Identification of a Motif within the 5′ Regulatory Region of pS2 Which Is Responsible for AP-1 Binding and TCDD-Mediated Suppression†

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ABSTRACT: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds modulate several endocrine systems by altering hormone synthesis, enhancing ligand metabolism, and down-regulating receptor levels/binding activity. Previous studies have demonstrated that TCDD inhibits the 17β-estradiol (E2)-induction of pS2, a human breast cancer prognostic marker. This inhibition occurs at the gene expression level and is Ah receptor (AhR)-mediated. Analysis of the 5′ regulatory region has identified three motifs which resemble dioxin response element (DRE) core sequences. pS2-regulated luciferase deletion constructs identified the DRE-like motif located at −527 to −514 as being required for TCDD-mediated suppression. A point mutation within this core motif (T-518C) abolished the inhibition by TCDD while UV-induced protein−DNA cross-linking and competitive gel retardation assays demonstrated AhR complex binding to this motif. Further study of this region also revealed an adjacent putative AP-1 site, diverging by one base pair from the consensus sequence. Gel retardation assays using TPA-treated MCF-7 cell nuclear extracts showed an induced complex binding to the AP-1-like site. Competition studies and antibody supershifts confirmed that the retarded complex consists of AP-1-like proteins. pS2-regulated luciferase constructs containing mutations specific to the AP-1-like motif greatly diminished the inducibility in response to E2. These results suggest that an interaction between AhR complexes and AP-1-like proteins may be responsible for TCDD-mediated inhibition of E2-induced pS2 expression.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[1] and related halogenated aromatic hydrocarbons elicit a diverse spectrum of sex-, strain-, age-, and species-specific responses. These effects include a wasting syndrome, tumor promotion, immunotoxicity, hepatotoxicity, teratogenesis, enzyme induction, and the modulation of endocrine systems (Goldstein & Safe, 1989; Safe, 1993). The biological and toxic responses elicited by TCDD are believed to be mediated by the aryl hydrocarbon (Ah) receptor (AhR), a ligand-dependent transcription factor which is a member of the basic helix−loop−helix-PAS (bHLH-PAS) family (Burbach et al., 1992; Ema et al., 1992). Studies investigating the induction of the gene encoding cytochrome P4501A1, *CYP*1A1, suggest that the mechanism of action of TCDD is similar to that proposed for steroid hormones (Swanson & Bradfield, 1993; Whitlock, 1990). Ligand binding to the AhR releases heat shock protein 90 (Hsp90), enabling the receptor to heterodimerize with the AhR nuclear translocator protein (ARNT) (Perdew, 1988; Reyes et al., 1992; Whitelaw et al., 1993). The resulting complex exhibits high affinity for DNA and binds to specific motifs located within the 5′ regulatory region of target genes. AhR complex binding causes changes in chromatin structure, increasing the accessibility of the promoter to transcriptional machinery required for gene transcription (Durrin & Whitlock, 1989; Morgan & Whitlock, 1992; Robertson et al., 1994; Wu & Whitlock, 1992). Consequently, alterations in target gene transcription have been proposed as the mechanism responsible for the effects elicited by TCDD and related compounds (Whitlock, 1990). Such alterations in gene expression have also been proposed as a mechanism in carcinogenic promotion (Pitot, 1995).

An examination of TCDD-inducible genes including *CYP*1A1, *CYP*1A2, glutathione *S*-transferase, and quinone reductase has revealed that AhR complexes bind to specific response elements referred to as dioxin responsive elements (DREs) or xenobiotic responsive elements (XREs) (Denison et al., 1989; Favreau & Pickett, 1991; Fujisawa-Sehara et al., 1988; Hapgood et al., 1989; Nebert & Jones, 1989; Quattrochi et al., 1994; Rushmore et al., 1990). Results from studies examining protein−DNA interactions have identified an invariant core sequence, CGTG, which is essential for both AhR complex binding and AhR-mediated transcriptional activation (Neuhold et al., 1989; Saatcioglu et al., 1990; Shen & Whitlock, 1989; Watson & Hankinson, 1992; Wu & Whitlock, 1993). Nucleotides adjacent to the core have also been shown to dramatically affect AhR-complex binding

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[1] Abbreviations: AhR, aryl hydrocarbon (Ah) receptor; ARNT, Ah receptor nuclear translocator; BrdU, 5′-bromodeoxyuridine; DMSO, dimethyl sulfoxide; DRE, dioxin response element; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; Luc, luciferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element; TRE, TPA response element; TPA, phorbol 13-myristylacetate.

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(Bank et al., 1992; Yao & Denison, 1992); however, no relationship between the strength of DNA binding and AhR-mediated inducibility has yet been observed (Lusska et al., 1993; Shen & Whitlock, 1992). These results indicate that binding is not sufficient to cause induction of a target gene. It has also been suggested that AhR complex binding to a nonfunctional response element could cause the disruption of the gene transcriptional machinery, resulting in transcriptional repression (Lusska et al., 1993).

In addition to the binding of transcription factors to nonfunctional response elements, transcriptional regulation may also be mediated by protein−protein interactions, a mechanism that has been extensively studied with the nuclear receptor superfamily (Stein & Yang, 1995; Yang-Yen et al., 1990). Interactions involving the glucocorticoid receptor and the general transcription factor, AP-1, demonstrate that these interactions can occur between members of different transcription factor families (Diamond et al., 1990; Jonat et al., 1990). The similarity in the mechanisms of action between the AhR and the nuclear receptor superfamily suggests that protein−protein interactions could also occur with bHLH-PAS proteins, resulting in the modulation of gene expression. In fact, the AhR has been shown to be able to modulate cathepsin D gene transcription by interfering with the DNA binding ability of an estrogen receptor (ER)/Sp1 complex (Krishnan et al., 1995). Other studies detailing AhR-mediated modulations of gene transcription have also been reported; however, the exact mechanism for these interactions has not been fully elucidated (Masten & Shiverick, 1995; Zacharewski et al., 1994).

We have recently shown that TCDD inhibits the E2 induction of pS2 expression in MCF-7 human breast cancer cells (Zacharewski et al., 1994). The mature, processed pS2 protein is a 6.5 kDa polypeptide expressed in ER-positive breast tumors, but has also been detected in normal gastric epithelium (Luqmani et al., 1989; Rio et al., 1988). The pS2 protein contains the "trefoil" disulfide loop structure which is common to a new family of growth factor-like peptides that includes pancreatic spasmolytic polypeptide (PSP) and spasmolysin (Sands & Podolsky, 1996; Thim, 1989). Although there is no known physiological function for pS2, detection in breast tumors has been reported to identify patients who are more likely to respond to antihormonal therapy (Foekens et al., 1990; Koerner et al., 1992; Rio & Chambon, 1990; Schwartz et al., 1991). Therefore, pS2 is considered a positive prognostic marker for hormone-dependent breast cancer since its presence is indicative of functional ERs.

The 5′ regulatory region of pS2 contains an imperfect estrogen response element which is responsible for its induction by E2. Since pS2 gene regulation is a primary transcriptional event mediated by E2, pS2 provides an excellent model to investigate interactions between the AhR and the ER. Previous studies examining the TCDD-mediated suppression of E2-induced pS2 expression have demonstrated that suppression occurs at the transcriptional level and requires functional AhR complexes (Zacharewski et al., 1994). In this report, we describe the identification of a motif within the 5′ regulatory region of pS2 which is required, but not sufficient, for TCDD-mediated suppression. This motif is also capable of binding a TPA-inducible complex and appears to play a critical role in E2-induced pS2 gene expression.

