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Specific protein–DNA interactions at a xenobiotic-responsive element: Copurification of dioxin receptor and DNA-binding activity

(2,3,7,8-tetrachlorodibenzo-*p*-dioxin/glucocorticoid receptor/cytochrome P-450c/glucocorticoid-responsive element)

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ABSTRACT **Upon binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (called dioxin or TCDD), the dioxin receptor exhibits increased affinity for the cell nucleus *in vivo* and for DNA *in vitro*. To define the recognition sequence of the dioxin receptor and its relationship with that of the glucocorticoid receptor, oligonucleotides derived from dioxin-responsive elements of the rat cytochrome P-450c gene were tested for their ability to form specific protein–DNA complexes in a gel retardation assay. We found that a previously defined sequence motif that is similar to the glucocorticoid-responsive element and exhibits strong enhancer activity in response to dioxin receptor ligands bound a dioxin-inducible factor with high specificity but was not recognized by the DNA-binding domain of the glucocorticoid receptor. Binding to this element was only observed in nuclear extracts of wild-type mouse hepatoma cells in a time- and dose-dependent manner and not in nuclear extracts from a nonresponsive mutant cell line deficient in DNA binding of the dioxin receptor. The specific DNA-binding activity in wild-type nuclear extracts comigrated in a Superose size-exclusion column and cosedimented on sucrose gradients with the *in vivo* labeled dioxin receptor. These experiments strongly suggest that the dioxin receptor is a sequence-specific DNA-binding protein and is not only biochemically but also functionally similar to the steroid receptor family.**

The effect of dioxin on specific cytochrome P-450c gene transcription is mediated by an intracellular receptor protein to which dioxin and related compounds bind with high affinity and selectivity (1, 2). In analogy to the mechanism of action of steroid hormones, binding of dioxin to its receptor induces an increased affinity of the receptor for nuclear target sites *in vivo* and for nonspecific DNA *in vitro* (ref. 3 and references therein). Several biochemical properties of the dioxin receptor are similar to those of the glucocorticoid receptor (refs. 4 and 5). However, the dioxin and glucocorticoid receptors do not appear to share any common ligand-binding specificity (6), and the endogenous ligand for the dioxin receptor, if any, has not yet been identified. Although steroid hormone receptors are known to activate gene expression by binding to specific hormone-dependent enhancers, little is known about the function of the dioxin receptor. Attempts to determine whether the dioxin receptor binds directly to specific DNA sequences have been hampered by difficulties in purifying the receptor (7). While this work was in progress, dioxin-inducible protein–DNA interactions with an unidentified sequence motif in the 5′ flank of the murine cytochrome P_1-450 gene were reported (8).

In the rat cytochrome P-450c gene, two classes of sequence elements have been defined by deletion analysis to mediate dioxin induction of gene expression. The "drug regulatory elements" (DREs; ref. 9) exhibit a rather weak enhancer activity, whereas the second class of elements, referred to as "xenobiotic-responsive elements" (XREs), express strong enhancer activity in response to dioxin receptor ligands (10). However, it has not been established whether the dioxin receptor itself binds directly to these specific DNA sequences. We have used nuclear extracts from wild-type mouse hepatoma cells and a mutant cell line deficient in nuclear accumulation (11) and DNA-binding of the dioxin receptor (3) to address this question. We demonstrate here that a dioxin-inducible factor specifically recognizes the XRE motifs *in vitro* and that the XRE motifs are not recognized by the glucocorticoid receptor. Moreover, by exploiting the ligand-binding properties of the dioxin receptor, we present strong evidence that the XRE-specific factor represents the dioxin receptor.

MATERIALS AND METHODS

Materials and Cells. 2,3,7,8-Tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin ([^{3}H]TCDD; 46 Ci/mmol; 1 Ci = 37 GBq) was a gift from S. Safe (Texas A & M University). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was supplied by C. Rappe (Umeå, Sweden). The Hepa 1c1c7 cells, a subclone of the mouse hepatoma line Hepa 1, and the mutant line c4 derived from it were maintained as described (3).

