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Molecular Mechanism of Inhibition of Estrogen-Induced Cathepsin D Gene Expression by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) in MCF-7 Cells

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17β-Estradiol (E2) induces cathepsin D mRNA levels and intracellular levels of immunoreactive protein in MCF-7 human breast cancer cells. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) alone does not affect cathepsin D gene expression in this cell line; however, in cells cotreated with TCDD and E2, TCDD inhibited E2-induced cathepsin D mRNA levels, the rate of gene transcription, and levels of immunoreactive protein. The inhibitory responses were observed within 30 to 120 min after the cells were treated with TCDD. TCDD also inhibited E2-induced secreted alkaline phosphatase activity in aryl hydrocarbon (Ah)-responsive MCF-7 and wild-type mouse Hepa 1c1c7 cells cotransfected with the human estrogen receptor (hER) and the pBC12/S1/pac plasmid, which contains the 5′ promoter region (−296/+57) of the cathepsin D gene and an alkaline phosphatase reporter gene. The E2-responsive ER/Sp1 sequence (−199 to −165) in the cathepsin D 5′ region contains an imperfect GTGCGTG (−175/−181) xenobiotic responsive element (XRE); the role of this sequence in Ah responsiveness was investigated in gel electrophoretic mobility shift assays and with plasmid constructs containing a wild-type ER/Sp1 oligonucleotide or a mutant ER/Sp1-"*XRE*" oligonucleotide containing two C→A mutations in the XRE sequence (antisense strand). In plasmid constructs which contained a chloramphenicol acetyltransferase reporter gene and the wild-type ER/Sp1 promoter sequence, E2-induced chloramphenicol acetyltransferase activity and mRNA levels were inhibited by TCDD whereas no inhibition was observed with the mutant ER/Sp1-"*XRE*" plasmids. Electrophoretic mobility shift assays showed that the nuclear or transformed cytosolic Ah receptor complex blocked formation of the ER-Sp1 complex with the wild-type but not the ER/Sp1 mutant oligonucleotide. Moreover, incubation of the wild-type bromodeoxyuridine-substituted ER/Sp1 oligonucleotide with the nuclear Ah receptor complex gave a specifically bound cross-linked 200-kDa band. These data demonstrate that Ah receptor-mediated inhibition of E2-induced cathepsin D gene expression is due to disruption of the ER-Sp1 complex by targeted interaction with an overlapping XRE.

The aryl hydrocarbon (Ah) receptor protein is expressed in laboratory animals and humans and in mammalian cells in culture (54, 65). Although the endogenous ligand(s) for this receptor has not been identified, several different classes of compounds reversibly bind the Ah receptor, and these include the polynuclear and polyhalogenated aromatic hydrocarbons (42, 54, 57, 59, 65, 67). 3-Methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are prototypical aromatic hydrocarbons which exhibit high-affinity binding ($K_d \leq 1$ nM) for the Ah receptor (44, 58) and have been utilized for characterizing Ah receptor-mediated responses.

TCDD induces a broad spectrum of biochemical and toxic responses including a wasting syndrome, immune suppression, hepatotoxicity, developmental and reproductive toxicity, carcinogenicity, dermal toxicity, alteration of endocrine response pathways, and modulation of diverse enzyme activities (26, 29, 59, 73, 82). The induction of *CYP1A1* gene expression by TCDD and 3-methylcholanthrene has been extensively investigated, and the results indicate that the Ah receptor acts as a nuclear ligand-induced transcription factor (13, 29, 73, 82). After treatment of animals or cells with TCDD, there is a rapid formation of a heterodimeric nuclear Ah receptor complex (19) which consists of the ligand-binding protein (7) and the Ah receptor nuclear translocator (Arnt) protein (31). The unbound Ah receptor is associated with heat shock protein 90 (48, 55, 60, 61), and the subcellular distribution of these proteins in the absence of ligand is currently being investigated (60, 61). The nuclear Ah receptor complex interacts with cognate genomic sequences (dioxin or xenobiotic responsive elements [DREs and XREs, respectively]) in the 5′-promoter region of the *CYP1A1* gene and transactivates *CYP1A1* gene expression (15–17, 27, 34, 70, 83). There is evidence that this transactivation process is comparable for induction of *CYP1A1* gene expression and induction of the expression of other members of the Ah gene battery, namely CYP1A2, glucuronosyl transferase, aldehyde-3-dehydrogenase, glutathione *S*-transferase *Ya* gene, and NAD(P)H:menadione oxidoreductase (1, 35, 53, 56, 62, 64). Other studies also report that enhanced expression of other genes by TCDD is due to posttranscriptional processes (20).

TCDD also decreases gene expression and/or the respective activities of the encoded proteins. Phosphoenolpyruvate carboxy kinase, glucose-6-phosphatase, and tryptophan 2,3-dioxygenase activities and mRNA levels are decreased in rats treated with an acutely toxic dose (125 μg/kg of body weight) of TCDD (72). Rat uterine c-*fos* and epidermal growth factor

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receptor mRNA levels are also decreased (2, 3), and decreased epidermal growth factor receptor mRNA levels or binding activity has been observed in several animal species and mammalian cells (33, 36, 43). TCDD inhibits several estrogen (E2)-induced responses in the rodent uterus (21, 66) and mammary gland including the development and formation of mammary tumors in female Sprague-Dawley rats and B6D2 mice (22, 32, 38). The antiestrogenic effects of TCDD and related compounds have also been reported in Ah-responsive MCF-7 human breast cancer cells (22–25, 30, 40). TCDD and related compounds inhibit E2-induced cell proliferation, progesterone receptor protein and mRNA levels, postconfluent focus production and extracellular tissue plasminogen activator activity, procathepsin D, and cathepsin D. This study will utilize the E2-regulated cathepsin D gene as a model for investigating the mechanism of action of TCDD as an antiestrogen.

Cathepsin D is expressed in human mammary cancer cells and tumors, and levels of this protein are used as a negative prognostic indicator for disease-free survival for women with breast cancer (71, 74, 75). Treatment of MCF-7 cells with E2 results in the induction of cathepsin D gene expression and increased intra- and extracellular levels of procathepsin D and cathepsin D (8–11, 47, 52, 76, 79–81). Cavailles et al. (10) have reported that cathepsin D gene transcription is initiated at five start sites (I to V); E2 induces transcription from start site I, and this response is dependent on an intact TATA sequence between −40 to −44. The 5′-proximal flanking region of the cathepsin D gene does not contain a classical palindromic estrogen-responsive element (ERE); however, deletion analysis experiments show that a promoter fragment from −365 to −122 was required for E2 responsiveness (4, 9, 10). More recent data confirmed the importance of this sequence and suggested that E2 regulation of this promoter sequence was complex and appeared to require cooperative interactions with other *trans*-acting nuclear factors such as Sp1 (4). An imperfect ERE half-site and an Sp1 binding site were identified in the cathepsin D promoter between −199 to −165 (41), and this site was protected in DNase I footprinting assays using E2-induced nuclear extracts (4). Research in this laboratory showed that incubation of nuclear extracts from E2-treated MCF-7 cells with a synthetic [^{32}P]ER/Sp1 oligonucleotide (−199 to −165) resulted in formation of an ER-Sp1 complex which could be detected by gel electrophoretic mobility shift assays. Subsequent studies with wild-type and mutant ER/Sp1 oligonucleotides in electrophoretic mobility shift and transient transfection assays showed that formation of an ER-Sp1 complex was required for the E2-induced response (41). The results reported in this study demonstrate that TCDD inhibits E2-induced cathepsin D gene expression and chloramphenicol acetyltransferase (CAT) activity in MCF-7 cells transiently transfected with an ER/Sp1-tk-CAT construct. The data indicate that the inhibitory effects of TCDD are associated with direct interaction of the nuclear Ah receptor complex with an XRE strategically located between the ER and Sp1 response elements. Thus, the nuclear Ah receptor complex acts as a negative transcriptional factor, and the results illustrate a molecular model for interaction between the Ah receptor- and ER-mediated endocrine pathways.

