Regulation of Constitutive Gene Expression through Interactions of Sp1 Protein with the Nuclear Aryl Hydrocarbon Receptor Complex†

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ABSTRACT: The region of residues −145 to −119 (CD/L) of the cathepsin D gene promoter contains a GC-rich motif that binds Sp1 protein and an adjacent pentanucleotide (CACGC) that corresponds to the core sequence of a dioxin responsive element (DRE) and binds the aryl hydrocarbon receptor (AhR)−AhR nuclear translocator (Arnt) complex. This Sp1(N)$_4$DRE(core) motif has been identified in promoters of several genes in which Sp1 plays an important role in basal gene expression. In transient transfection assays with MCF-7 human breast cancer cells using wild-type pCD/L and constructs mutated in the core DRE (pCD/L$_{m1}$) and Sp1 (pCD/L$_{m2}$) sites, it was shown that both motifs were required for maximal basal activity. The requirements for AhR−Arnt interactions with Sp1 protein for maximal activity of pCD/L were confirmed in wild-type MCF-7 and Hepa 1c1c7 cells and Arnt-deficient Hepa 1c1c7 cells using antisense Arnt and Arnt expression plasmids. The functional interactions of Sp1 with AhR−Arnt were paralleled by physical interactions showing that AhR−Arnt and Sp1 proteins were co-immunoprecipitated and AhR−Arnt enhanced Sp1−[^{32}P]CD/L binding in electrophoretic mobility shift assays. The physical and functional interactions of Sp1 with AhR−Arnt proteins bound to the Sp1(N)$_4$DRE(core) motif were also dependent on the proximity of these sites, and both the activity and the extent of Sp1−DNA binding decreased as the number of intervening nucleotides increased from 4 to 20. These studies show that regulation of basal expression of some genes by Sp1 may also require interactions with AhR−Arnt.

The Sp1 protein is widely expressed in mammalian cells and plays an important role as a basal transcription factor in regulating gene expression (*1*, *2*). Sp1 protein recognizes GGGCGG or closely related promoter sequences (GC boxes), and interaction with one or more GC-rich elements in many gene promoters can significantly enhance gene expression (*3*). Sp1 physically interacts with TATA binding protein-associated factors (TAFs) including TAF$_{II}$110 (*4*) and the TFIID component TAF$_{II}$55 (*5*) to enhance transactivation. Additionally, there are multiple examples of physical and/or functional interactions of Sp1 with other DNA-bound transcription factors, including retinoblastoma protein (RB), E2F1, GATA, p53, OTF, E1A, sterol regulatory element binding protein, AP-1, and EGR-1 (*6*−*24*). Sp1 also interacts with the glucocorticoid receptor (GR) (*25*) and estrogen receptor (ER).[1] For example, Sp1 interactions with Sp1(N)$_x$ estrogen response element half-site (ERE$^1/_2$) motifs have been characterized in the creatine kinase B, c-myc, retinoic acid receptor α, cathepsin D, and heat shock protein 27 gene promoters (*26*−*29*).

Recent studies in this laboratory have demonstrated that the Sp1 and ER proteins physically interact and estrogen-induced transactivation can be observed through ER−Sp1 interactions with GC-rich elements in which the ER binds Sp1 protein but not DNA (*30*). Estrogen-responsive GC-rich sequences have now been identified in the c-fos protooncogene, cathepsin D, and retinoic acid receptor α1 gene promoters (*31*−*33*). Functional PR−Sp1 interactions through a GC-rich element in the p21 gene promoter have also been reported (*34*) and both ER−PR action through Sp1 sites are similar to ER−AP-1 interactions through AP-1 elements (*35*, *36*).

Recent studies in this laboratory characterized a unique E2-responsive GC-rich site in the cathepsin D gene promoter which is dependent on interactions with the heterodimeric aryl hydrocarbon receptor (AhR)−AhR nuclear translocator (Arnt) complex that is bound to an adjacent core pentanucleotide dioxin responsive element (DRE; GCGTG) (*32*). Sp1 protein regulates basal expression of several mammalian genes through interactions with GC-rich promoter sequences; moreover, Sp1-mediated activity is often dependent on interactions with other nuclear factors. The Sp1(N)$_4$DRE-(core) motif is common in many gene promoters, and this study utilizes this motif (residues −145 to −126) in the cathepsin D gene promoter as a model for investigating the influence of AhR−Arnt on basal activity mediated by the Sp1 protein. The results show that the high basal activity

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[1] Abbreviations: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; ATCC, American Type Culture Collection; CAT, chloramphenicol acetyltransferase; DRE, dioxin responsive element; ER, estrogen receptor; ERE, estrogen responsive element; ERE$^1/_2$, ERE half-site; FCS, fetal calf serum; PMSF, phenylmethanesulfonyl fluoride; PR, progesterone receptor; TLC, thin-layer chromatography.