Gel Shift Assay. Nuclear extracts were prepared exactly as described by Dignam *et al.* (12). The protein concentration as determined by the method of Bradford (13) was 4 ± 1 μg/μl. Nuclear extracts (10 μg of protein) were incubated for 15 min at 4°C in 15 mM Hepes, pH 7.9/0.5 mM dithiothreitol/3 mM $MgCl_2$/4 mM spermidine/15% (vol/vol) glycerol/60 mM NaCl/0.15 mM EDTA/0.4 μg of sonicated salmon sperm DNA per μl/0.1 μg of poly[d(I·C)] per μl/0.1 μg of tRNA per μl (final volume, 19 μl). The ^{32}P-end-labeled probe (20,000 cpm; 20 fmol) was added in the absence or presence of unlabeled synthetic competitor oligonucleotide (1 μl) as shown in the figures, and the incubations were continued for an additional 20 min at 20°C. Protein–DNA complexes were then immediately analyzed on a 4% polyacrylamide gel in 50 mM Tris·HCl, pH 8.4/380 mM glycine/2 mM EDTA, followed by autoradiography. The 12-kDa DNA-binding domain of the glucocorticoid receptor was purified after expression of the cDNA in *Escherichia coli* (14). Gel shifts were performed by incubating 120 ng of this protein for 30 min at 20°C in 20 mM Tris·HCl, pH 8.0/20% (vol/vol) glycerol/60 mM NaCl/1 mM EDTA/5 mM dithiothreitol/100 ng of insulin per μl containing radioactive probe in the absence or presence of unlabeled

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Abbreviations: GRE, glucocorticoid-responsive element; DRE, drug regulatory element; XRE, xenobiotic-responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

competitor. The final incubation volume was 25 μl. Protein–DNA complexes were analyzed as described above.

Oligonucleotides. Complementary single-stranded oligonucleotides were synthesized with an Applied Biosystem DNA synthesizer, purified by HPLC, and hybridized. The concentrations were determined spectrophotometrically. The oligonucleotide GRE (for glucocorticoid responsive element; see Fig. 2) is derived from the rat tyrosine aminotransferase gene (15). XRE 1, XRE 2, and DRE (see Fig. 2) are from the 5′ flanking region of the rat cytochrome P-450c gene (9, 10). The sequences of the oligonucleotides NS1–NS4, which do not contain GREs, XREs, or DREs, are given below.

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NS1: 5′-dGGATCCACCCTGTCTCATGAATATGCAAATCAGGTGAG-3′
           dTGGGACAGAGTACTTATACGTTTAGTCCACTCCCTAGG

NS2: 5′-dGGATCCCAGGTACCAGGGCCGTGAGTTCTG-3′
           dGTCCATGGTCCCGGCACTCAAGACCCTAGG

NS3: 5′-dGATCCGCCTTATTTTAGAAACGCAAATTGTCCAGGTGTTGTTTTGCTCAGTAGAG-3′
         dGCGGAATAAAATCTTTGCGTTTAACAGGTCCACAACAAAACGAGTCATCTCCTAG

NS4: 5′-dATGAATATGCAAATCAGGTG-3′
       dTACTTATACGTTTAGTCCAC
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Gel Permeation Chromatography. High-performance gel permeation chromatography of nuclear extract was performed at 4°C on a prepacked Superose 12 HR column (10 × 300 mm) (3). A total of 0.5 ml (2.2 mg of protein; 20,000 dpm) was applied to the column equilibrated in 25 mM Hepes, pH 7.9/1.5 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/500 mM NaCl. The flow rate was 30 ml/hr.

Safety. Since TCDD and TCDF are toxic, special handling procedures were followed as described (3).

RESULTS

We used a gel retardation assay to study nuclear proteins that interact with XREs. Nuclear extracts were prepared from untreated and TCDD- or TCDF-treated wild-type and variant hepatoma cells. After incubation of nuclear extracts with ^{32}P-labeled oligonucleotide XRE 1, the protein–DNA complexes were separated by gel electrophoresis and visualized by autoradiography. A dioxin-inducible protein–DNA complex was detected after incubation with wild-type cell extracts but not with variant cell extracts [Fig. 1, complex (arrow) 1]. The appearance of the complex was dependent on the dose and time of TCDF treatment, maximum response occurring with 100 nM TCDF after 1 hr (Fig. 1, compare lanes 3, 8, 9, and 11). TCDD was a more potent inducer of complex 1 than TCDF, in accordance with its greater potency to induce cytochrome P-450c (16) (Fig. 1, lanes 7 and 10). Two additional complexes (Fig. 1, complexes 2 and 3) were detected in both wild-type and variant extracts, and their presence was independent of TCDF treatment. At present we do not know whether the factors associated with complexes 2 and 3 are involved in the cellular response to TCDD.

