in contrast to the failure to restore receptor activity by removing 1,10-phenanthroline in the absence of Zn\(^{2+}\), as pointed out above.

DNA Binding Protects the Ah Receptor from Inhibition by 1,10-Phenanthroline—In contrast to the substantial inhibition of the receptor when 1,10-phenanthroline was added to nuclear extracts prior to the DNA binding reaction, inhibition was decreased substantially when the receptor was bound to XRE1 prior to treatment with 1,10-phenanthroline (Fig. 9). This was not due to protection by poly(dI-dC)-poly(dI-dC) or to interference by any other component of the binding reaction. 1,10-Phenanthroline efficiently inhibited the DNA-binding activity of the receptor in the presence of all reaction components (Fig. 9, compare lanes 1 and 2 with lanes 5 and 6), as long as it was added prior to the addition of \(^{32}\)P-labeled DNA (XRE1-40) (Fig. 9, compare lanes 5 and 6 with lanes 7 and 8).

Influence of Divalent Cations on the DNA-binding Activity of the Ah Receptor—Several divalent cations were tested for their direct effect on the DNA-binding activity of the Ah receptor. CoCl\(_2\) or CdCl\(_2\), included at 1 mM in mobility shift reactions, completely abolished the binding of the Ah receptor to XRE1 (Fig. 10, compare lanes 1 and 2 with lanes 3 and 7). ZnCl\(_2\), CaCl\(_2\), MgCl\(_2\), and MnCl\(_2\) at 1 mM did not measurably

**Fig. 6. Inhibition of the Ah receptor DNA-binding activity by 1,10-phenanthroline and protection from inhibition by Zn\(^{2+}\).** A, a nuclear extract was incubated at 23°C for 15 min with 0–5 mM 1,10-phenanthroline (lanes 1–7) or 15 min with 1 mM divalent cation followed by 15 min with 2 mM 1,10-phenanthroline (lanes 8–11). DNA binding activity was measured by mobility shift method. B, the effect of the nonchelating isomer 4,7-phenanthroline on the binding of the Ah receptor to XRE1-40 was assessed. Numbering system is as in Panel A, except that the effects of Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) were not tested, and 4,7-phenanthroline was used in place of 1,10-phenanthroline.
Fig. 7. Oxalic acid inhibits the DNA-binding activity of the Ah receptor and divalent cations protect. A, nuclear extract, containing the Ah receptor, was incubated for 15 min at room temperature with 0-3.0 mM oxalic acid (lanes 1-10). B, nuclear extract, containing the Ah receptor, was incubated with 1 mM divalent cation for 15 min (lanes 3-6), after which oxalic acid was added to 2 mM and incubation continued for another 15 min. XRE1-40 binding activity was measured by the standard mobility shift method.

AFFECT the DNA-binding activity of the Ah receptor (Fig. 10, lanes 2, 4, 5, and 6).

**DISCUSSION**

Fig. 11 summarizes the interactions between the Ah receptor and the Ah response element XRE1. Both mobility shift competition experiments using mutant oligonucleotide competitors and methylation interference footprinting yielded precise information concerning the participation of specific bases in DNA-protein interactions, while ortho-phenanthroline/Cu²⁺ footprinting provided inherently less precise information. The results obtained by this procedure were, however, consistent with those of the former two techniques.

The results of all three mapping procedures in conjunction with the data of Denison et al. (1988b) and Fujisawa-Sehara et al. (1988) can be used to construct a more complete picture of the binding domain of the receptor.⁴ By comparing several different natural Ah receptor binding domains, these two groups have identified a core binding domain whose sequence is 5'-CAGCA/TA-3'. These investigators also presented evidence that high affinity binding and gene regulatory function require bases other than those present in the core-binding domain.

Fujisawa-Sehara et al. (1988) have presented an extended consensus sequence that suggests possible candidates for bases important to Ah receptor binding: GNNGC/GC/AN-GNNCNTNTCAGC/TA. The competition mobility shift experiments presented in Fig. 2 provide further evidence concerning the importance of specific nucleotides within this sequence. The cytosine at position 10 in this sequence is probably important to receptor binding, because mutant M1, in which this cytosine is replaced with guanine, binds the Ah receptor very weakly. The base substitutions at positions 9 and 11 in M1 probably do not contribute substantially to differences between M1 and XRE1, because the bases at these positions are not conserved in other known XREs. The thymines at positions 12 and 14 in the above consensus sequence can be replaced with adenines without altering binding. Thus, even though the thymine at position 12 is highly conserved in the natural sequences examined by the others, it may not be essential for receptor binding. This is also the case for the adenine at position 21.