MATERIALS AND METHODS

Chemicals, biochemicals, cells, oligonucleotides, and plasmids. TCDD, [^{3}H]TCDD (32 Ci/mmol), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were synthesized in this laboratory (>98%). RPMI 1640, DME-F12 (2906), controlled processed serum replacement (CPSR-2), transferrin, bovine serum albumin, antibiotic-antimycotic solution, and E2 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fetal calf serum was obtained from JRH Biosciences (Kansas City, Mo.). An ELSA cath-D radioimmunoassay (RIA) kit was purchased from CIS-US Inc. (Bedford, Mass.). α-Naphthoflavone (αNF) was purchased from the Aldrich Chemical Co. (Milwaukee, Wis.). [γ-^{32}P]ATP (3,000 Ci/mmol), [^{3}H]17β-estradiol (130 Ci/mmol), and [α-^{32}P]UTP (800 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). All other chemicals and biochemicals were of the highest purity available from commercial sources.

The human estrogen receptor (hER) expression plasmid was provided by Ming-Jer Tsai (Baylor College of Medicine, Houston, Tex.). The pB12/pL/pac (Seap-pac) plasmid containing the cathepsin D promoter (−296/+57) fused to an alkaline phosphatase gene was used to study the promoter activity (63). The pBC12/S1/pac (S1) plasmid containing no promoter and the pBC12/RSV/pac (RSV) plasmid containing a Rous sarcoma virus promoter fused to the alkaline phosphatase gene were used as negative and positive controls, respectively. The above plasmids were generous gifts from Andrej Hasilik, Münster, Germany (with permission from B. R. Cullen, Duke University, Durham, N.C.). A 2.6-kb *Bam*HI fragment containing the entire *arnt* cDNA was isolated from the pMB5/NEO-M1-1 expression vector (kindly supplied by O. Hankinson, University of California at Los Angeles) and religated in the pcDNA1/NEO expression vector (Invitrogen). The direction of the *arnt* cDNA was confirmed by *Pvu*II restriction mapping. The correct bacterial clone was amplified, and the plasmid containing antisense *arnt* cDNA was purified and used in the experiment. The MCF-7 cells were purchased from the American Type Culture Collection (Rockville, Md.); the wild-type and class II mutant Hepa 1c1c7 cells were kindly provided by J. P. Whitlock, Jr. (Stanford University). This mutant cell line expresses the cytosolic Ah receptor, but TCDD does not induce formation of the nuclear Ah receptor complex (50).

The ER/Sp1 and ER/Sp1-"*XRE*" oligonucleotides were synthesized by DNA Technologies Laboratory, Texas A&M University. The complementary strands were annealed, and the 5′ overhangs were used for cloning into the thymidine kinase-CAT vector (37). Ligation products were transformed into *Escherichia coli* DH5α cells, and clones obtained were verified by restriction mapping. Plasmids containing cDNA probes for Northern (RNA) analysis of cathepsin D and β-tubulin mRNA levels were obtained from the American Type Culture Collection. A cDNA for CAT mRNA was kindly provided by D. O. Peterson (Texas A&M University). Quantitation of radiolabeled bands after separation by electrophoretic and chromatographic assays was determined with a Betagen Betascope 603 blot analyzer. The following oligonucleotides were synthesized and used in this study: ER/Sp1 oligonucleotide (antisense strand), 5′-GATCCTGGG CG**GGGCA**ACCTCGGGCACGCACAGCGCCCG**GGCGGG**GGGCGGG GA-3′; ER/Sp1-"*XRE*" oligonucleotide (antisense strand), 5′-GATCCTGGGC G**GGGCA**ACCTCGGG**CAAGAAC**AGCGCCCG**GGCGGG**GGGCGGGGA-3′; DRE, 5′-GATCTGGCTCTT**CTCACG**CAACTCCG-3′; DRE′ (mutant), 5′-GA TCCCAGGCTCTT**CTACATC**AACTCCGGGGC-3′; and cross-linking primer sequence, TCCCCGCCCCCCGCC.

RIA for cathepsin D. The commercially available ELSA cath-D kit consisted of ELSA tubes coated with the anti-cathepsin-D monoclonal antibody (8, 39); 300 μl of monoclonal ^{125}I-labeled anti-cathepsin-D antibody and then 50 μl of sample (medium or cytosolic extract) were added to each tube. This mixture was then incubated at 25°C with shaking in an incubator shaker for 3 h. The tubes were then washed three times with 3 ml of Tween 20 solution after the contents in the tube were removed by aspiration. The tubes were then measured for bound ^{125}I in a Packard gamma scintillation counter. Quantitation of cathepsin D in the samples was based on a standard curve obtained as outlined in the instructions provided in the kit.

Northern blot analysis of cathepsin D mRNA. Cathepsin D mRNA levels were measured by using a 1.2-kb *Eco*RI fragment of the human cathepsin D cDNA. β-Tubulin mRNA levels were measured by using a 1.1-kb *Eco*RI fragment of human β-tubulin cDNA. Total RNA was isolated by the guanidinium isothiocyanate-acid phenol extraction method (12). Total RNA (20 μg) was separated in a 1.2% agarose–1 M formaldehyde gel in 20 mM sodium phosphate–2 mM EDTA, transferred onto a nylon membrane by capillary action, and bound to the membrane by UV cross-linking. The cDNAs were labeled with [α-^{32}P]dCTP by using a Random Primers DNA labeling system (Bethesda Research Laboratories) and added at 1×10^6 to 5×10^6 cpm/ml of hybridization solution (5× SSPE [1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA; pH 7.4], 1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, 5× Denhardt's solution). Hybridizations were performed in roller bottles at 65°C for 24 h. Nonspecifically-bound probe was removed by two 15-min washes at 20°C in 1× SSPE, two 45-min washes at 65°C in 0.1× SSPE–1% SDS, and one 20-min wash at 20°C in 1× SSPE. Membranes were stripped of probe by boiling for 20 min in 0.01× SSPE–0.5% SDS. Bands were quantitated with a Betagen Betascope 603 blot analyzer imaging system. Four separate experiments were carried out for each treatment group, and the results are expressed as means ± standard deviations (SD).

Nuclear run-on assays. MCF-7 cells (5×10^7) were harvested, resuspended in 5 ml of 1.5 M sucrose buffer plus 0.1% Brij 58, and homogenized with 10 to 20 strokes in a Dounce homogenizer. The homogenate was diluted to 15 ml with 1.5 M sucrose and centrifuged at 10,000 rpm for 20 min at 4°C. The nuclear pellet was resuspended in 0.5 ml of nucleus storage buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.3], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, 50% glycerol). The concentration of nuclei was determined by diluting an aliquot in 0.5% (wt/vol) trypan blue and counting with a hemocytometer. Aliquots of 2 ×

10^6 nuclei were then stored in liquid nitrogen until the transcription elongation assay was performed. The nuclear transcription run-on assay was performed essentially as described elsewhere (45). Briefly, nuclei were isolated at the appropriate times after treatment with TCDD and incubated with [α-^{32}P]UTP (200 μCi) and unlabeled ATP, CTP, and GTP (0.5 mM for each nucleotide). The radiolabeled RNA transcripts were isolated and hybridized to excess (10 μg) of denatured cDNA immobilized onto a nylon membrane by using a slot blot apparatus (Hoefer PR600). The membranes were exposed for 5 weeks, visualized by autoradiography, and quantitated by a Molecular Dynamics 300A scanning laser densitometer.

Preparation and transformation of cytosolic extracts. MCF-7 cells were grown in DME-F12 medium supplemented with 10% fetal bovine serum (2× dextran-coated charcoal). Cells were harvested and washed with Hanks solution. Cells were washed in HEDG buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol [pH 7.6]) and incubated in HED buffer (HEDG buffer without glycerol) for 10 min at 4°C. The cells were then centrifuged at 800 × *g* for 10 min, followed by homogenization using a Teflon pestle-drill apparatus in 1 ml of HEDG buffer. The cell suspension was centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was subjected to ultracentrifugation at 4,000 × *g* for 30 min at 4°C. Supernatant was obtained and protein content was quantitated by the Bradford assay (6). Cytosolic extract (1 mg of protein per ml) was incubated with 20 nM TCDD for 2 h at 25°C. Transformed cytosol (100 μl) was incubated with nuclear extracts from MCF-7 cells and analyzed by electrophoretic mobility shift assays using ER/Sp1 and ER/Sp1-"*XRE*" oligonucleotides.