The sequence-specificity of DNA binding of the TCDF-inducible factor(s) was analyzed by competition for binding to ^{32}P-labeled XRE 1 using unlabeled competitor oligonucleotides. The sequences of the various oligonucleotides representing XREs 1 and 2, DRE, and a GRE are compared in Fig. 2. Complex 1 was found to be highly specific for XREs 1 and 2 (Fig. 3*A*, lanes 3 and 6). We examined the relative affinity of DNA sequences for the protein(s) involved in the formation of complex 1 in more detail (Fig. 3*B*) and found that XRE 1 competed more efficiently for binding than did XRE 2. Oligonucleotides containing GRE and DRE did not compete with XRE 1 for binding, even at a 250-fold molar excess (Fig. 3*B*). When XRE 2 was used as the probe in direct binding experiments, a specific inducible complex was detected with nuclear extracts from wild-type cells. The relative mobility of this complex was identical to that of complex 1 observed with XRE 1 as probe, indicating that both XRE sequence motifs are recognized by the same factors.

To determine whether the glucocorticoid receptor interacts with XREs, we tested the ability of unlabeled XREs to compete for binding of the glucocorticoid receptor DNA-binding domain to ^{32}P-labeled GRE by the gel retardation assay (Fig. 4). We observed no competition with XRE 1 (Fig. 4, lanes 6–8), XRE 2, DRE, or NS1-4 (data not shown). The binding of the receptor protein to DNA was found to be highly specific for GREs in this assay (Fig. 4, lanes 3–5). We also performed incubations of nuclear extract with ^{32}P-labeled GRE and of receptor protein with ^{32}P-labeled XRE 1. Gel retardation analysis of these incubations did not reveal any protein–DNA complexes (data not shown).

To elucidate whether the dioxin-inducible protein–DNA complex 1 contains the dioxin receptor, we treated cells with [^{3}H]TCDD, fractionated nuclear extract from these cells by gel permeation and heparin–Sepharose chromatography or gradient ultracentrifugation, and monitored the position of the specifically bound [^{3}H]TCDD and XRE-specific DNA-binding activities, respectively. Nuclear extracts were prepared from wild-type and variant cells treated with 1 nM [^{3}H]TCDD for 1 hr in the absence or presence of a 100-fold molar excess of unlabeled TCDF. The [^{3}H]TCDD detected in the nuclear extract of wild-type cells could be completely

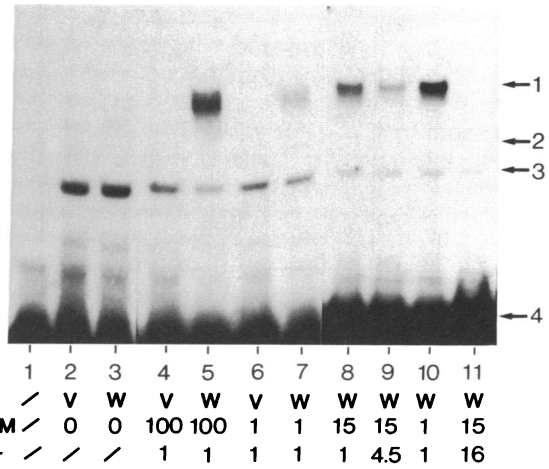

FIG. 1. Binding of nuclear extract protein from wild-type cells to XRE 1: dependence on time and dose of TCDF induction. Wild-type (lanes W) and variant (lanes V) cells were treated for different times and with different amounts of TCDF (lanes 4–9 and 11) or TCDD (lane 10) at 37°C. Nuclear extracts were prepared, and the gel shift assay was performed with ^{32}P-labeled XRE 1. Lane 1 shows the probe without protein. Arrows 1–3 show protein–DNA complexes, and arrow 4 shows the free probe.