The methylation interference analysis described in Fig. 4 also contributes information that clarifies the consensus sequence for the interaction of the receptor with the Ah response element.⁵ The four positions at which methylation interferes with binding of the receptor to XRE1 all fall within the central core binding domain (bases 15, 17, 18, and 19), confirming the importance of this region. Methylation at position 10 did not interfere substantially with receptor binding. In contrast, competition mobility shift experiments, in conjunction with the comparisons of native Ah receptor-binding domains by Denison et al. (1988b) and Fujisawa-Sehara et al. (1988), showed that this position was important for binding. Since methylation of guanines altered only major groove contacts at position 10, it may be that minor groove, but not major groove, contacts are important for the binding of the receptor at this position.

The first 6 bases of the extended consensus sequence of Fujisawa-Sehara et al. (1988) are not included in the ortho-phenanthroline/Cu²⁺ protection footprint and therefore are unlikely to be of great importance for receptor binding. The ortho-phenanthroline/Cu²⁺ footprint extends four bases in the 3’ direction beyond the above consensus sequence, yet competition experiments with both synthetic oligonucleotides (Fig. 2) and natural sequences (Fujisawa-Sehara et al., 1988) indicate little sequence specificity in this region for receptor binding. Perhaps protection in this region is not due to base-specific interactions with the DNA-binding domain, but to steric hindrance by other domains of the receptor molecule.

In conclusion, a revised version of the consensus sequence for DNA recognition by the Ah receptor would be as follows: CNA/TNA/TCAGCG/TA/T. Binding of the receptor seems to be strongly dependent on the cytosine at position 1 and CACGC at positions 6-10. At present we know that either thymine or adenine at positions 3, 5, 11, and 12 support binding. Synthesis and testing of other oligonucleotides will be necessary to determine whether strong binding is preserved when guanine or cytosine is present at these positions.

We have investigated the possible role of metal ions in the DNA-binding function of the Ah receptor. Our findings, suggesting that a divalent metal ion, probably Zn²⁺, is an essential cofactor for the DNA-binding activity of the receptor, can be summarized as follows: 1) treatment of the receptor with two chelators of divalent metal ions, 1,10-phenanthroline and

⁴ While this paper was under review, others reported mobility shift (Denison et al., 1988; Neuhold et al., 1989) and footprinting (Shen and Whitlock, 1988; Neuhold et al., 1989) results that confirm the findings reported here and that support the proposed DNA recognition consensus sequence for the Ah receptor.
Ah Receptor DNA-binding Activity

FIG. 8. Reversal of 1,10-phenanthroline inhibition by dialysis in the presence of Zn$^{2+}$. Nuclear extract, containing the Ah receptor, was left untreated (C) or incubated at 23°C with 2 mM 1,10-phenanthroline or 2 mM 4,7-phenanthroline for 15 min, as indicated. DNA-binding activity was measured by the mobility shift method either directly (lanes 1–6) or after dialysis for 3 h at 4°C (lanes 7–14). The dialysis buffer contained 0.1 M KCl, 25 mM HEPES, pH 7.5, and 1 mM diethiothreitol, except lanes 11 and 12, which also contained 0.1 mM ZnCl$_2$.