Electrophoretic mobility shift assays. Complementary strands of the synthetic ER/Sp1 and mutated ER/Sp1-"*XRE*" oligonucleotides were labeled at the 5′ end by using T4 polynucleotide kinase and [γ-^{32}P]ATP (68). Nuclear extracts (5 μg) from MCF-7 and HeLa cells treated with dimethyl sulfoxide (DMSO) (<0.1%) and other appropriate chemicals were incubated in TEGD buffer with 1 μg of poly(dI-dC) for 15 min at 20°C to bind nonspecific DNA-binding proteins. Following addition of 1 nM ^{32}P-labeled specific oligonucleotide, the mixture was incubated for 15 min at 20°C. For competition with specific unlabeled oligonucleotides, the unlabeled oligonucleotide in appropriate excess was incubated for 5 min prior to the addition of the labeled oligonucleotide. Reaction mixtures were loaded onto a 5% polyacrylamide gel (acrylamide/bisacrylamide ratio, 30:0.8) and run at 110 V in 0.09 M Tris–0.09 M borate–2 mM EDTA, pH 8.3. The relative intensities of the retarded complexes were quantitated by the Betagen Betascope 603 blot analyzer imaging system, visualized by autoradiography, and also quantitated with a model 300A scanning laser densitometer (Molecular Dynamics).

Cloning, transient transfection, CAT, and alkaline phosphatase assays. Cloning of the ER/Sp1 and the mutant ER/Sp1-"*XRE*" oligonucleotides into the thymidine kinase-CAT vector at the *Bam*HI and *Hin*dIII sites was carried out as previously described (37, 41). Transient transfection of the CAT plasmids and the Seap-pac, S1, RSV, and hER plasmids was carried out essentially as described elsewhere (37, 41). MCF-7 cells were cotransfected with a Polybrene (200-μg/ml) solution containing 5 to 20 μg of appropriate plasmid DNA and the hER plasmid. After 6 h, the cells were shocked with 15% glycerol in Hanks solution for 75 s, washed twice with the same solution, and grown in DME-F12 (without phenol red)–5% stripped fetal bovine serum. Cells were dosed 12 h after being shocked with appropriate chemicals dissolved in DMSO; control cells were treated with DMSO alone (<0.1%). Two days later the cells were removed by manual scraping, cell extracts were obtained, and 100 μg of protein extract was used to determine CAT activity as previously described (28, 37). Levels of acetylated product were quantitated by a Betagen Betascope 603 blot analyzer imaging system and visualized by autoradiography. Secreted alkaline phosphatase activity was determined essentially as described elsewhere (5, 63). One milliliter of medium was obtained at the appropriate times and incubated for 10 min at 65°C. The medium was then centrifuged at 10,000 × *g* for 5 min, and 900 μl of the supernatant was mixed with an equal amount of Seap buffer (5). This mixture was then warmed in a 37°C water bath separately, along with freshly prepared substrate (120 mM *p*-nitrophenyl phosphate). The two solutions were mixed with constant vortexing and incubated in the 37°C water bath for 6 h. The product of the alkaline phosphatase reaction was quantitated with a spectrophotometer at an optical density at 405 nM. The activity from each sample was compared with the standard curve obtained by using a serial dilution of alkaline phosphatase enzyme activity (5).

Cross-linking studies. For cross-linking studies, 10 pmol of the synthetic oligonucleotide ER/Sp1 was annealed to 10 pmol of a cross-linked primer sequence. The annealed template was end filled with the Klenow fragment of DNA polymerase in the presence of 0.1 μM dGTP, dATP, and bromodeoxyuridine (BrdU) and 1 μM [^{32}P]dCTP (68) and was designated the BrdU-substituted DRE oligonucleotide. Nuclear extracts (10 μg) from MCF-7 cells treated with appropriate chemicals (i.e., E2, E2 plus TCDD, or [^{3}H]TCDD) were incubated with the BrdU-substituted ^{32}P-labeled DRE for 15 min at 20°C following a 15-min incubation at 20°C with 400 ng of poly(dI-dC) in HEGD buffer or for 10 min followed by a 5-min incubation at 20°C with unlabeled excess competitor. The incubation mixtures were irradiated by using a FOTODYNE UV transilluminator at >205 nm for 30 min at 20°C. Samples were then mixed with 20 μl of an SDS loading buffer, heated to 95°C for 5 min, and then subjected to electrophoresis on SDS–6% polyacrylamide gels. ^{32}P-labeled ligand-Ah receptor-DRE complexes were resolved by autoradiography of the dried gel. When 10 nM [^{3}H]TCDD (±2 μM TCDF) was used in the experiments, the resulting gels were cut into eight (1.2-cm) slices and extracted with Solvable (Dupont, Boston, Mass.) and radioactivity was determined by liquid scintillation counting. Molecular weights of UV cross-linked nuclear and transformed ligand-Ah receptor-ER/Sp1 complexes were calculated from ^{14}C-methylated protein standards obtained from Amersham Corp. (Arlington Heights, Ill.).

Electrophoretic mobility shift assays using in vitro-translated proteins. Plasmids containing the full-length Ah receptor and Arnt cDNAs were used to in vitro transcribe and translate the corresponding proteins in a rabbit reticulocyte lysate kit (Promega, Madison, Wis.). DNA binding assays were performed by assembling the appropriate in vitro-translated proteins in 20 mM HEPES–5% glycerol–100 mM potassium chloride–5 mM magnesium chloride–0.5 mM dithiothreitol–1 mM ethylene diaminetetraacetic acid in a final volume of 25 μl. The labeled oligonucleotides (30,000 cpm) were then added to the reaction mixtures in the presence of 1 μg of poly(dI-dC), and the mixtures were incubated for 15 min at 25°C. Reaction mixtures were immediately analyzed by gel electrophoretic mobility shift assays as described above. Gels were dried, and protein-DNA binding was visualized by autoradiography.

Statistical analysis. All the experiments were carried out at least in triplicate, and the results were expressed as means ± SD. Statistical significance was determined by analysis of variance using Scheffe's post hoc test.

RESULTS

Effects of TCDD on E2-induced cytosolic immunoreactive cathepsin D. MCF-7 cells were treated with 1 nM E2 alone for 24 h; cotreated cells were also treated with 1 nM E2 for 24 h, and 1 nM TCDD was added 2, 4, 6, and 12 h prior to harvesting of the cells and isolation of cytosolic extracts. Extracts were analyzed for immunoreactive cathepsin D by using a commercially available RIA kit to detect and quantitate intracellular levels of cathepsin D as previously described (39). The results in Fig. 1 (top) illustrate that E2 alone induced cathepsin D levels (1.8-fold); TCDD alone also slightly increased levels of this protein compared with control (DMSO-treated) cells. In cells cotreated with 1 nM E2 for 24 h and TCDD for 2, 4, 6, or 12 h, there was a significant decrease in immunoreactive cathepsin D at the earliest time point.

Effects of TCDD on E2-induced cathepsin D mRNA levels and rate of transcription. The results in Fig. 1 summarize the time-dependent decrease in E2-induced cathepsin D mRNA levels by TCDD. Treatment of MCF-7 cells for 24 h with 10 nM E2 resulted in a threefold increase in cathepsin D mRNA levels. In cells cotreated with 10 nM E2 for 24 h and 10 nM TCDD for 1, 2, 4, 8, or 12 h, there was significant decrease in cathepsin D mRNA levels at the earliest time point. TCDD alone caused some initial increase in cathepsin D mRNA levels. The results in Fig. 2 summarize the effects of E2 and TCDD plus E2 on the rate of cathepsin D gene transcription in nuclear run-on assays. E2 caused a 6.8-fold increase in cathepsin D mRNA levels, whereas in cells cotreated with E2 plus TCDD a significant decrease in E2-induced mRNA levels was observed 30 or 60 min after addition of TCDD. A Northern blot of cathepsin D mRNA levels after treatment with DMSO, E2, or TCDD plus E2 is shown in Fig. 2C. These data demonstrate the E2 inducibility of cathepsin D gene expression as previously reported (11, 41, 47, 76, 80). Moreover, like ICI 164,384, an antiestrogen which blocks formation of the ER homodimer (14), TCDD also inhibits E2-induced cathepsin D gene expression. However, the inhibitory effects of TCDD in the nuclear run-on assay were observed within 30 min (Fig. 2), whereas the effects of ICI 164,384 occurred 2 to 4 h after treatment (41).