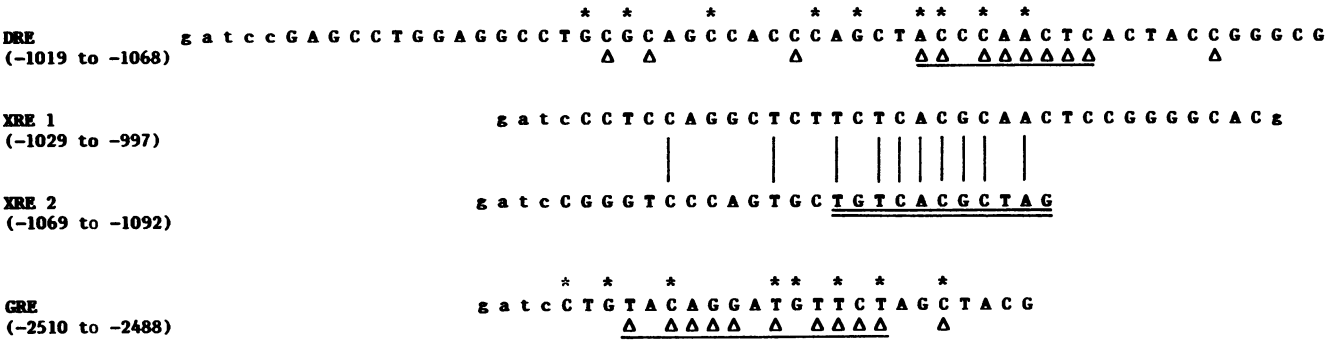

FIG. 2. Comparison of DREs, XREs, and GREs. Sequences of coding or noncoding strands of the synthesized oligonucleotides were compared. For simplicity only one strand of each double-stranded oligonucleotide is shown. Lowercase letters indicate flanking restriction enzyme linker sequences. Vertical lines represent bases in XRE 1 common to XRE 2 and a horizontal double line highlights the region of greatest homology between XRE 1 and XRE 2. Triangles and stars indicate bases in DRE or GRE common to XRE 1 and XRE 2, respectively. A horizontal single line highlights the regions of DRE and GRE with greatest homology to XREs.

eliminated by competition with unlabeled TCDF and corresponded to 5000 ± 1000 molecules of dioxin receptor per cell. Variant cell nuclear extracts contained 20 ± 5% of the total [^{3}H]TCDD present in the wild-type extracts. Sucrose density gradient ultracentrifugation of wild-type extracts revealed a 7.0 ± 0.2-S species that bound [^{3}H]TCDD in a saturable manner (data not shown). Gel permeation chromatography identified a species with a Stokes radius of 6.9 ± 0.2 nm associated with [^{3}H]TCDD (Fig. 5*A*, fraction 11). Thus, the macromolecular species associated with [^{3}H]TCDD in our nuclear extracts from wild-type cells represents the dioxin receptor, and the results agree well with previous data on the abundance of nuclear receptor from murine hepatoma cells (17, 18).

Gel retardation analysis of individual fractions from the sucrose gradients (data not shown) and the eluate from the gel permeation column (Fig. 5*B*, fraction 11) showed that the XRE-specific DNA-binding species (complex 1 in Figs. 1, 3, and 5*B*) comigrated with the TCDD receptor. A large TCDD-binding aggregate was eluted in the void volume (Fig. 5*A*) (19). We did not observe DNA binding of this aggregate, possibly because of masking of the DNA-binding domain of the XRE-specific factor. When the wild-type nuclear extract was chromatographed on heparin-Sepharose, the [^{3}H]-TCDD–receptor complex was retained on the column in 50 mM NaCl and was eluted at 350 mM NaCl. Gel retardation analysis of the individual fractions from the heparin-Sepharose eluate again demonstrated coelution of specific [^{3}H]TCDD- and XRE-binding activities (data not shown). Taken together, these data provide strong evidence that the dioxin receptor participates in the formation of a protein–DNA complex specific for DNA fragments containing XREs.

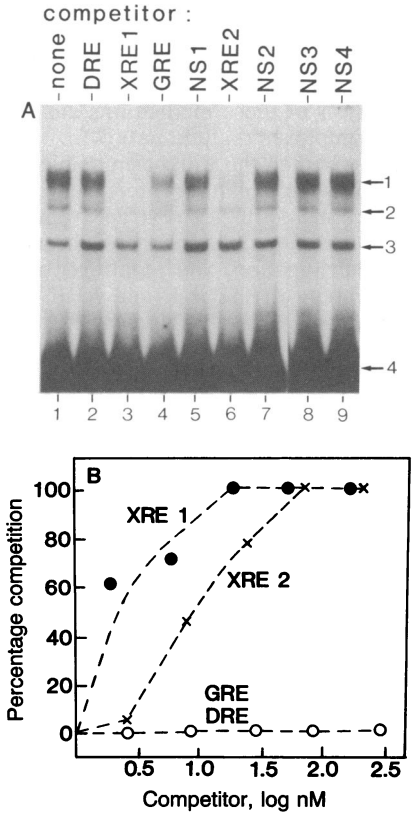

FIG. 3. Sequence-specific binding of the dioxin-inducible factor to XRE 1. (*A*) Nuclear extracts were prepared from wild-type cells induced for 1 hr with 100 nM TCDF. Gel retardation assays were performed with radioactive XRE 1 in the absence or presence of a 25-fold molar excess of unlabeled competitor as shown. Arrows 1–4 are as for Fig. 1. (*B*) The gel retardation assay was performed as described above except that increasing concentrations of competitors XRE 1, XRE 2, GRE, or DRE were used. The protein–DNA complexes corresponding to those indicated by arrow 1 in *A* were excised from the dried gel and assayed by liquid scintillation spectroscopy. The relative radioactivity present in complex 1 in the absence or presence of competitor is expressed as the percentage competition.