oxalic acid, prevented the binding of the receptor to XRE1; 2) inhibition by 1,10-phenanthroline and oxalic acid could be prevented almost completely by the presence of excess Zn$^{2+}$, suggesting that inhibition was due to chelation; 3) the abilities of other divalent cations to prevent inhibition by 1,10-phenanthroline or oxalic acid were strongly correlated with their affinities (Vallec and Wacker, 1970) for these chelators; 4) the inhibition by 1,10-phenanthroline was relieved after extensive dialysis in the presence of Zn$^{2+}$ but not by dialysis in its absence; 5) in contrast, inhibition by the nonchelating isomer 4,7-phenanthroline was not prevented by Zn$^{2+}$ or other divalent cations and was reversed almost completely by dialysis in the absence of Zn$^{2+}$. The ability to restore receptor activity by dialysis to remove 1,10-phenanthroline in the presence of Zn$^{2+}$, but not in its absence, indicated that the removal of a metal ion by 1,10-phenanthroline may cause a reversible change in the receptor that prevents its binding to XRE1. Although these experiments suggest that this metal
ion is probably Zn\textsuperscript{2+}, another metal ion for which Zn\textsuperscript{2+} can substitute, and which we have not tested, may be the natural cofactor for receptor function.

Although the data presented show that Zn\textsuperscript{2+} is an essential cofactor for Ah receptor DNA-binding activity, they do not directly indicate whether Zn\textsuperscript{2+} is tightly bound to the receptor or is in rapid equilibrium with the solvent. We tested other chelators for their effect on the DNA-binding activity of the Ah receptor. Interestingly, EDTA and CDTA, which are known to chelate Zn\textsuperscript{2+} effectively, did not inhibit binding of the receptor to XRE1, even at high concentrations. Both of these compounds are structurally quite different from 1,10-phenanthroline and oxalic acid. The fact that structurally diverse chelators vary in their ability to inactivate the receptor and that this variation is not correlated with their affinities for Zn\textsuperscript{2+} (e.g. EDTA, which is noninhibitory, has a much higher affinity for Zn\textsuperscript{2+} than does oxalic acid, which is inhibitory) indirectly suggests that the essential Zn\textsuperscript{2+} is not in rapid equilibrium with the solvent. It is more likely to be sequestered such that access to it by chelators is sterically constrained, and, therefore, dependent upon the structure of the chelator used. The finding that association of the receptor with DNA protects from inhibition by 1,10-phenanthroline is also consistent with the possibility that Zn\textsuperscript{2+} is bound to the receptor. Direct evidence bearing upon this point must await purification of sufficient quantities of the Ah receptor to analyze its divalent cation content and the chemical nature of the association of this ion with the receptor.

We have found that Cd\textsuperscript{2+} and Co\textsuperscript{2+} directly inhibited Ah receptor binding to XRE1. The mechanism by which Cd\textsuperscript{2+} and Co\textsuperscript{2+} inhibit is unknown at this time. It is thought that the steroid receptor binds Zn\textsuperscript{2+} through cysteine sulfhydryls. If the Ah receptor is a member of the same receptor family, it is possible that Cd\textsuperscript{2+} and Co\textsuperscript{2+} inhibit the Ah receptor by displacing the physiologic divalent cation from the receptor and forming a complex with the receptor that is incapable of binding DNA. Alternately they may interact with other cysteines that are required for the DNA-binding activity of the receptor.

Many structural and functional similarities (Hannah et al., 1986; Okey et al., 1979, 1980; Wilhelmsson et al., 1986) based on hydrodynamic properties and signal transduction, as well as differences based on genetic findings (Yamamoto et al., 1976; Whitlock, 1987), have been observed between the Ah receptor and the steroid receptors, particularly the glucocorticoid receptor. Based on the extensive similarities reported, it has been suggested that the Ah receptor belongs to the steroid and thyroid hormone receptor superfamily (Evans, 1988). The members of this family have a multidomain structure, the most highly conserved of which is the DNA-binding domain with zinc-finger motifs analogous to those of transcription factor TFIIIA (Evans, 1988). Zn\textsuperscript{2+} has been shown to be an essential cofactor for both the glucocorticoid receptor (Freedman et al., 1988) and the estrogen receptor (Sabbah et al., 1987). Here, we have presented parallel findings for the Ah receptor, showing that the sequence-specific DNA-binding activity of this receptor requires a metal ion, most likely Zn\textsuperscript{2+}. These results are consistent with the hypothesis that the Ah receptor is a member of the same receptor superfamily. Cloning, sequencing, and expression of the cDNA for the Ah receptor, in conjunction with mutagenesis and gene transfer experiments, will be required to conclusively establish whether or not the Ah receptor is a member of this receptor superfamily.

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