Inhibition of E2-induced cathepsin D promoter activity in MCF-7, wild-type, and mutant mouse Hepa 1c1c7 cells. The effects of TCDD on cathepsin D promoter activity were determined in MCF-7 human breast cancer cells and wild-type and mutant mouse Hepa 1c1c7 cells cotransfected with the Seap-pac and hER plasmids and treated with E2, TCDD, or E2 plus TCDD (Table 1). Because of the relatively high expression of

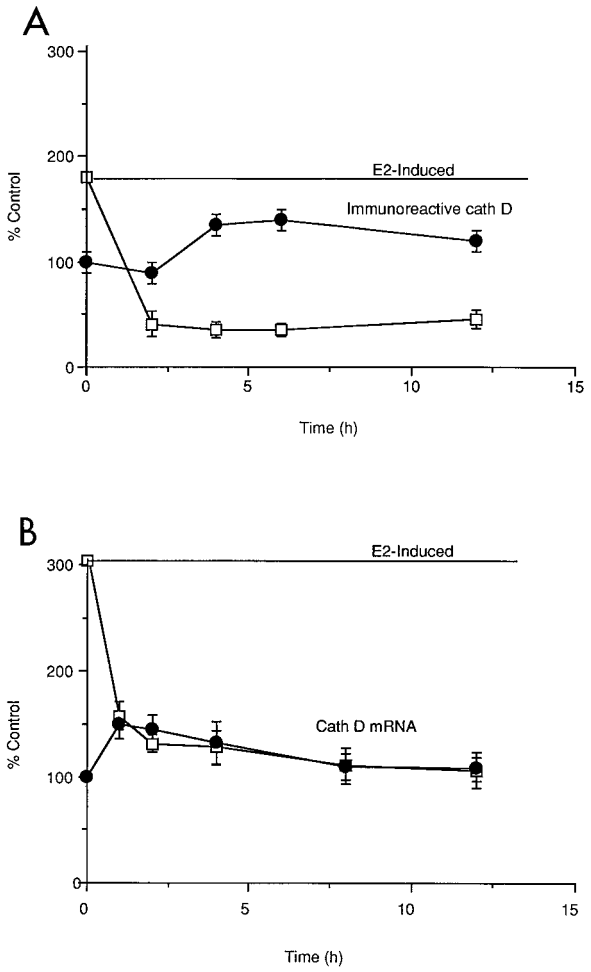

FIG. 1. RIA of immunoreactive cathepsin D (cath-D) using cytosolic cathepsin D levels (top) and cathepsin D mRNA levels (bottom) in MCF-7 cells. Levels of immunoreactive cathepsin D (A) were determined after treatment of cells with 1 nM E2 alone for 24 h and after treatment with 1 nM TCDD alone (●) for 2, 4, 6, and 12 h. The effect of cotreatment on immunoreactive cathepsin D was determined in cells treated with 1 nM E2 for 24 h and TCDD for 1, 2, 4, 6, and 12 h (□). The amount of immunoreactive cathepsin D in control cells was determined after treatment with DMSO (vehicle control) for 24 h and was assigned a value of 100%. The amount of protein in femtomoles per milligram was determined by plotting a standard curve as indicated in the instructions for the RIA kit. The results are expressed as means ± SD for at least three determinations for each time point. E2 significantly induced cathepsin D levels at all time points ($P < 0.05$), and TCDD significantly inhibited the E2-induced response at all time points ($P < 0.05$). Cathepsin D mRNA levels (B) were determined in cells treated with 1 nM E2 alone for 24 h and with 10 nM TCDD alone (●) for 1, 2, 4, 8, and 12 h. Cathepsin D mRNA levels were also determined in MCF-7 cells cotreated with 10 nM E2 for 24 h plus 10 nM TCDD (□) for 1, 2, 4, 8, and 12 h. Treatment of cells with DMSO for 24 h gave control cathepsin D mRNA levels, which were assigned a value of 100%. Cathepsin D mRNA was isolated, visualized by Northern blot analysis, and quantitated by a Betagen Betascope 603 blot analyzer as described in Materials and Methods. Cathepsin D mRNA levels were normalized relative to β-tubulin mRNA for each treatment group.

the recombinant plasmid, cotransfection of the hER expression plasmid was required for E2 responsiveness. Cotransfection of the hER plasmid has previously been reported with plasmid constructs derived from 5′-promoter sequences of the cathepsin D, pS2, and progesterone receptor genes (9, 10, 41, 69, 85). In MCF-7, wild-type, and mutant Hepa 1c1c7 cells cotransfected with the Seap-pac and hER plasmids, 10 nM E2 induced an 8.4-, a 6.9-, and a 6.4-fold increases in secreted alkaline phosphatase activity, respectively. For cells cotreated with 10 nM E2 for 48 h plus 10 nM TCDD for different periods, TCDD caused a time-dependent decrease in secreted alkaline phosphatase activity in MCF-7 and wild-type Hepa 1c1c7 cells whereas no inhibitory effects were observed in class II mutant Hepa 1c1c7 cells. In MCF-7 cells treated with 10 nM TCDD alone and transfected with the Seap-pac plasmid, alkaline phosphatase activity was not significantly induced. Ten nanomolar TCDD was the optimum concentration for inhibition of E2-induced activity in all the transient transfection studies, and this concentration was not cytotoxic or growth inhibitory. Ten nanomolar E2 was also the optimal concentration which was used in all the transient transfection studies.

CAT assays and CAT mRNA levels. An ER/Sp1 sequence in the cathepsin D promoter (−199 to −165) has been cloned into a thymidine kinase-CAT plasmid (41), and the effects of E2, TCDD, and E2 plus TCDD on CAT activity in MCF-7 cells transiently cotransfected with this plasmid and the hER plasmid were determined (Fig. 3). The results show that E2 induced CAT activity (lane 2) and TCDD inhibited the E2-induced response (lane 3). In addition, cotreatment of MCF-7 cells with αNF (lanes 5 and 6), an Ah receptor antagonist, or cotransfection with a plasmid expressing antisense Arnt (lanes 7 through 10) resulted in reversal of the TCDD-mediated decrease in E2-induced CAT activity (lanes 5 and 9, respectively). TCDD inhibits E2-induced ER/Sp1 binding (lanes 2 and 3), and αNF reverses this inhibitory effect (lane 5). A plasmid containing mutations in the XRE (ER/Sp1-"*XRE*"-tk-CAT) was utilized to determine the role of this sequence in mediating the effects of TCDD (lanes 11 through 14). E2 induced CAT activity in the transient transfection assay using the mutant ER/Sp1-"*XRE*"-tk-CAT plasmid (lane 12), but TCDD did not significantly inhibit E2-induced CAT activity (lane 13) with this plasmid. These results show that the mutant ER/Sp1-"*XRE*"-tk-CAT plasmid retains E2 responsiveness; however, TCDD did not inhibit E2-induced CAT activity. These data indicate that an intact XRE is required for TCDD responsiveness.

In parallel experiments with MCF-7 cells, the effects of 10 nM TCDD on E2-induced CAT mRNA levels were also determined by Northern blot analysis (Table 2). The cells were treated with 10 nM E2 for 24 h and 10 nM TCDD for 2 h. The results show that in cells transiently transfected with the wild-type ER/Sp1-tk-CAT plasmid, E2 induced CAT mRNA levels and TCDD inhibited the E2-induced response. E2 also induced CAT activity in cells transiently transfected with the mutant ER/Sp1-"*XRE*"-tk-CAT plasmid; however, TCDD did not inhibit the hormone-induced response. These data also demonstrate that the plasmid-containing mutants with mutations in the XRE are not responsive to the effects of TCDD.