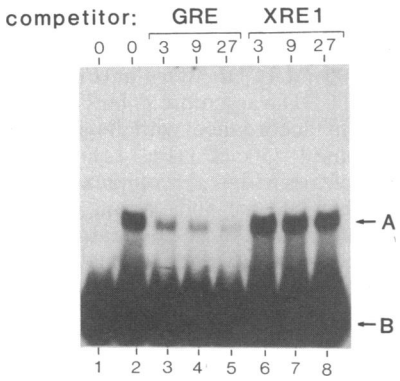

FIG. 4. DNA-binding domain of the glucocorticoid receptor does not bind to XREs. Purified expressed DNA-binding fragment of the glucocorticoid receptor was analyzed by the gel shift assay using ^{32}P-labeled GRE as the radioactive probe in the absence or presence of increasing molar excesses of competitor DNA as shown. Arrow A marks the GRE-specific protein–DNA complex, and arrow B shows the free probe. The first lane shows an incubation in the absence of receptor protein.

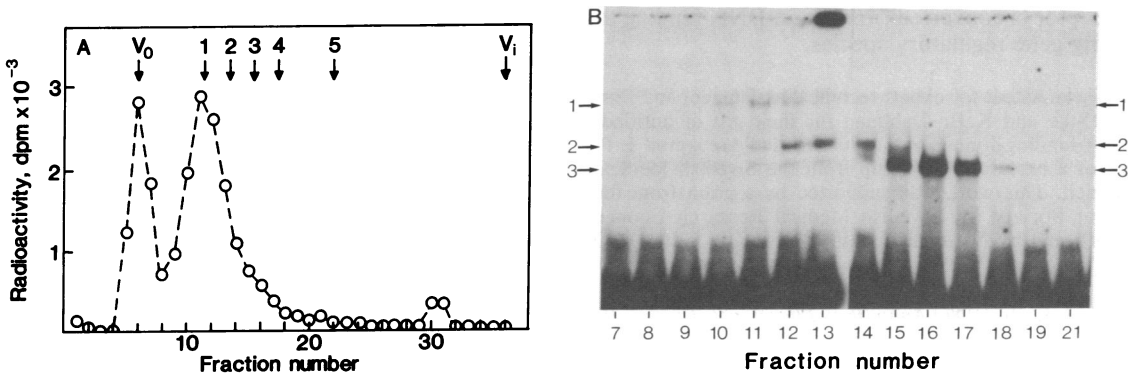

FIG. 5. High-performance gel permeation chromatography of nuclear extract: coelution of the [^{3}H]TCDD–receptor complex and specific DNA-binding activity. Nuclear extract prepared from wild-type cells induced with 1 nM [^{3}H]TCDD for 1 hr was analyzed on a Superose 12 column as described. Individual fractions were analyzed by liquid scintillation counting (*A*) and by the gel retardation assay (*B*) using ^{32}P-labeled XRE 1 as the probe and 2 μl of each fraction per incubation. Note that the increase in complex 3 relative to complex 1 (compared to Figs. 1 and 3) is the result of the decreased concentration of poly[d(I-C)] (20 ng/μl) used to increase the sensitivity of the assay. Protein–DNA complexes marked with arrows 1–3 have the same relative mobility as those in Figs. 1 and 3. The column was calibrated with blue dextran (V_0), ^3H_2O (V_i), and the following proteins (Stokes radius in parentheses): 1, β-galactosidase (6.9 nm); 2, ferritin (6.15 nm); 3, aldolase (4.8 nm); 4, bovine serum albumin (3.5 nm); and 5, myoglobin (2.0 nm).