MATERIALS AND METHODS

Materials. ICI 164,384 was a gift from Dr. A. Wakeling (ICI Pharmaceuticals, England). Phenol red-free Dulbecco's minimal essential medium powder and media supplements were purchased from Gibco/BRL (Burlington, Ontario, Canada). Fetal bovine serum, protease inhibitors, and [γ-^{32}P]-dATP were purchased from ICN (Mississauga, Ontario, Canada). D-Luciferin was purchased from Molecular Probes (Eugene, OR). The AhR-specific antibody was kindly provided by Dr. Larry Stanker (Texas A&M University, College Station, TX). The *jun* antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest quality available from commercial sources.

Cell Culture, Transfections, and Reporter Gene Assays. MCF-7 (obtained from Dr. L. Murphy, University of Manitoba, Winnipeg, Manitoba) and Hepa 1c1c7 wild type (obtained from Dr. O. Hankinson, UCLA) cells were maintained in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, sodium bicarbonate, glucose, HEPES, nonessential amino acids, vitamin supplement solution, sodium pyruvate, lipoic acid, vitamin B12, zinc sulfate, and glutamine. The medium was also augmented with gentamycin, penicillin/streptomycin, and amphotercin B. Cells were grown at 37 °C in a 4% CO_2 humidified environment. Transient transfections and luciferase reporter gene assays were performed as previously described (Zacharewski et al., 1994). Briefly, MCF-7 cells were plated on 60 mm dishes in media supplemented with 5% dextran-coated charcoal-treated fetal bovine serum. After attachment and growth for 6 h, the cells were transfected with 4 μg of pCH110 (β-galactosidase expression vector, Pharmacia), 7.5 μg of pS2-regulated luciferase reporter vector, 1 μg of HEGO [ER expression vector, kindly provided by Dr. P. Chambon (Tora et al., 1989)] or Gal4-HEGO [GAL4-HEGO chimeria, kindly provided by Dr. P. Chambon (Berry et al., 1990)], and 2.5 μg of pBS (carrier DNA, Stratagene) per dish using the calcium phosphate coprecipitation method (Sambrook et al., 1989). Twenty hours following transfection, the plates were washed, the medium was replaced, and the cells were treated as indicated. The cells were harvested 24 h following treatment, and the luciferase assay was performed as described in Brasier et al. (1989). The reference plasmid, pCH110, was cotransfected as an internal control in order to correct for variations in transfection efficiency and was measured using standard protocols (Sambrook et al., 1989). Statistical significance was determined by performing the Student's T test.

Constructs. The −1100/−87 and −405/−393 pS2Luc reporter genes have been previously described (Zacharewski et al., 1994). The −429/−87pS2Luc reporter gene was prepared by excising the *Sac*I fragment from −1100/−87pS2Luc and religating the cut vector. To generate the other deletions, −1100/−87pS2Luc was digested with *Kpn*I/*Hind*III, and the fragment containing the pS2 regulatory region was ligated into pTL2 and henceforth is referred to as −1100/−87pTL2. This construct was partially digested with *Bst*XI and *Mlu*I, and then blunt-ended and ligated. The *Kpn*I/*Hind*III fragment was excised and inserted into the pGL2 vector to create −537/−87pS2Luc. The −1100/−391pS2Luc reporter gene was constructed by partially digesting −1100/−87pTL2 with *Dra*III and *Hind*III. The

resulting cut vector was blunt-ended and ligated, and the *Kpn*I/*Bam*HI fragment was inserted into pGL2 digested with *Kpn*I/*Bgl*II. The −540/−323pS2Luc construct was prepared by ligating the −540/−86pS2Luc *Kpn*I/*Hind*III fragment into pTL2. This construct was digested with *Pst*I and *Hind*III; the cut ends were filled in and ligated. The *Kpn*I/*Bam*HI fragment was then excised and inserted into pGL2 digested with *Kpn*I/*Bgl*II. The motif I-Luc reporter gene was prepared from oligonucleotides complementary to the pS2 regulatory region from positions −523 to −514. The oligonucleotides contained two copies of the motif I sequence and when annealed generated compatible *Kpn*I/MluI ends that could be inserted into pGL2. The motif I/ERE-Luc reporter gene was prepared using oligonucleotides which contained two copies of the vitellogenin A2 ERE. Complementary oligonucleotides were annealed to generate *Nhe*I and *Bgl*II compatible ends and ligated into the motif I-Luc reporter gene. PCR was used to prepare mutpS2Luc by introducing a C to T point mutation which was incorporated into the forward primer at position −518. The −535/−87pS2(17m)-Luc [also referred to as wtpS2(17m)Luc] construct containing the 17-mer was prepared by oligo-mediated site-directed mutagenesis. Single-stranded DNA of the −537/−87pS2Luc construct was annealed to an oligonucleotide containing the 17-mer in place of ER domain C. The primer was then extended with T4 DNA polymerase and closed with T4 DNA ligase. The −535/−87pS2(17m)Luc construct is identical to −535/−87pS2 except that the pS2 ERE has been replaced by the GAL4 recognition sequence 17-mer. (mutAP-1)pS2-(17m)Luc was prepared from −535/−87pS2(17m)Luc by PCR using a forward primer which contained CA to TG point mutations at positions −513 to −512. The pS2 regulatory region was amplified between positions −535 and −87 using a forward primer which contained either a point mutation or the wild-type sequence with a *Kpn*I add-on and a reverse primer with a *Bam*HI add-on. All constructions were verified by restriction enzyme digestions and DNA sequencing.

Cytosol. Hepa 1c1c7 cells were brought to 90% confluency in 100 mm^2 tissue culture dishes (Sarstedt), washed twice with phosphate-buffered saline (PBS), and detached from the flasks using trypsin (0.5%). Cells were pelleted, resuspended in HEGD buffer (25 mM HEPES, 3 mM EDTA, 1 mM dithiothreitol, and 10% glycerol), homogenized (Ten Broeck), and ultracentrifuged (Beckman TL-100) at 104000*g* for 1 h at 4 °C. Guinea pig cytosol was obtained from Dr. M. S. Denison (University of California at Davis) and was prepared as described (Helferich, 1991).

Nuclear Extracts. AP-1 nuclear extracts were prepared according to the method described by Dignam et al. (1983). Briefly, MCF-7 cells were treated with 125 nM TPA or DMSO 90 min prior to harvesting. Cells were washed twice with PBS and harvested. The cells were incubated in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT] and homogenized with a Dounce homogenizer. The homogenate was initially centrifuged at 400*g* for 10 min, and then at 25000*g* for 20 min. The crude nuclear pellet was then resuspended in buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT] and homogenized with a Dounce homogenizer. The homogenate was then centrifuged at 25000*g* and dialyzed for 5 h in buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5mM PMSF, and 0.5 mM DTT]. The dialysate was centrifuged at 25000*g* and frozen in liquid nitrogen until use.