Electrophoretic mobility shift assays of nuclear extracts, transformed cytosol from MCF-7 cells, and in vitro-translated proteins using wild-type and mutant ER/Sp1 oligonucleotides. The results in Fig. 4 illustrate the pattern of retarded bands in electrophoretic mobility shift assays using the wild-type ER/Sp1 and the mutant ER/Sp1-"*XRE*" oligonucleotides in a titration experiment using TCDD (20 nM)- or DMSO-transformed cytosol from MCF-7 cells as previously reported (51). Incubation of wild-type [^{32}P]ER/Sp1 oligonucleotide (Fig. 4) with nuclear extracts from MCF-7 cells treated with DMSO, 10 nM E2, 10 nM TCDD alone, or 10 nM E2 (24 h) plus 10 nM TCDD for 1 h showed that relatively low levels of ER/Sp1 binding were observed in cells treated with DMSO and TCDD whereas E2 induced ER/Sp1 binding and TCDD rapidly inhibited the E2-induced response. In cells cotreated with E2, TCDD, and 1 μM αNF, the effects of TCDD on formation of

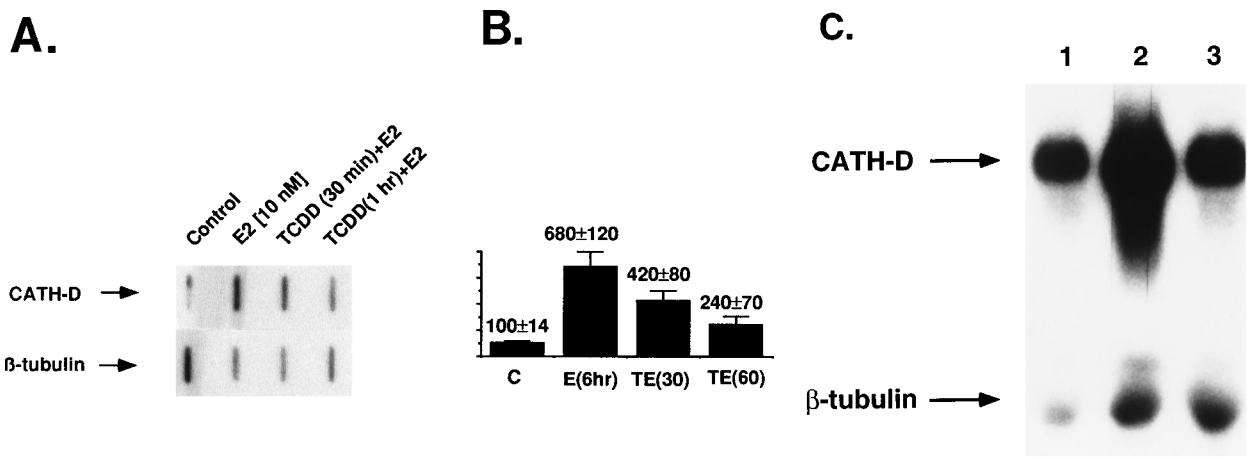

FIG. 2. Effect of 10 nM TCDD on the rate of E2-mediated cathepsin D (CATH-D) gene expression. (A) MCF-7 cells were treated with DMSO (control), 10 nM E2 (24 h), 10 nM TCDD (30 min) plus 10 nM E2, or 10 nM TCDD (1 h) plus 10 nM E2. Cotreated cells were treated with E2 for 24 h, and TCDD was added 30 min or 1 h prior to the end of this period. Cells in the various treatment groups were harvested at appropriate times, and nuclei were obtained and subjected to the nuclear run-on assay as described in Materials and Methods. The amount of newly transcribed mRNA was quantitated by slot blot analysis using a scanning laser densitometer. (B) Cathepsin D mRNA levels were normalized relative to β-tubulin in mRNA and expressed as means ± SD for three separate determinations. Cathepsin D mRNA levels in the different treatment groups were compared with levels in control cells, which were assigned a value of 100%. E2 significantly induced cathepsin D gene transcription after treatment for 6 h; TCDD significantly inhibited the E2-induced response within 30 [TE(30)] or 60 [TE(60)] min after addition ($P < 0.05$). (C) RNA was isolated from MCF-7 cells treated with DMSO (lane 1), 10 nM E2 alone for 24 h (lane 2), or 10 nM E2 for 24 h plus 10 nM TCDD for 1 h (lane 3). The Northern blot was visualized by autoradiography and quantitated by a Betagen Betascope blot analyzer as described for Fig. 1. Cathepsin D mRNA levels were normalized to β-tubulin mRNA for each treatment group, and the relative cathepsin D mRNA levels were 100 ± 12 (lane 1), 301 = 60 (lane 2), and 158 ± 16 (lane 3). The levels in cells treated with 10 nM TCDD alone were 156 ± 14 (data not shown). The results are expressed as means ± SD for three separate determinations. There was a significant decrease ($P < 0.05$) in cathepsin D mRNA levels in the cotreated cells compared with those treated with E2 alone.

the ER/Sp1 retarded band were reversed (data not shown). Coincubation of nuclear extracts from E2-induced MCF-7 cells with cytosol from MCF-7 cells transformed with DMSO resulted in minimal effects on ER/Sp1 binding, whereas coincubation with cytosol transformed with 20 nM TCDD resulted in a significant loss of ER/Sp1 binding. The same experiment was repeated with the mutated ER/Sp1-"*XRE*" oligonucleotide (Fig. 4), and the binding of nuclear extracts from E2-treated cells was not decreased after coincubation with DMSO- or TCDD-transformed cytosol; the intensities of the retarded ER-Sp1 complex were comparable after the mutant ER/Sp1-"*XRE*" was incubated with nuclear extracts from MCF-7 cells treated with 10 nM E2 or 10 nM E2 plus 10 nM TCDD for 1 h. These results demonstrate that the transformed or nuclear Ah receptor complexes blocked formation of the ER-Sp1 complex and this inhibitory response was dependent on an intact XRE sequence.

TABLE 1. Inhibition of E2-induced alkaline phosphatase activity by TCDD in MCF-7 and Hepa 1c1c7 cells transfected with the Seap-pac and hER plasmids[a]

Treatment (h)	Alkaline phosphatase activity (% of control)		
	MCF-7	Wild-type Hepa 1c1c7	Class II mutants
DMSO (48)	100 ± 1	100 ± 9	100 ± 14
E2 (48)	845 ± 89	690 ± 30	640 ± 100
E2 (48) + TCDD (1)	782 ± 87		
E2 (48) + TCDD (2)	629 ± 30[b]	435 ± 51[b]	501 ± 70
E2 (48) + TCDD (4)	515 ± 47[b]		
E2 (48) + TCDD (6)	311 ± 30[b]	316 ± 38[b]	657 ± 47
E2 (48) + TCDD (12)	245 ± 28[b]	268 ± 9[b]	595 ± 72
E2 (48) + TCDD (24)	153 ± 13[b]	115 ± 16[b]	601 ± 99
S1[c]	53 ± 13	37 ± 3	22 ± 14
RSV[d]	1,533 ± 240	1,250 ± 17	860 ± 72

[a] Cells were treated with 10 nM E2 alone for 48 h, and, in the cotreatment group, cells were treated with 10 nM E2 for 48 h and 10 nM TCDD was added at different time points prior to the end of the 48-h period. The cells were cotransfected with the Seap-pac and hER plasmids, and secreted alkaline phosphatase activity was determined as described in Materials and Methods. Treatment of cells with 10 nM TCDD alone for 12 h and the Seap-pac plasmid did not result in increased alkaline phosphatase activity compared with DMSO treatment; however, in cells transfected with the hER and Seap-pac plasmids and treated with 10 nM TCDD, there was a significant increase in alkaline phosphatase activity in MCF-7, wild-type, and mutant Hepa 1c1c7 cells (data not shown). The Ah receptor-independent responses were not further investigated.
[b] Significantly lower ($P < 0.05$) than the value for cells treated with E2 alone.
[c] Negative control.
[d] Positive control.