DISCUSSION

It has been shown previously that a segment of the 5′ flanking region of the rat cytochrome P-450c gene comprising nucleotides −844 to −1140 from the start of transcription is essential not only for directing dioxin-inducible expression from the homologous cytochrome P-450c promoter but also to confer dioxin inducibility on the heterologous simian virus 40 promoter (10, 20). Within this cytochrome P-450c 5′ flanking fragment, two regions of about 30–40 nucleotides, referred to as XREs 1 and 2, have been defined that are essential and sufficient for dioxin-inducibility in hepatoma cells (10). Moreover, the XRE motif is conserved in the equivalent human and mouse genes (see ref. 10 and references therein). In this paper, we have characterized a factor that specifically recognizes these sequences *in vitro*. This factor exhibits properties that are indistinguishable from those of the dioxin receptor by several criteria. First, the nuclear factor has a high affinity for XREs only after treatment of mouse hepatoma Hepa 1c1c7 cells with TCDD or TCDF, two well-known inducers of cytochrome P-450c and ligands of the dioxin receptor. Similarly, pretreatment of target cells with cytochrome P-450c-inducing compounds is necessary for nuclear translocation of the dioxin receptor (16); more significantly, DNA binding *in vitro* of the dioxin receptor, as assessed by retention of the receptor on calf thymus DNA-cellulose, is a ligand-dependent event (21). In the case of steroid hormone receptors, the hormone is required for induction of a DNA-binding form of the glucocorticoid receptor both *in vivo* (22) and *in vitro* (23).

A second similarity between the XRE-specific factor and the dioxin receptor is that the factor is not inducible by dioxin in mutant cells derived from the mouse hepatoma cell line Hepa 1c1c7. The c4 mutant cells are deficient in induction of cytochrome P-450c gene expression when exposed to TCDD but exhibit normal dioxin-receptor ligand-binding characteristics. However, the ligand–receptor complexes are unable to accumulate in the cell nucleus *in vivo* (11) and to bind to calf thymus DNA *in vitro* (3). The kinetics and dose dependency for induction of specific DNA-binding activity in wild-type cells closely parallel those for nuclear translocation of the dioxin receptor complex and for induction of cytochrome P-450c transcription (1, 24). Thus, it is possible to correlate the DNA-binding activity of the XRE-specific factor with that of the dioxin receptor. Somatic cell hybridization experiments have shown that the defect in the c4 mutant cell line results from a mutation at a single complementation group, which affects the normal functioning of the dioxin receptor (25). This mutation may result in a defect in the dioxin receptor or in another factor that mediates binding of the dioxin receptor to specific DNA regulatory elements and possibly its nuclear translocation. Therefore, from our present results we cannot exclude the possibility that the XRE-specific binding of the dioxin receptor is mediated via such a putative factor. We are currently attempting to purify the factor(s) involved in the XRE-specific protein–DNA complexes.

Finally, the XRE-specific factor cosediments and comigrates in chromatography with dioxin receptor. From the sedimentation coefficient and Stokes radius, it is possible to calculate a relative molecular mass of the nuclear Hepa 1c1c7 dioxin receptor of 192–216 kDa. This is slightly larger than the value of 176 kDa reported by others for the nuclear dioxin receptor in murine hepatoma cells (18). The cytosolic form of the rat and mouse dioxin receptor is a 95- to 100-kDa protein, as determined both in solution (4) and under denaturing conditions by photoaffinity labeling (7). The larger molecular mass of the nuclear form of the dioxin receptor is consistent with a homodimeric configuration of the protein. It also could reflect a complex between the dioxin receptor and another nuclear factor(s), as discussed above. In the case of steroid hormone receptors, it seems plausible that they interact with DNA as dimers, given the background of the apparent dyad symmetry of several hormone-responsive elements (reviewed in ref. 26, Sophia Y. Tsai, Nancy L. Weigel, Karin Dahlman, Jan Carlstedt-Duke, Ming-Jer Tsai, J.-Å. G., and Bert W. O'Malley, unpublished data). However, this dimerization is believed to require the presence of DNA, as opposed to the stable 192- to 216-kDa complex of the dioxin receptor.

In spite of the suggested sequence similarity between GREs and XREs (10), we could not detect any affinity of an expressed, DNA-binding glucocorticoid receptor fragment for the XRE motifs. The crude biochemical and functional similarities between the dioxin receptor and steroid hormone receptors (see Introduction) are intriguing. The determination of the structure of the dioxin receptor by either purification or cloning will be necessary to determine its degree of relatedness to the gene family of nuclear receptors including not only steroid receptors but also thyroid hormone and retinoic acid receptors (26). However, the identification of a target sequence for the ligand-activated dioxin receptor should permit a detailed analysis of functional properties of the protein as well as an investigation of the mechanism by

which the receptor is converted from a non-DNA-binding to a DNA-binding gene regulatory species.

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