Gel Retardation Assay. The following complementary oligonucleotides were synthesized and used in band shift assays: (i) −998 to −973 of the *CYP*1A1 regulatory region (site D; 5′-gatctctTCTCACGCaActccgag-3′ and 5′-gatcctcg-gagTtGCGTGAGAaga-3′, henceforth termed wtDRE) (Lusska et al., 1993); (ii) −530 to −508 of the pS2 regulatory region (5′-gggatta**c**aGCGTGAG**c**cactgc-3′ and 5′-gcagtg**g**CT-CACGCt**g**taatccc-3′, henceforth termed motif I) (Berry et al., 1989); (iii) −530 to −508 of the pS2 regulatory region with a T to C point mutation at position −518 which is in the motif I sequence (henceforth termed mut-motif I); (iv) the consensus AP-1 sequence (5′-cgcttgaTGACTCAgccggaa-3′); and (v) the mutated AP-1 sequence (5′-cgcttgaTGACT-**tg**gccggaa-3′) (Lee et al., 1987). Nucleotides which vary from the consensus DRE sequence are underlined and in boldface type. Individual oligonucleotides (100 nM) were end-labeled with [γ-^{32}P]dATP (83 μCi) in the presence of T4 polynucleotide kinase (9 units) for 1 h at 37 °C. Additional aliquots of kinase (4.5 units) were added at 20 min intervals during the incubation. Complementary oligonucleotides were then annealed in 100 mM NaCl, loaded onto a 15.5% nondenaturing polyacrylamide gel, and electrophoresed to separate free [γ-^{32}P]dATP from labeled oligonucleotides. Annealed complementary oligonucleotides were visualized by exposure to Kodak X-OMAT AR film, and the resulting band was excised and extracted in TE8 (10 mM Tris, 1 mM EDTA, pH 8).

The assay was performed as previously described (Denison et al., 1988). Cytosol from Guinea pig liver (16 mg of protein/mL) and extracts from mouse Hepa 1c1c7 wild-type cells (5 mg of protein/mL) were transformed by incubation at 24 °C for 2 h with 20 nM TCDD. Transformed cytosol (50−80 μg) or nuclear extract (5−20 μg) was incubated for 15 min with 375 ng of poly(dIdC) (Boehringer Mannheim, Montreal, Canada) in HEGD buffer containing 64 mM KCl (mouse) or 96 mM KCl (Guinea pig or nuclear extract). For competition assays, unlabeled annealed oligonucleotides were added during the initial incubation. Labeled annealed oligonucleotide (100 000 cpm, 0.5 ng) was then added and incubated for an additional 15 min. Samples were loaded onto a preelectrophoresed 5% nondenaturing polyacrylamide gel run using 1× TBE running buffer (90 mM Tris, 120 mM boric acid, and 4 mM EDTA, pH 8). The gel was then dried, and specific DNA−protein interactions were localized following exposure to Kodak X-OMAT AR film. Quantitation of band intensity was performed using a laser densitometer (LKB 2222-020 UltroScan XL).

UV Cross-Linking Studies. Nuclear extracts were prepared from MCF-7 cells (obtained from ATCC, Rockville, MD) treated with 10 nM E2, 10 nM TCDD, or 10 nM E2 plus 10 nM TCDD for 1 h at 37 °C. After incubation, the cells were harvested by trypsinization and transferred to a 50 mL conical tube and centrifuged at 1000*g* for 10 min at 4 °C. Cell pellets were washed twice with 40 mL of HEGD buffer. The resulting pellet was resuspended by gentle vortexing with 3 mL of HED buffer (same as HEGD with no glycerol) followed by centrifugation at 1000*g* for 10 min at 4 °C. The pellet was then resuspended in 750 μL of HEGD and homogenized with 20−30 passes of a Teflon pestle/drill apparatus in a 3 mL Wheaton homogenizing tube. Nuclei were found to be intact as determined by microscopic

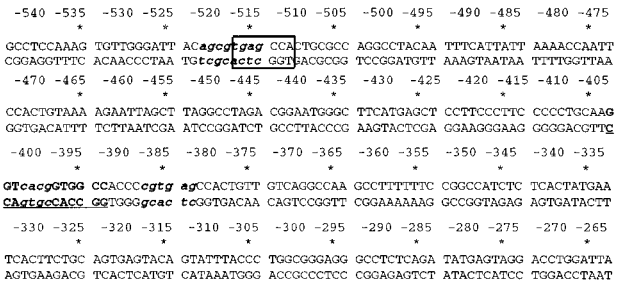

FIGURE 1: Location of DRE core motifs within the pS2 promoter. The DNA sequence of the pS2 promoter from −545 to −265. The boldface, underlined sequence between positions −405 and −393 indicates the pS2 ERE. DRE-like motifs are in boldface, lower case italics with the cores located at positions −521 to −517 (I), −402 to −399 (II), and −388 to −385 (III). The putative AP-1-like motif is boxed and is located at positions −518 to −512.

examination after dye exclusion staining with typan-blue. The homogenate was transferred to a centrifuge tube by addition of 2 mL of HEGD buffer and centrifuged at 1000*g* for 10 min at 4 °C. The pellet was then resuspended by dropwise addition of 150 μL of HEGD buffer containing 0.5 M KCl (pH 7.6) and incubated at 4 °C for 1 h. The suspension was transferred to a 5 mL polycarbonate ultracentrifuge tube and centrifuged at 105000*g* for 30 min. The resulting supernatant was used as the nuclear extract.

The oligonucleotide, 5′-GTGTTGGGATTACAGCGTGAGCCACTGCGCCAGGCCTAC-3′ (10 pmol) was annealed with the complementary 6 bp primer 5′-GTAGGC-3′ (10 pmol) and end-filled with the Klenow fragment of DNA polymerase in the presence of 0.1 μM each of dGTP, dATP, and 5′-bromodeoxyuridine (BrdU) and 1 μM [α-^{32}P]-dCTP as described (Sambrook et al., 1989). Nuclear extracts (10 μg) were incubated with 400 ng of poly(dIdC) in HEGD buffer for 15 min incubation at 20 °C followed by the addition of BrdU-substituted ^{32}P-labeled DRE for 15 min at 20 °C. For immunodepletion studies, the nuclear extracts were preincubated with 200 ng of either AhR antibody or IgG control for 1 h at 25 °C prior to incubation with the BrdU-substituted ^{32}P-labeled DRE. The gel mobility shift assay products were UV-irradiated on a UV transilluminator (>205 nm for 20 min at 20 °C). Samples were then mixed with 20 μL of SDS-loading buffer, heated to 95 °C for 5 min, and subjected to electrophoresis on a 10% SDS−polyacrylamide gel. TCDD-inducible protein−DNA complexes were visualized by autoradiography following exposure of the dried gel to Kodak X-OMAT AR film. The molecular weight of the complex was determined by comparison to ^{14}C-methylated protein standards obtained from Amersham Corp. (Arlington Heights, IL).