Figure 5A illustrates the results of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of nuclear extracts from MCF-7 cells cross-linked to a BrdU-ER/Sp1 oligonucleotide. Nuclear extracts from DMSO-treated cells did not form a cross-linked band (lane 2); incubation of nuclear extracts from cells treated with 10 nM TCDD plus 10 nM E2 and [^{32}P]BrdU-ER/Sp1 gave a 200-kDa cross-linked band (lane 3), and coincubation with a 100-fold excess of unlabeled DRE (lane 4) resulted in decreased formation of this band. Competition with a mutated DRE oligonucleotide (lane 5) did not decrease the intensity of the cross-linked band. Only weak, specifically bound cross-linked bands were observed with extracts from cells treated with 10 nM E2 or 10 nM TCDD alone (data not shown). In parallel experiments, cells were treated with 10 nM E2 and 10 nM [^{3}H]TCDD in the presence or absence of a 200-fold excess of unlabeled TCDF. After cross-linking and photoaffinity labeling followed by SDS-PAGE, the gels were sliced, and the specifically-bound radioactivity (^{3}H) was localized in the 200-kDa cross-linked band (Fig. 5B).

The binding of the Ah receptor complex to the ER/Sp1 oligonucleotide was further investigated with an Ah receptor complex formed by reconstituting the Ah receptor and Arnt proteins expressed in vitro by using the rabbit reticulocyte lysate system (Fig. 6). Direct binding of the reconstituted Ah receptor complex to [^{32}P]ER/Sp1 was not detected (data not

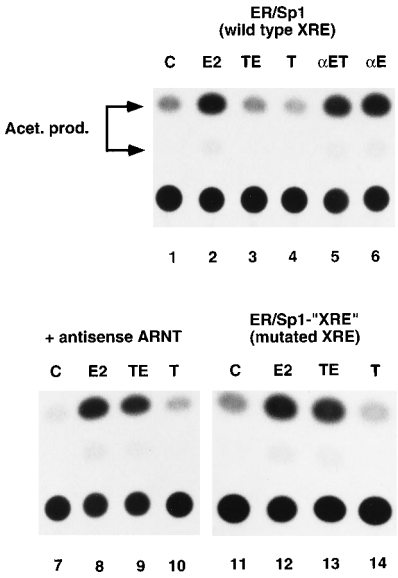

FIG. 3. Effects of TCDD, αNF, and antisense Arnt on E2-induced ER/Sp1-tk-CAT activity: requirement for a functional nuclear Ah receptor-Arnt complex and intact XRE sequence. MCF-7 cells were transiently transfected as described in Materials and Methods. Briefly, cells were seeded in DME-F12 media containing fetal bovine serum stripped twice, and were grown to 60% confluency. ER/Sp1-tk-CAT plasmids (10 μg) along with the hER plasmid (5 μg) or Arnt antisense plasmid (10 μg) were mixed in a solution containing Polybrene and added to the media. Cells were shocked after 12 h by using 15% glycerol in Hanks solution and dosed with DMSO (C), 10 nM E2, 10 nM TCDD plus 10 nM E2 (TE), 10 nM TCDD alone (T), 10 nM E2 plus 1 μM α-NF (αE), or 10 nM TCDD plus 10 nM E2 plus 1 μM α-NF (αET). After 48 h, cells were harvested and assayed for CAT (Acet.) activity. CAT activities relative to that for the control (lane 1, 100% ± 9%) were as follows: lane 2, 364 ± 29; lane 3, 102 ± 14; lane 4, 95 ± 12; lane 5, 372 ± 40; lane 6, 375 ± 35; lane 7, 100 ± 14; lane 8, 405 ± 28; lane 9, 325 ± 30; lane 10, 132 ± 12. Lanes 7 through 10, cells transfected with both the wild-type ER/Sp1-tk-CAT plasmid and the antisense Arnt expression plasmid. In a separate experiment, the mutant ER/Sp1-"*XRE*"-tk-CAT plasmid was utilized as described above, and relative CAT activities in cells treated with DMSO (assigned a value of 100%), E2, TCDD plus E2, and TCDD alone were 100 ± 14, 298 ± 30, 302 ± 42, and 95 ± 14, respectively (lanes 11 through 14). CAT activities in experiments using antisense Arnt DNA or 1 μM αNF were not significantly different from control values (data not shown). Results are expressed as means ± SD for three separate experiments.

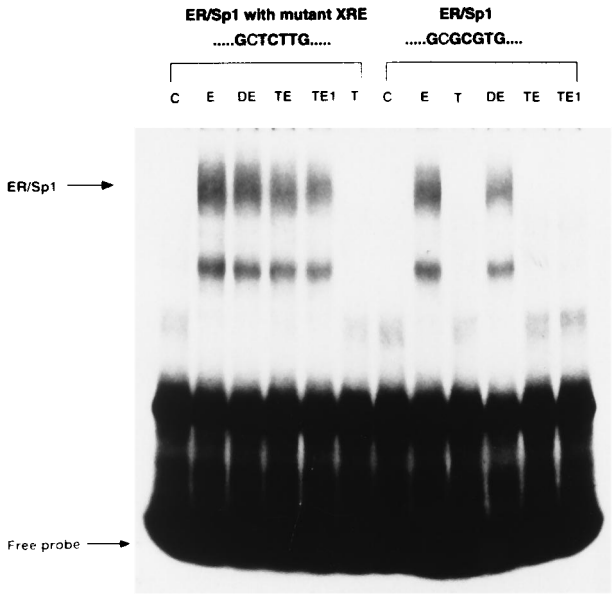

FIG. 4. Effect of TCDD on ER/Sp1 binding: role of the XRE. Nuclear extracts from MCF-7 cells treated with DMSO (lanes C), 10 nM E2 alone for 24 h (lanes E), 10 nM TCDD for 1 h plus 10 nM E2 for 24 h (lanes TE1), or 10 nM TCDD alone for 1 h (lanes T) were isolated, incubated with the mutant [^{32}P]ER/Sp1-"*XRE*" or wild-type [^{32}P]ER/Sp1 oligonucleotide, and analyzed by electrophoretic mobility shift assays as described in Materials and Methods. Cytosolic extracts were obtained from MCF-7 cells and transformed in vitro with 20 nM TCDD or DMSO for 2 h at 25°C as previously described (51). Cytosol (100 μl) transformed with TCDD (lanes TE) or DMSO (lanes DE) was incubated with nuclear extracts from E2-treated cells plus [^{32}P]ER/Sp1 or mutant [^{32}P]ER/Sp1 oligonucleotide and analyzed by electrophoretic mobility shift assay. The retarded ER/Sp1 bands (arrow) were visualized by autoradiography and quantitated by a Molecular Dynamics 300A laser densitometer. The intensity values in lanes 2 through 12 relative to the control band (lane 1, 100% ± 15%) were 510 ± 42, 525 ± 30, 470 ± 22, 440 ± 35, 50 ± 7, 90 ± 9, 444 ± 42, 65 ± 17, 330 ± 23, 95 ± 11, and 80 ± 10 (means ± SD for three separate determinations), respectively. The intensities of the ER-Sp1 complexes in lanes 6, 7, 9, 11, and 12 were all significantly lower ($P < 0.05$) than that observed for complexes with extracts from E2-treated cells and the mutant (lane 2) or wild-type (lane 8) ER/Sp1 oligonucleotide. Previous studies have determined the binding specificity of the ER/Sp1 band (41).

shown), but a specifically bound complex was formed after incubation with [^{32}P]DRE (lane 1). The intensity of this retarded band was not decreased after coincubation with a 100-fold excess of ERE (lane 3), 100- or 400-fold excess of ER/Sp1-"*XRE*" (lanes 6 and 7), or 100-fold excess of mutated DRE (data not shown). In contrast, competition with 100-fold excess DRE (lane 2) or 100- or 400-fold excess of ER/Sp1 (lanes 4 and 5) decreased formation of the specifically bound DRE-Ah receptor complex retarded band. These results with in vitro-expressed Ah receptor and Arnt proteins confirm the interaction of the Ah receptor-Arnt heterodimer with the XRE sequence located within the cathepsin D ER/Sp1 oligonucleotide.