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associated with binding of Sp1 protein to the GC-rich element requires physical interactions with the nuclear AhR−Arnt complex bound to the adjacent core DRE. The AhR has been extensively characterized as a ligand-induced transcription factor (*37*, *38*); however, this study defines an endogenous role for the AhR−Arnt in the absence of exogenous ligand. The nuclear AhR−Arnt in combination with Sp1 regulates basal activity via binding to an $Sp1(N)_4$DRE(core) sequence, and this motif ($N = 1-6$) is also present in promoters of many other genes.

MATERIALS AND METHODS

Chemicals, Cells, Antibodies, and Oligonucleotides. MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in minimum essential medium (MEM) with phenol red and supplemented with 10% FCS and 0.2× antibiotic/antimycotic solution, 0.035% sodium bicarbonate, 0.011% sodium pyruvate, 0.1% glucose, 0.238% Hepes, and 0.0000006% insulin. Cells were incubated in an air/carbon dioxide (95:5) atmosphere at 37 °C and passaged every 3−5 days without becoming confluent. Cells were grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME/F12) medium without phenol red and 2.5% stripped fetal calf serum (FCS) for 1 day before transfection. Wild-type and Arnt-deficient Hepa-1 cells were kindly provided by J. P. Whitlock, Jr. (Stanford University, Palo Alto, CA). DME/F12 without phenol red, phosphate-buffered saline (PBS), acetyl CoA, 100× antibiotic/antimycotic, and ellipticine solution were purchased from Sigma Chemical Co. (St. Louis, MO). FCS was obtained from Intergen (Purchase, NY). MEM was purchased from Life Technologies (Grand Island, NY). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly[d(I-C)], restriction enzymes, T4 polynucleotide kinase, and ligase were purchased from Boehringer Mannheim (Indianapolis, IN) or Promega (Madison, WI). Oligonucleotides were synthesized and purchased from the Gene Technologies Laboratory (Texas A&M University), Genosys Biotechnologies, Inc. (Woodlands, TX), or Life Technologies (Grand Island, NY). Sp1 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). AhR antibody was prepared and kindly provided by C. Holtzapple and L. Stanker (USDA Agricultural Research Service Laboratory, College Station, TX). AhR and Arnt expression plasmids were kindly provided by C. A. Bradfield (Northwestern University, Evanston, IL) and O. Hankinson (University of California, Los Angeles, CA), respectively. The TNT T7/SP6 Coupled Reticulocyte Lysate System and Sp1 protein were purchased from Promega. Plasmid preparation kits were purchased from Qiagen (Santa Clarita, CA). Polyacrylamide (40%) was obtained from National Diagnostics (Atlanta, GA). Flexible plates for thin-layer chromatography (TLC) were purchased from Whatman Ltd. (Maidstone, Kent, England). All other chemicals and biochemicals were the highest quality available from commercial sources.

The oligonucleotides that were used are given below and used throughout the paper. The Sp1 and core DRE sites are underlined; the mutated bases are denoted with an asterisk, and restriction enzyme linker sequences are italicized. The numbers in parentheses indicate the positions within the 5′-promoter region of the cathepsin D gene (*39*, *40*). The numbering is based on +1 being the first nucleotide of the translation codon as described previously (*40*): 5′-*GATCC*GCTCG CCCCGCCCG ATCGAATA-3′ [consensus Sp1 oligo (antisense strand)], 5′-*GATCC*GCTCG CCCCGCT-*T*CG ATCGAATA-3′ [$Sp1_m$ oligo (antisense strand)], 5′-*AGCTT*CCGCC CCGCCCCGCG CACGCCGGCC GC*G*-3′ [CD/L (residues −119 to −145) oligo (sense strand)], 5′-*AGCTT*CCGCC CCGCCCCGCG A*AA*A*CCGGCC GC*G*-3′ [CD/L_{m1} (residues −119 to −145) oligo (sense strand)], 5′-*AGCTT*CA*T*T*T*T* CA*T*A*CA*A*GCG CACGCCG-GCC GC*G*-3′ [CD/L_{m2} (residues −119 to −145) oligo (sense strand)], 5′-*AGCTT*CA*T*T*T*T* CA*T*A*CA*A*GCG A*AA*A*CCGGCC GC*G*-3′ [CD/L_{m3} (residues −119 to −145) oligo (sense strand)], 5′-CTCCGGTCCT TCTCACG-CAA CGCCTGGGCA-3′ [DRE (sense strand) (*41*)], 5′-CTCCGGTCCT TCTA*C*A*T*CAA CGCCTGGGCA-3′ [DRE_m (sense strand)], 5′-GATCATCGAT AACC-3′ [Link 1 (sense strand)], 5′-*AGCTT*CCGCC CCGCCCCG*AG ATCT*CGCACG CCGGCCGC*G*-3′ [CD/L_6 (sense strand)], 5′-*AGCTT*CCGCC CCGCCCCGAG ATCGATCTCG CA-CGCCGGCC GC*G*-3′ [CD/L_{10} (sense strand)], 5′-*AGCT-T*CCGCC CCGCCCCGAG ATCATCGATA ACCGATC-TCG CACGCCGGCC GC*G*-3′ [CD/L_{20} (sense strand)], and 5′-*GATCC* (TCTCACGCAA)$_9$ *A*-3′ [$(DRE)_9$].