RESULTS

Deletion Analysis of the 5′ Regulatory Region of the pS2 Gene. Previous studies have demonstrated that TCDD-mediated suppression of E2-induced pS2Luc activity requires cis-acting elements within the 5′ regulatory region of pS2 (Zacharewski et al., 1994). Sequence analysis of the regulatory region from −700 to −1 identified three DRE core motifs (CGTG) at positions −520 to −517, −402 to −399, and −388 to −385 (Figure 1). Deletion constructs were prepared to investigate the role of each DRE-like motif while maintaining the E2-inducibility of the reporter gene (Figure 2A,B). Constructs were transiently transfected into

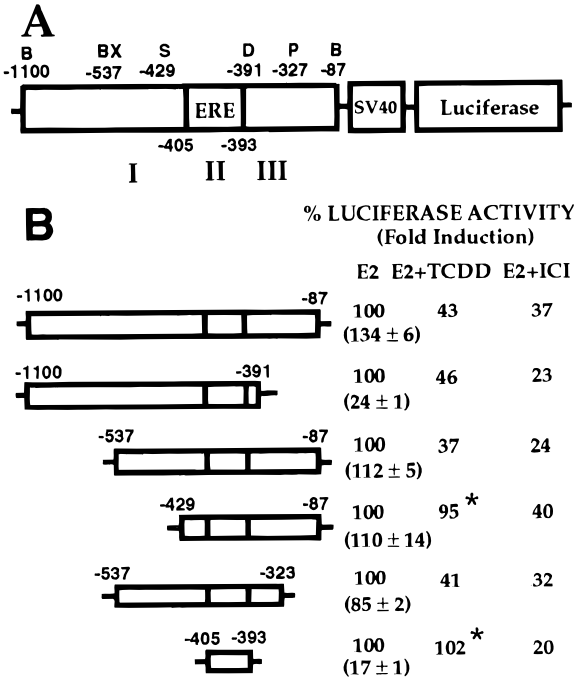

FIGURE 2: Deletion analysis of the pS2 promoter. Fragments of the pS2 regulatory region were subcloned upstream of a SV40 promoter linked to the firefly luciferase cDNA within the pGL2 vector. (A) Schematic diagram of the pS2 promoter indicating the relative positions of the pS2 ERE and DRE-like cores I, II and III and the location of restriction enzyme sites used for the constructions (B = *Bam*HI, BX = *Bst*XI, S = *Sac*I, D = *Dra*III, and P = *Pst*I). (B) Schematic diagram of the pS2 promoter deletion constructs. Constructs were transiently transfected into MCF-7 cells as described under Materials and Methods and treated with 1 nM E2, 1 nM E2 plus 10 nM TCDD, or 1 nM E2 plus 10 nM ICI 164,384. The fold induction level refers to the ratio of luciferase activity of treated cells compared to cells treated with 100 nM ICI 164,384 (Bondy & Zacharewski, 1993). The values are averages of four measurements (two samples taken from each plate, each treatment performed on duplicate plates) and are reported as a percentage relative to the maximum induction observed with 1 nM E2. Transfection efficiency between treatments was normalized using a constitutively expressed β-galactosidase expression vector. Variation in β-galactosidase activity was less than 10% within assays and less than 15% between experiments. The asterisk indicates values that are not significantly different from E2-treated cells ($p < 0.05$). Experiments were performed a minimum of 3 times.

MCF-7 cells and treated with either 1 nM E2, 1 nM E2 plus 10 nM TCDD, or 1 nM E2 plus 10 nM ICI 164,384. The results are reported as fold induction levels relative to cells treated with a 100 nM aliquot of the pure anti-estrogen ICI 164,384 (Bondy & Zacharewski, 1993).

All constructs exhibited increased levels of luciferase activity following treatment with 1 nM E2 (Figure 2B). However, −1100/−391pS2Luc and −405/−393pS2Luc exhibited significantly lower fold induction levels compared to the −1100/−87pS2Luc construct (24- and 17- fold vs 134-fold) due to the deletion of elements between positions −428 and −324 which contribute to both constitutive and E2-induced gene expression (Berry et al., 1989). The induction of deletion constructs (−429/−87pS2Luc and −405/−393pS2Luc) which lack DRE-like motif I was not affected following cotreatment of cells with E2 plus TCDD, whereas luciferase activity was suppressed by the "pure" anti-estrogen ICI 164,384. In contrast, TCDD inhibited E2-induced pS2Luc activity by approximately 60% in all constructs possessing motif I (−1100/−87pS2Luc, −1100/−391pS2Luc,

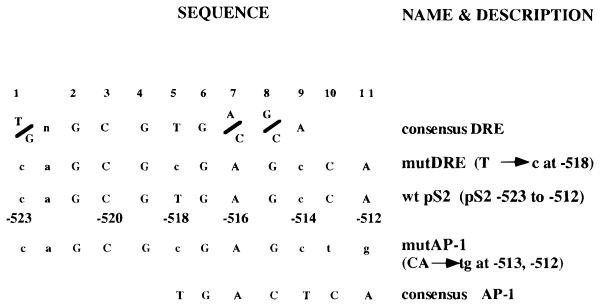

FIGURE 3: Consensus and mutated response elements. The consensus dioxin response element (DRE), DRE-like motif I with DRE core mutations, the DRE-like motif I sequence located within the pS2 regulatory region from position −523 to position −514, the DRE-like motif I with AP-1 mutations, and the consensus AP-1 sequence. Capital letters indicate bases which are in agreement with the consensus DRE sequence while bases in lower case (positions 1 and 9) indicate differences. These oligonucleotides were used in gel retardation assays and in the construction of the −518pS2Luc and mutAP-1pS2(17m)Luc reporter genes.

−537/−87pS2Luc, and −537/−323pS2Luc). This level of suppression was comparable to results obtained following treatment with E2 plus 10 nM ICI 164,384. The pS2Luc activity of the deletion constructs remained unchanged following treatment of 10 nM TCDD alone; however, 10 nM ICI 164,384 was capable of inhibiting reporter gene induction attributable to serum-borne estrogens (data not shown) (Bondy & Zacharewski, 1993).

Examination of Motif I. In order to examine the role of DRE-like motif I in TCDD-mediated suppression of E2-induced pS2Luc activity, a T to C point mutation was introduced at position −518 of the pS2 regulatory region (Figure 3). Previous studies have shown that oligonucleotides containing mutations in the DRE core eradicate enhancer function and do not form TCDD-inducible protein−DNA complexes (Bank et al., 1992; Shen & Whitlock, 1992; Yao & Denison, 1992). E2 induction of the −535/−87pS2Luc (wild type) and −518pS2Luc (point mutation) constructs were 139- and 122-fold, respectively (Figure 4). Cotreatment of −535/−87pS2Luc with E2 plus TCDD or E2 plus ICI 164,384 resulted in a 68% and 84% reduction in luciferase activity, respectively. In contrast, the −518pS2Luc reporter gene was not affected by cotreatment with TCDD while ICI 164,384 inhibited luciferase induction by 80%. TCDD alone did not affect reporter gene activity whereas induction by serum-borne estrogens was inhibited by 10 nM ICI 164,384 (data not shown).

The inhibitory action of motif I was further analyzed in isolation and in tandem with a consensus ERE. Two copies of motif I (position −523 to −514) were inserted upstream of a luciferase reporter gene vector and transiently transfected into MCF-7 and Hepa 1c1c7 wild-type cells. Treatment with 1−100 nM TCDD did not cause induction of luciferase activity in either cell line (data not shown). Experiments with a similar reporter gene construct containing two copies of wtDRE (*CYP*1A1 site D) exhibited 3-fold induction following treatment with 10 nM TCDD (data not shown). Similarly, TCDD did not induce a tandem DRE-like motif I/ERE-regulated luciferase reporter gene (data not shown), while 1 nM E2 increased luciferase activity 22-fold. In cotreatment experiments, TCDD did not suppress DRE-like motif I/ERE-luciferase activity while ICI 164,384 inhibited E2-induced luciferase activity by 60% (Figure 4).