TABLE 2. Inhibition of E2-induced CAT mRNA levels by TCDD in MCF-7 cells transiently cotransfected with the hER and ER/Sp1-tk-CAT or ER/Sp1-"*XRE*"-tk-CAT plasmids[a]

Treatment (h)	Relative % CAT mRNA	
	ER/Sp1-tk-CAT	ER/Sp1-"*XRE*"-tk-CAT
DMSO	100 ± 20	100 ± 15
E2 (10)	278 ± 30	327 ± 21
TCDD (2)	120 ± 12	114 ± 15
E2 (10) + TCDD (2)	95 ± 19[b]	348 ± 31

[a] Cells were cotransfected with the hER and ER/Sp1-tk-CAT or mutant ER/Sp1-"*XRE*"-tk-CAT plasmids and treated with 10 nM E2 for 10 h and 10 nM TCDD for 2 h prior to the end of the 10-h incubation period as described in Materials and Methods. CAT mRNA levels were determined by Northern blot analysis (standardized relative to β-tubulin mRNA), and the relative CAT mRNA levels are given as means ± SD for three separate determinations.

[b] Significantly lower ($P < 0.05$) than the value for cells treated with E2 alone.

DISCUSSION

Several studies report that TCDD and related compounds inhibit a number of E2-induced responses (2, 3, 22–25, 30, 32, 38, 40, 66, 84a, 85) including secretion of procathepsin D and cathepsin D in MCF-7 cells. Studies using wild-type Ah-responsive and mutant Ah-nonresponsive MCF-7 cells showed that formation of a transcriptionally active nuclear Ah receptor was required for inhibition of E2-induced secretion of procathepsin D by TCDD (51). Moreover, there was a good correlation between the structure-Ah receptor binding affinities for several Ah receptor agonists and their structure-dependent inhibition of this E2-induced secretory protein (40). This study further investigates the molecular mechanism of action of

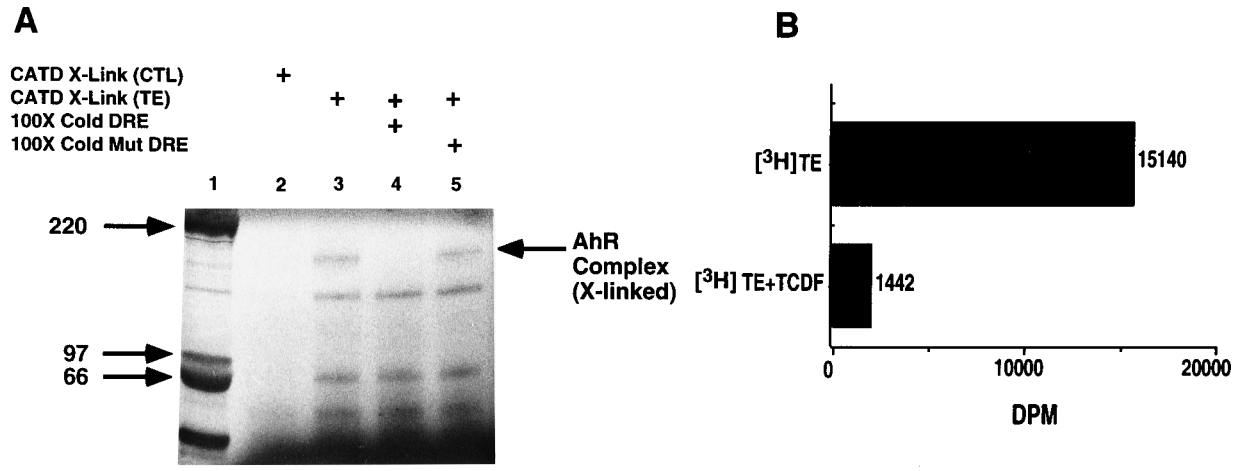

FIG. 5. Cross-linking of the nuclear Ah receptor complex with BrdU-ER/Sp1. (A) The BrdU-ER/Sp1 oligonucleotide was incubated with nuclear extracts from cells treated with DMSO (control [CTL]) (lane 2), TCDD plus E2 (TE) (lane 3), or TCDD plus E2 in the presence of 100-fold excess of unlabeled wild-type DRE (lane 4) or mutant (Mut) DRE (lane 5). Incubation, cross-linking, and electrophoresis of the cross-linked complexes were carried out as described in Materials and Methods. The 200-kDa cross-linked (X-linked) Ah receptor complexes (arrow) were visualized by autoradiography and quantitated by using a Sharp SX-330 scanner and Scanalytics (Billerica, Mass.) ZERO-Dscan software. Molecular weights were determined with ^{14}C-labeled protein standards purchased from Amersham Corp. (lane 1). The band intensity values in lanes 3 to 5 relative to the control (lane 2, 100% ± 37%) were 404 ± 14, 121 ± 2, and 346 ± 5, respectively; the results are means ± SD for three determinations. CATD, cathepsin D. (B) Nuclear extracts were obtained as described in Materials and Methods from MCF-7 cells treated with 10 nM [^{3}H]TCDD plus 10 nM E2 alone or in combination with a 200-fold excess of TCDF. Incubation, cross-linking, and electrophoresis were carried out, and the regions corresponding to the cross-linked complex were excised for the two treatment groups, extracted with Solvable (Dupont), and counted in a liquid scintillation counter. The values obtained were 15,140 dpm ([^{3}H]TCDD plus E2) and 1,442 dpm ([^{3}H]TCDD plus E2 plus TCDF). Background levels (520 dpm) were subtracted to give the net dpm values for the 200-kDa band. The only radioactive (^{3}H) band on the gel was associated with the photoaffinity-labeled 200-kDa cross-linked band.

TCDD as an antiestrogen and utilizes the E2-responsive cathepsin D gene as a model.

The results summarized in Fig. 1 and 2 demonstrate that E2 induced intracellular immunoreactive cathepsin D levels, cathepsin D mRNA levels, and the rate of cathepsin D gene transcription in nuclear run-on assays. In cells cotreated with E2 plus TCDD, all of the induced responses were significantly inhibited within 0.2 to 2 h after treatment with TCDD, suggesting that TCDD induces an early gene product which affects cathepsin D gene transcription at some level or there is a direct interaction of the nuclear Ah receptor complex with protein and/or DNA elements involved in transactivation of this gene.

Deletion analysis of the 5′-flanking region of the cathepsin D gene identified several nonconsensus ERE sequences and an E2-responsive region from −252 to −124 (4). The Seap-pac plasmid containing the −296/+59 5′-flanking sequence from the cathepsin D gene (63) was used to determine the effects of TCDD on E2-induced secreted alkaline phosphatase activity in cells transiently transfected with this plasmid (Table 1). In Ah-responsive human MCF-7 and wild-type mouse Hepa 1c1c7 cells transiently transfected with the Seap-pac and hER plasmids, E2 induces secreted alkaline phosphatase activity and TCDD inhibited the induced response within 2 h after treatment. In contrast, TCDD did not inhibit E2-induced alkaline phosphatase activity in the class II mutant Hepa 1c1c7 cell line, which expresses the Ah receptor but does not form transcriptionally active nuclear Ah receptor complexes (50). These results confirm that the antiestrogenic response requires a functional nuclear Ah receptor complex and genomic sequences within the −296/+59 region are sufficient for mediating the TCDD-induced inhibitory response.

Results of previous studies suggest that formation of ER-Sp1 complexes regulates E2-induced transactivation of the c-*myc*, creatine kinase B, and cathepsin D genes (18, 41, 84). An ER-Sp1 complex, GGGCGGn_{23}ACGGG, was previously identified (−199 to −165) in the cathepsin D promoter (41), and further analysis of this sequence has identified an XRE, namely