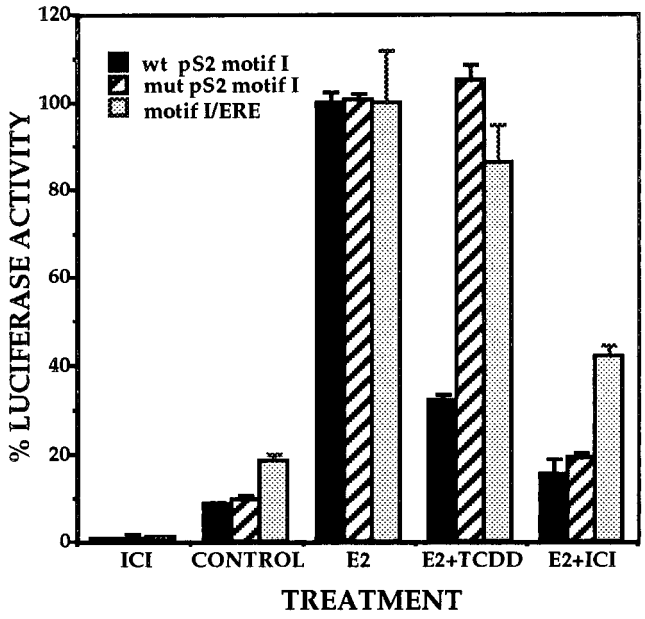

FIGURE 4: Analysis of the DRE-like motif I. Constructs were transiently transfected into MCF-7 cells as described under Materials and Methods. The wild-type motif I construct refers to the wild-type motif I linked to the firefly luciferase gene. The mut motif I construct refers to a construct that contains the p52 5′ regulatory sequence with a mutation introduced into position −518 of the DRE-like core linked to the firefly luciferase gene. The motif I/ERE construct refers to a construct which contains motif I linked to two copies of the vitellogenin A2 ERE and the firefly luciferase gene. Cells were treated with 1 nM E2, control (DMSO), 1 nM E2 plus 10 nM TCDD, or 1 nM E2 plus 10 nM ICI 164,384. The values are averages of four measurements (two samples taken from each plate, each treatment performed on duplicate plates) and are reported as a percentage relative to the maximum induction observed with 1 nM E2. Transfection efficiency between treatments was normalized using a constitutively expressed β-galactosidase expression vector. Variation in β-galactosidase activity was less than 10% within assays and less than 15% between experiments. Brackets indicate the standard error of the mean. Experiments were performed a minimum of 3 times.

TCDD-Inducible Protein−DNA Complexes. The sequence similarity of motif I (position −523 to −514) to the consensus DRE and the conservation of the DRE core suggest that TCDD-mediated suppression of E2-induced pS2Luc activity may involve binding of AhR complexes to motif I (Figure 3). Gel retardation assays were performed using nuclear extracts and transformed cytosol to determine if AhR complexes could directly bind to an oligonucleotide containing motif I (positions −530 to −508). Several assay conditions were used including using larger double-stranded oligonucleotides (positions −535 to −498), varying salt and poly(dIdC) concentrations, and adding zwitterionic detergents (Hassanain et al., 1993). Although the motif differs from the consensus sequence at only two positions outside the required core sequence, no TCDD-inducible complex was observed in direct binding gel retardation assays (data not shown). However, TCDD-inducible protein−DNA complexes were observed in UV cross-linking studies using BrdU-substituted, ^{32}P-labeled, double-stranded probes and nuclear extracts (Figure 5). The complex was effectively competed with a 200-fold excess of unlabeled wtDRE (site D) (Figure 5A), and was immunodepleted with an AhR-specific antibody but not with the IgG control antibody (Figure 5B). Close examination of Figure 5B indicates that

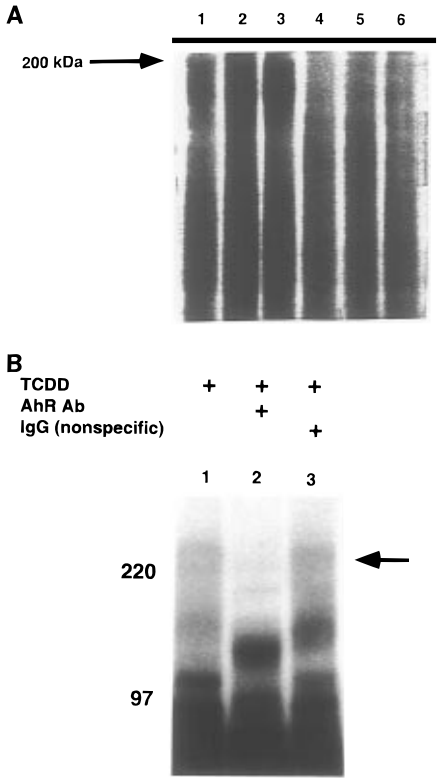

FIGURE 5: UV-induced cross-linking of AhR complexes to the DRE-like motif I. Nuclear extracts were prepared from MCF-7 cells following treatment with 10 nM E2 (lanes 1 and 4), 10 nM E2 plus 10 nM TCDD (lanes 2 and 5), or TCDD alone (lanes 3 and 6) as described under Materials and Methods. Extracts (10 μg) were incubated with [^{32}P]BrdU-motif I and subjected to UV irradiation and SDS−PAGE analysis. The arrows indicate the position of the complexes. Lanes 4−6 in panel A correspond to samples incubated with a 200-fold excess of wtDRE (site D) oligonucleotide, 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′, prior to incubation with the ^{32}P-labeled BrdU-motif I. Panel B illustrates the effect of an AhR-specific antibody (lane 2) amd a control IgG antibody (lane 3) on complex formation.

the induced complex actually consists of two bands, one band at approximately 220 kDa and a second band at a slightly higher molecular mass.

In addition, oligonucleotides containing motif I competed with the ^{32}P-labeled wtDRE double-stranded probe (Lusska et al., 1993) in competitive gel retardation assays (Figure 6). The band intensity of the induced complex decreased by approximately 40% following the addition of a 150-fold excess of unlabeled motif I competitor (Figure 6, lanes 8 and 20), while the band intensity decreased by approximately 90% at a 1.5-fold excess of unlabeled competitor containing the wtDRE sequence (Figure 6, lanes 3 and 15). Concentrations of motif I oligonucleotides greater than 150-fold caused significant decreases in nonspecific band intensities and therefore were not used (data not shown). Competitive gel retardation assays were also performed using oligonucleotides containing a mutation in the DRE-like region of motif I (Figure 3). This mutated motif I corresponds to sequence changes previously shown to abolish the ability of TCDD to suppress E2-induced pS2Luc activity in transient transfection assays (Figure 4) and was found not to significantly affect AhR complex−wtDRE interactions (Figure 6, lanes 11 and 12, 23 and 24). Comparable results were observed for TCDD-transformed Guinea pig liver cytosol and Hepa 1c1c7 cell extracts.

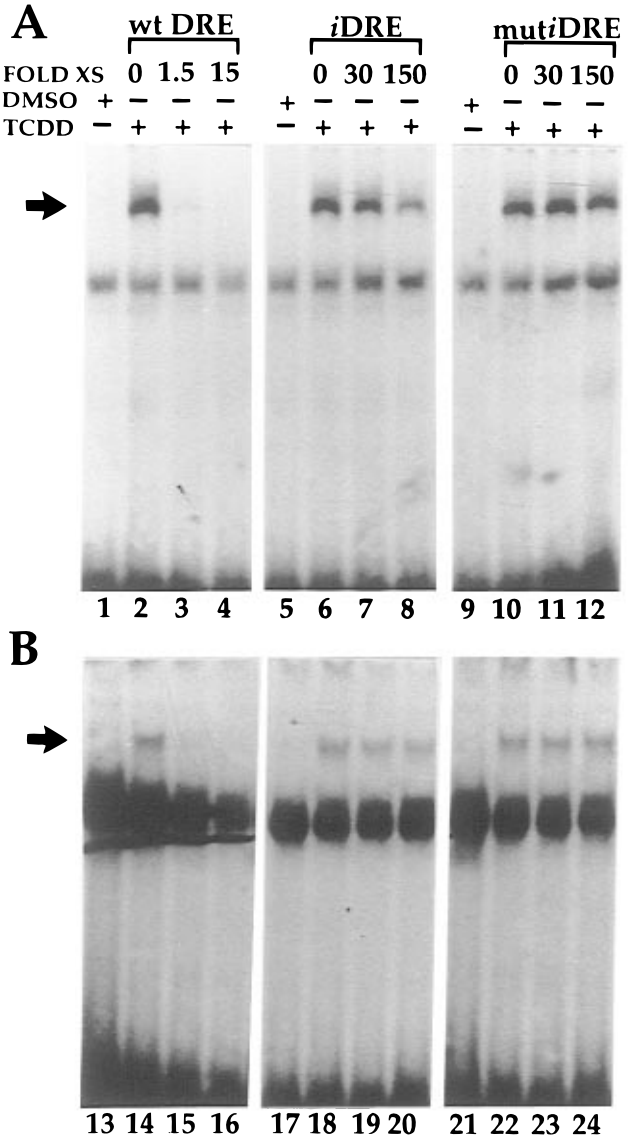

FIGURE 6: Competitive gel retardation analysis. A synthetic double-stranded oligonucleotide containing sequences complementary to the wtDRE was labeled with [γ-^{32}P]dATP and incubated with *in vitro* transformed (A) hepatic Guinea pig cytosol and (B) extracts from Hepa 1c1c7 wild type cells. Unlabeled oligonucleotides complementary to the wtDRE, motif I from positions −535 to −498, and motif I with a T to C point mutation at position −518 were used as the unlabeled competitor. The protein−DNA complexes were analyzed by gel electrophoresis, and bands were visualized by autoradiography. The arrow indicates the position of the TCDD-inducible protein−DNA complex. Competitor DNA is given in fold molar excess relative to the concentration of labeled DNA (2.5 ng).

TPA-Inducible Protein−DNA Complexes. The AP-1-like motif located between −518 and −512 of pS2 diverges from the consensus sequence by only one nucleotide, suggesting that AP-1-like factors may bind this motif resulting in synergistic interactions with the pS2 receptorsome. Gel retardation assays were performed using nuclear extracts prepared from TPA-treated MCF-7 cells to determine if AP-1-like factors were capable of binding to this motif (Figure 7). Retarded complexes were observed when the ^{32}P-labeled pS2 oligonucleotide was incubated with TPA-induced nuclear extracts (Figure 7, lanes 1 and 2) which decreased approximately 74% following the addition of excess, unlabeled pS2 motif (Figure 7, lane 3). Incubation with the mutpS2DRE

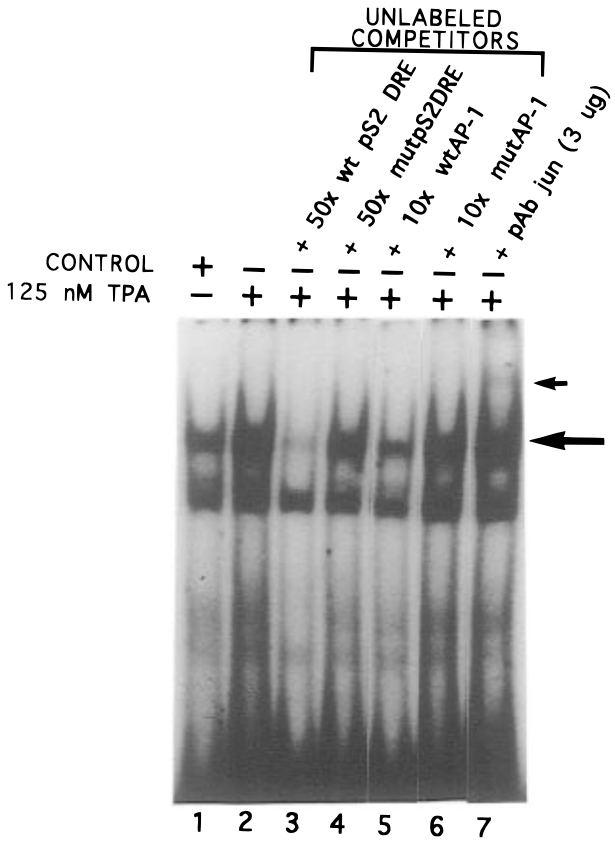

FIGURE 7: Competitive gel retardation analysis of the TPA-induced complex. A synthetic double-stranded oligonucleotide containing sequences complementary to the pS2 motif I sequence was end-labeled with [γ-^{32}P]dATP and incubated with TPA-induced nuclear extracts. The induced complex is identified by the large arrow. Lanes 1 and 2 illustrate complex formation using DMSO- and TPA-induced extracts, respectively. Excess unlabeled competitor DNA sequences [lane 3, pS2 motif I sequence; lane 4, mutated pS2 motif I sequence; lane 5, wild type AP-1 (TRE) sequence; lane 6, mutated AP-1 (TRE) sequence] were used to investigate the specificty of the resulting protein-DNA complexes. The formation of a *jun*-specific polyclonal antibody supershift is indicated by the small arrow (lane 7). Fold molar excess is calculated based on a labeled probe concentration of 2.5 ng.

oligonucleotide showed very slight competition (15% decrease vs lane 2) for the shifted complex compared to the wild-type pS2 sequence (Figure 7, lane 4). Unlabeled oligonucleotides containing the consensus AP-1 motif reduced complex intensity (approximately 46% decrease vs. lane 2) although it did not completely abolish complex formation (Figure 7, lane 5). Competition assays using the unlabeled probe containing a mutated AP-1 sequence (Lee et al., 1987) did not significantly compete (3% decrease vs lane 2) with the radiolabeled oligonucleotide (Figure 7, lane 6). Incubation of the reaction mixture with a commercial polyclonal antibody raised against *jun* family members was found to partially supershift the TPA-induced protein−DNA complex (Figure 7, lane 7). Incubation of this antibody with control nuclear extracts fails to produce a supershifted complex (data not shown).