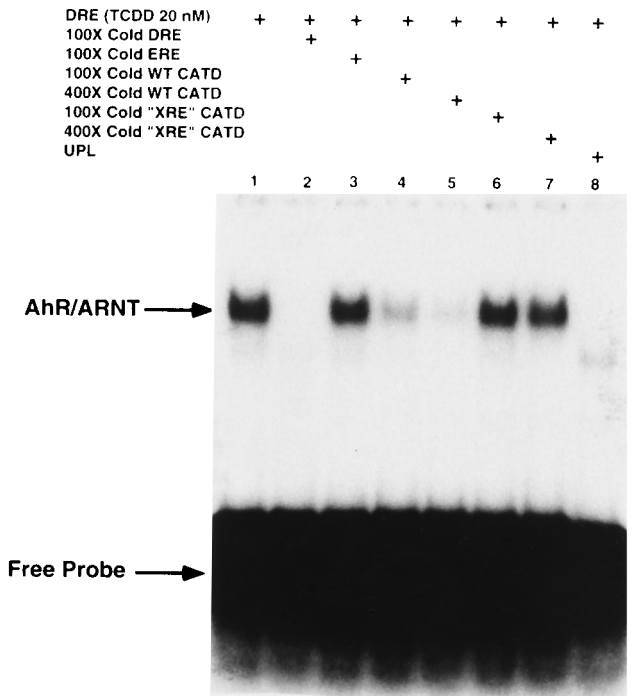

FIG. 6. Electrophoretic mobility shift assays using in vitro-translated Ah receptor and Arnt. In vitro-translated proteins were obtained as described in Materials and Methods. Ah receptor (3 μl) and Arnt (3 μl) or unprogrammed rabbit reticulocyte lysate (UPL) (6 μl) were incubated in the presence of 20 nM TCDD for 2 h at 25°C. The preincubated proteins were then subjected to gel electrophoretic mobility shift assay using a ^{32}P-labeled DRE oligonucleotide. The retarded band (lane 1) (arrow) was blocked with a 100-fold excess of an unlabeled DRE oligonucleotide (lane 2), 100-fold excess of an unlabeled ERE oligonucleotide (lane 3), 100- or 400-fold excess of an unlabeled ER/Sp1 (wild-type cathepsin D [WT CATD]) oligonucleotide (lanes 4 and 5), or 100- or 400-fold excess of an unlabeled ER/Sp1-"*XRE*" ("XRE" CATD) oligonucleotide (lanes 6 and 7). In the absence of ligand, a specifically bound, but less intense, retarded band (20 to 25% the intensity of the band in lane 1) was observed (data not shown).

GTGCGTG (−175/−181), located between the genomic sequences which bind the ER and Sp1 proteins. The potential role of this XRE as a critical target site for mediating the antiestrogenic effects of TCDD was therefore investigated by utilizing the wild-type ER/Sp1 and a mutant ER/Sp1-"*XRE*" oligonucleotide in electrophoretic mobility shift assays and in transient transfection assays in which these oligonucleotides were cloned into plasmids with a thymidine kinase promoter and a CAT reporter gene. The results in Fig. 4 illustrate that nuclear extracts from MCF-7 cells treated with 10 nM E2 formed a retarded ER-Sp1 complex with both wild-type ER/Sp1 (lane 8) and mutant ER/Sp1-"*XRE*" (lane 2) oligonucleotides. Electrophoretic mobility shift assays of nuclear extracts from cells treated with 10 nM E2 (24 h) plus 10 nM TCDD for 1 h showed that TCDD blocked formation of a retarded band with the wild-type ER/Sp1 (Fig. 4, lane 12) but not the mutant ER/Sp1-"*XRE*" (Fig. 4, lane 5) oligonucleotide. The role of the Ah receptor heterodimer in destabilizing formation of the ER-Sp1 complex was further investigated by incubating nuclear extracts from E2-treated MCF-7 cells with cytosolic extracts treated with DMSO or 20 nM TCDD, which transforms the cytosolic Ah receptor to an XRE-binding form indistinguishable from the nuclear Ah receptor complex (51). The transformed cytosolic Ah receptor decreased formation of the ER-Sp1 complex (Fig. 4, lane 11), whereas formation of the ER-Sp1 complex was not affected by addition of transformed Ah receptor complex using the mutant ER/Sp1-"*XRE*" oligonucleotide (Fig. 4, lane 4). Thus, disruption of the ER-Sp1 complex requires (i) the intact XRE sequence located between the ER and Sp1 genomic binding sites in the cathepsin D promoter and (ii) the presence of transformed or nuclear Ah receptor complex (T and TE1 lanes, respectively, with the ER/Sp1 oligonucleotide; Fig. 4).

The results of the gel electrophoretic mobility shift assays did not show a retarded band associated with direct binding of the transformed or nuclear Ah receptor complex with the wild-type ER/Sp1 oligonucleotide (Fig. 4). This may be due to increased protein-DNA dissociation which can result in loss of a retarded band in the assay system. Therefore, two additional experiments were performed to investigate binding of the Ah receptor complex to the DRE located within the ER/Sp1 oligonucleotide (Fig. 5 and 6). Cross-linking experiments with the BrdU-ER/Sp1 oligonucleotide showed that a specifically bound TCDD-induced 200-kDa cross-linked complex was formed only with nuclear extracts from MCF-7 cells treated with E2 plus TCDD (Fig. 5A, lane 3). Competition with excess unlabeled DRE oligonucleotide decreased formation of the cross-linked band (lane 4), whereas competition with mutant DRE had minimal effect (lane 5). In this study, only one major specifically bound cross-linked 200-kDa band was observed, and this was consistent with results obtained with MCF-7 and T47D breast cancer cells (78). Previous studies have demonstrated that UV irradiation of the nuclear Ah receptor complex bound with [^{3}H]TCDD results in photoaffinity labeling of the Ah receptor (77). In parallel cross-linking experiments using the BrdU-ER/Sp1 oligonucleotide and nuclear extracts from cells treated with [^{3}H]TCDD plus E2, the specifically bound radioactivity (^{3}H) was localized in the 200-kDa band (Fig. 5B). Thus, formation of the cross-linked complex (Fig. 5A) was induced by TCDD only in cells cotreated with E2, suggesting that E2-induced formation of the ER-Sp1 complex is required to facilitate accessibility and interaction of the Ah receptor complex with the XRE site; these data are consistent with results showing that disruption of the retarded ER-Sp1 band (Fig. 4) by the nuclear Ah receptor complex required an intact XRE and occurred only in extracts from cells cotreated with TCDD plus E2.

The data in Fig. 6 also confirm competitive binding of the reconstituted Ah receptor-Arnt heterodimer with in vitro-translated proteins. Unlabeled DRE (lane 2) and unlabeled ER/Sp1 (lanes 4 and 5) oligonucleotides competitively decrease formation of the retarded [^{32}P]DRE-Ah receptor complex. In contrast, competition with the ER/Sp1-"*XRE*" oligonucleotide (lanes 6 and 7), which contains mutations in the XRE sequence, does not decrease formation of the retarded band. Thus, in competitive binding studies, wild-type ER/Sp1 competitively decreased formation of the DRE-Ah receptor complex retarded band, confirming the competitive binding affinity of the ER/Sp1 oligonucleotide with the Ah receptor/Arnt heterodimer.

The results obtained in transient transfection studies using the wild-type ER/Sp1-tk-CAT and mutant ER/Sp1-"*XRE*"-tk-CAT plasmids (Fig. 3) complement the results of electrophoretic mobility shift assays and confirm that the antiestrogenic activity of TCDD requires a nuclear Ah receptor complex and an intact XRE sequence within the ER/Sp1 oligonucleotide. TCDD inhibited E2-induced CAT activity and mRNA levels (Table 2) in MCF-7 cells transiently transfected with the ER/Sp1-tk-CAT construct (Fig. 3, lane 3), whereas no inhibition of CAT activity or mRNA levels was observed with the mutant ER/Sp1-"*XRE*"-tk-CAT construct (Fig. 3, lane 13) containing C→A mutations in the XRE (antisense strand). In addition, αNF, an Ah receptor antagonist which inhibits formation of the nuclear Ah receptor complex (49) (Fig. 3, lane 5) and expression of antisense Arnt mRNA (Fig. 3, lane 9), blocked the antiestrogenic activity of TCDD.

In summary, the results of in vitro binding and induction assays using wild-type and mutated 5′-flanking ER/Sp1 sequences indicate that the core XRE sequence located between the ER and Sp1 genomic binding sites in the cathepsin D promoter is required for disruption of the E2-induced ER-Sp1 complex by TCDD. These data demonstrate a unique Ah receptor-mediated mechanism of action in which direct interaction of the nuclear Ah receptor complex with a strategically located XRE results in decreased transcription of an E2-induced gene. Binding to an XRE sequence may also be important for inhibition of E2-induced pS2 gene expression by TCDD (84a), and it is possible that similar mechanisms may play a role in cell-specific inhibition of other genes by Ah receptor agonists (82). The mechanisms associated with inhibition of other E2- and mitogen-induced genes by TCDD in breast cancer cell lines are unknown and are currently being investigated in this laboratory.

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