In order to ascertain the identity of the complex, a comparison of the mobility of the complex binding to the pS2 sequence and that binding to the TRE was investigated (Figure 8). Slight differences in band mobility were observed, with the complex binding to the pS2 motif migrating slightly further than the TPA-induced protein−TRE complex. However, *jun* polyclonal antibodies were

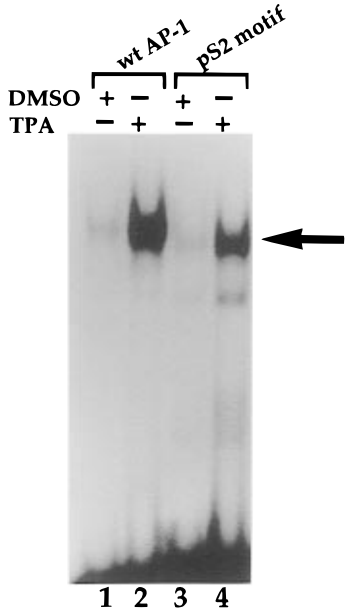

FIGURE 8: Gel retardation analysis using TPA-induced nuclear extracts. A synthetic double-stranded oligonucleotide containing sequences complementary to the consensus wild-type AP-1 sequence (TRE) (lanes 1 and 2) and the pS2 motif I (lanes 3 and 4) was end-labeled with [γ-^{32}P]dATP and incubated with DMSO- or TPA-induced MCF-7 nuclear extracts. The end-labeled oligonucleotide is indicated above each set of lanes. The TPA-induced protein−DNA complex is indicated by the arrow.

capable of supershifting both TPA-induced wtAP-1 and pS2 motif complexes (unpublished results; Figure 7, lane 7, respectively).

Conversion of pS2 ERE to the GAL4 Motif (17-mer) and Mutation of the AP-1-like Motif. Replacement of the pS2 ERE with a Gal4 response element (i.e., 17-mer) significantly reduced background luciferase activity and negated the use of the ICI 164,384 control. Treatment of MCF-7 cells transiently transfected with Gal4-HEGO and wtpS2(17m)-Luc with 1 nM E2 significantly induced (34-fold) luciferase activity, while cotreatment with 10 nM TCDD or 10 nM ICI 164,384 inhibited induction by 61% and 77%, respectively (Figure 9).

In order to investigate the significance of the AP-1-like sequence adjacent to motif I, in E2-induced pS2Luc activity, the (mutAP-1)pS2(17m)Luc reporter gene was constructed by introducing point mutations at positions −513 and −512 which converted CA to TG in the AP-1-like motif (TGAgc-**CA** to TGAgc**tg**; alterations are shown in boldface). These mutations do not affect the adjacent DRE core but have been previously shown to abolish AP-1−TRE complex formation in gel retardation assays. Treatment of MCF-7 cells transiently transfected with Gal4-HEGO and (mutAP-1)pS2-(17m)Luc with 1 nM E2 exhibited only 6-fold induction compared to the 34-fold observed with wtpS2(17m)Luc. Moreover, cotreatment of Gal4-HEGO and (mutAP-1)pS2-(17m)Luc transfected MCF-7 cells with 10 nM TCDD did not affect reporter gene activity while 10 nM ICI 164,384 decreased E2-induced luciferase activity by 45%.

DISCUSSION

Recent studies have demonstrated that TCDD inhibits E2 induction of pS2 expression at the transcription level (Zacharewski et al., 1994). This effect is dependent on

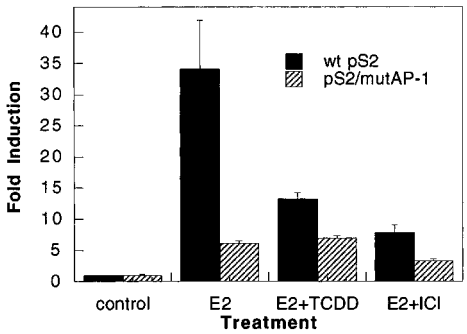

FIGURE 9: Analysis of the AP-1-like motif. Constructs were transiently transfected into MCF-7 cells as described under Materials and Methods. The wild-type pS2 construct refers to the wild-type pS2 motif I linked to the firefly luciferase gene [wtpS2(17m)Luc]. The pS2/mutAP-1 construct refers to a construct that contains point mutations introduced into positions −513 and −512 of the AP-1-like core linked to the firefly luciferase gene [mutAP-1(17m)Luc]. Cells were treated with 1 nM E2, control (DMSO), 1 nM E2 plus 10 nM TCDD, or 1 nM E2 plus 10 nM ICI 164,384. The values presented are from a representative experiment and are averages of four measurements (two samples taken from each plate, each treatment performed on duplicate plates). Results are reported as fold induction relative to the control (DMSO). Transfection efficiency between treatments was normalized using a constitutively expressed β-galactosidase expression vector. The coefficient of variance was less than 10% within assays and less than 15% between experiments. Error bars indicate the standard error of the mean. All values are significantly different than the control ($p < 0.01$). All values are significantly different than E2 ($p < 0.01$) aside from E2 plus TCDD for mutAP-1(17m)Luc ($p < 0.05$). Experiments were performed a minimum of 3 times.

functional AhR complexes and requires specific sequences within the pS2 promoter (Zacharewski et al., 1994). Previous studies have demonstrated that AhR complexes modulate gene transcription by binding to specific DRE sequences located within the 5′ regulatory region of a number of responsive genes (Hankinson, 1995; Swanson & Bradfield, 1993). Three DRE-like core sequences (CGTG) located at positions −520 to −517, −402 to −399, and −388 to −385 have been identified within the 5′ regulatory region of pS2. Deletion analyses of the pS2 regulatory region demonstrate that motif I, located at positions −520 to −517, is required for TCDD-mediated suppression of E2-induced pS2Luc activity (Figure 1). In contrast, ICI 164,384, an ER-mediated "pure antiestrogen" (Wakeling & Bowler, 1988), significantly decreased luciferase activity in all constructs. These results emphasize the different mechanisms of action used by TCDD and ICI 164,384 in inhibiting E2-induced responses.

The essential role of motif I was confirmed by introducing a T to C point mutation at position −518 which eradicated TCDD-mediated suppression of E2-induced pS2Luc activity (Figure 2). These results were consistent with previous studies that have shown oligonucleotides containing mutations in the DRE core were incapable of mediating direct binding to AhR complexes and were ineffective in competitive gel retardation assays (Bank et al., 1992; Shen & Whitlock, 1992; Yao & Denison, 1992). Thus, the effects of TCDD on pS2 expression, at least in part, appear to require the DRE-like motif. However, when taken outside of the context of the pS2 regulatory region, motif I was unable to mediate the anti-estrogenic activity of TCDD since cotreatment with E2 plus TCDD had no effect on E2-induced motif I/ERE-Luc activity (Figure 4). Therefore, although the motif facilitates the suppressive activity, other proteins appear to be necessary for TCDD-mediated suppression of E2-induced pS2 expression.

Motif I differs from the consensus DRE sequence at positions 1 and 9 (Lusska et al., 1993) (Figure 3). While individual nucleotide substitutions at either of these highly conserved positions have been previously shown to diminish TCDD-inducible protein−DNA complex formation, neither mutation was sufficient to abolish complex formation (Bank et al., 1992; Shen & Whitlock, 1992; Yao & Denison, 1992). Based on these studies, AhR complex−motif I interactions should occur since the integrity of the DRE core is maintained; however, the effect of two coexisting mutations outside of the core has not been systematically investigated. Although the inability to demonstrate direct binding between the AhR complex and the motif I sequence in gel retardation assays is problematic, competitive gel retardation assays and UV-induced protein−DNA complex formation indicate that interactions do occur between the TCDD-transformed cytosol and motif I (Figures 5A,B and 6A,B).

Other TCDD-repressed genes have also been reported to use a similar mechanism of action. For example, TCDD has been found to inhibit the expression of the human B lymphocyte surface marker CD19 (Masten & Shiverick, 1995). AhR-mediated repression was localized to a region of DNA within the 5′ regulatory region which contained a DRE-like motif (**a**gGCGTGac**c**) that, like the pS2 motif I, differed from the consensus DRE sequence at positions 1 and 9 (alterations from the consensus DRE are shown in boldface). Moreover, competitive gel retardation assays using a radiolabeled DRE probe from the human *CYP*1A1 gene (−968 to −997) also found that the CD19 DRE-like motif only partially inhibited the formation of TCDD-inducible protein−DNA complexes.

TCDD has also been shown to inhibit E2-induced cathepsin D expression which is regulated via an ER/Sp1 responsive site (−199 to −165) (Krishnan et al., 1995). Analysis of the ER/Sp1 motif identified another DRE-like motif (**g**cGCGTGcc**c**) with substitutions at positions 1 and 9 (alterations from the consensus DRE are shown in boldface). Mutations to the cathepsin D DRE-like motif abolished TCDD-mediated inhibition but did not affect E2-inducibility. In addition, gel retardation studies using TCDD-treated MCF-7 nuclear extracts were unable to demonstrate DNA binding by AhR complexes to the ER/Sp1 motif; however, UV cross-linking experiments have shown that a TCDD-inducible complex of approximately 200 kDa was formed with the ER/Sp1 motif. The complex was competed with excess wild-type DRE, suggesting AhR involvement, and is consistent with the studies involving pS2 DRE motif I. However, UV cross-linking indicates that the complex is actually compromised of two bands that have molecular masses of approximately 220 kDa, further suggesting the involvement of other proteins in addition to the AhR and ARNT.

Studies examining the effect of changes to DRE positions 1 or 9 found DNA binding and enhancer function can be dramatically affected by bases adjacent to the core and that *in vitro* examination of receptor−enhancer binding affinity is not predictive of function (Lusska et al., 1993; Shen & Whitlock, 1992). Consistent with these results, TCDD treatment did not induce a DRE-like motif I-regulated luciferase reporter gene, suggesting that nucleotide changes at positions 1 and 9 may contribute to the negative nature

of this regulatory element (data not shown). Together, the results strongly suggest that the DRE-like motif I acts as a mediator of TCDD-mediated repression of E2-induced pS2Luc activity.

Examination of pS2 motif I has also identified an imperfect TPA response element (TRE) that is capable of forming a retarded complex with nuclear extracts prepared from TPA-treated MCF-7 cells. However, TPA-induced protein−TRE and protein−pS2 complexes exhibited differences in migration using gel retardation assays. This variation in mobility suggests that the protein composition of the complexes binding to the sites may differ or have different tertiary structures. Both bands were supershifted with a *jun* polyclonal antibody, indicating that a *jun*-related protein is contained within the protein−DNA complex. AP-1 complexes can be composed of either *jun* homodimers or *jun*/*fos* heterodimers and can consist of several different *fos* and *jun* isoforms (Halazonetis et al., 1988; Rauscher et al., 1988). Studies have shown that all *jun* homodimers or *jun* /*fos* heterodimer isoform complexes bind the consensus TRE, albeit with different affinities (Angel et al., 1987; Ryseck & Bravo, 1991). Moreover, some dimers have demonstrated higher affinity for nonconsensus TREs. These studies suggest that *jun/fos* complexes could be capable of weak interactions with the AP-1-like sequence within the pS2 motif I.

AP-1 protein complexes have also been found to interact with other transcription factor families (e.g., ATF/CREB and NF-AT) at nonconsensus motifs (Hai & Curran, 1991; Nolan, 1994) and have been implicated, with other proteins, in binding to the antioxidant response element (ARE) (Favreau & Pickett, 1991; Li & Jaiswal, 1992). Furthermore, studies have demonstrated that ATF-1 and/or CREB-1 constitutively bind to the response element of HIF-1, a bHLH-PAS family member (Kvietikova et al., 1995). These interactions have been shown to affect gene transcription in both a positive and a negative fashion and suggest that interactions between several transcription factor families may be involved in TCDD-mediated inhibition of E2-induced pS2 expression.

pS2 expression is contingent on the formation of multiprotein/DNA complexes referred to as receptorsomes (Schuh & Mueller, 1993). Receptorsome formation is strictly dependent upon the ER which is believed to stabilize the complex into an active transcriptional form. AP-1 proteins have also been implicated in receptorsome formation, suggesting that pS2 expression is regulated by a series of associations between regulatory factors (Nunez et al., 1989; Schuh & Mueller, 1993). Mutations to the AP-1-like portion of motif I confirmed the importance of this sequence in potentiating the action of E2 on pS2Luc expression (Figure 9). Similar complexes have also been reported to regulate the expression of other genes including *CYP*1A1 (Robertson et al., 1994; Wu & Whitlock, 1992), supporting the theory that gene regulation is an intricate scenario involving diverse components that include general and specific transcription factors, chromatin, and the nuclear matrix (Diamond et al., 1990; Miner et al., 1991; Pearce & Yamamoto, 1993; Struhl, 1991). Studies investigating protein interactions between steroid receptors and AP-1 dimers at composite response elements have clearly demonstrated that the components of a complex can influence gene regulation in a negative or positive fashion (Diamond et al., 1990; Pearce & Yamamoto, 1993). Consequently, TCDD-mediated suppression may result from interactions between AhR complexes and transcription factors which adversely affect the stability and activity of receptorsomes. Studies demonstrating AhR complex binding to DREs resulting in alterations in chromatin structure further support this hypothesis (Morgan & Whitlock, 1992; Robertson et al., 1994; Wu & Whitlock, 1992, 1993). Furthermore, AhR complexes also inhibit DNA binding of ER/Sp1 complexes that are required for cathepsin D expression (Krishnan et al., 1995), implying that the AhR may suppress pS2 transcription via a similar mechanism. Therefore, AhR-mediated repression may result from competitive interactions for DNA binding sites with several different transcription factors other than the ER. *In vivo*, AhR complex binding to the DRE-like motif I may also be significantly enhanced by novel protein−protein interactions which could lead to repression of gene transcription as has also been reported with other weak transcription factor−DNA interactions (TenHarmsel et al., 1993). For example, ER-mediated interleukin-6 (IL-6) gene repression has been shown to occur in the absence of DNA binding, even though the ER DNA binding domain is required for this effect (Ray et al., 1994; Stein & Yang, 1995). Therefore, AhR complex binding to the DRE-like motif of pS2 may initiate changes in chromatin structure which destabilize receptorsome formation or reduce ERE accessibility which results in transcriptional repression. Consequently, the anti-estrogenic activity of TCDD and related compounds may be attributed to the disruption of regulatory cross-talk between distinct transcriptional factors resulting in the repression of pS2 gene transcription.

In summary, results from this study strongly indicate that the anti-estrogenic effect of TCDD on E2-induced pS2 expression is mediated by a DRE/AP-1-like motif located within the 5′ regulatory region. This motif is required for TCDD-mediated suppression; however, it does not mediate suppressive activity when taken outside of the context of the pS2 promoter. These findings suggest that specific protein−protein interactions may occur *in vivo* which result in decreased gene transcription. Therefore, TCDD and related compounds exert their anti-estrogenic effects not only by increasing hormone metabolism (Gierthy et al., 1993; Spink et al., 1990) and down-regulating ER binding activity (Romkes et al., 1987) and receptor protein concentrations (Zacharewski et al., 1991) but also through the inhibition of E2-induced gene expression.

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