Cloning. The pBLTATA-CAT plasmid was made by digesting the pBLCAT2 vector with *Bam*HI and *Xho*I to remove the thymidine kinase promoter, and the double-stranded E1B-TATA oligonucleotide containing complementary 5′-overhangs was then inserted into the corresponding sites (*30*). Wild-type CD/L and its mutants CD/L_{m1}, CD/L_{m2}, and CD/L_{m3} were cloned into the pBLTATA-CAT plasmid at the *Hin*dIII and *Bam*HI sites to give pCD/L, pCD/L_{m1}, pCD/L_{m2}, and pCD/L_{m3}, respectively. Full-length cDNA of human AhR was removed from AhR expression vector phuAHR (C. A. Bradfield, Northwestern University) by digesting with *Kph*I and *Sal*I restriction enzymes and ligated into pcDNA3.1 (Invitrogen Corp., San Diego, CA) using *Kpn*I and *Xho*I sites. Human Arnt cDNA was removed from the Arnt expression vector (O. Hankinson, University of California) and cloned into pcDNA 3.1 using the *Bam*HI site. For generating the plasmid with six additional pairs between the Sp1 and DRE core sites (pCD/L_6), the CD/L_6 oligonucleotide carrying an additional *Bgl*II site between the Sp1 site and DRE core sequence was ligated into the pBL/TATA vector using *Hin*dIII and *Bam*HI sites. The mutant with an additional 10 base pairs (pCD/L_{10}) was created by cutting the newly introduced *Bgl*II site of the 6 bp mutant (pCD/L_6), filling in the site with the Klenow polymerase, and religating the plasmid. To construct the mutant with an additional 20 bp (pCD/L_{20}), the newly introduced *Bgl*II site of the 6 bp mutant was cut, and a 14 bp oligonucleotide (Link 1) was ligated into the site. pCD(−365/−10), pCD-(−208/−161), $pCD(-208/-161)_{m1}$, and pCD(−145/−101) were previously described (*32*, *42*), and pCD(−145/−101) utilized a synthetic oligonucleotide insert with a mutant core DRE at residues −130 to −126 (CACGC → AAAAC). $pCD-(-365/-10)_{m1}$ contained mutations at the upstream (residues −183 to −179) and downstream (residues −130 to −126) core DREs. Site-directed mutagenesis at residues −130 to −126 was carried out in the pCD12/PL/SEAP vector containing residues −365 to −10 of the cathepsin D gene

promoter insert (*27, 39*) and using a unique *Bgl*II site for selection. The selection and core DRE mutant primers were as follows: selection, 5′-GTCCCGGCCGATCTGATCA-CATGGCTGACTA-3′ (antisense, with the mutations underlined); and mutant, 5′-GGCGCGGCCGGCGTACACG-GGGGCGGGGCGG-3′ (residues −118 to −146, antisense, with the mutations underlined).

The mutation was confirmed by DNA sequencing, and the DNA fragment was removed, separated by electrophoresis, eluted from the agarose gel, and ligated into pBluescript (Stratagene, LaJolla, CA). The selection primer was designed from the pBluescript sequence in which an *Xba*I site was deleted. The selection and core DRE (residues −183 to −179) mutant primers were as follows: selection, 5′-GATCCACTAGTTATAAAGCGGCCGCC-3′ (sense, with the mutations underlined); and mutant, 5′-CGCCCGGGCGC-TATACATGCCCGAGGTT-3′ (residues −198 to −169, sense, with the mutations underlined).

The second core DRE mutation was confirmed by DNA sequencing, and the resulting 355 bp sequence from the cathepsin D gene promoter was removed from pBluescript and religated into pBLCAT3 (ATCC) to give pCD(−365/−10)$_{m1}$ (mutated in core DRE sites of residues −130 to −126 and −183 to −179). All the ligation products were transformed into DH5α competent *Escherichia coli* cells; plasmids were isolated, and correct clonings were confirmed by restriction enzyme mapping and DNA sequencing using the Sequitherm cycle sequencing kit from Epicenter Technologies (Madison, WI). Plasmid preparation for transfection was achieved by alkaline lysis followed by two cesium chloride gradient centrifugations or by the Qiagen Plasmid Mega Kit.

Transient Transfection and CAT Assays. Cultured MCF-7 cells were transiently transfected by the calcium phosphate method with 10 μg of reporter plasmids and 5 μg of pcDNA3.1 or β-galactosidase expression plasmid. Three hours prior to transfection, the medium was replaced with 5 mL of the charcoal-stripped DME/F12 medium. Cells in each Petri dish were transfected with 1 mL of transfection cocktail containing appropriate plasmids, 50 μL of 2.5 M $CaCl_2$, and 500 μL of 2× HBS (pH 7.05). After incubation for 14−16 h at 37 °C in air/CO_2 (95:5), cells were washed once with 5 mL of PBS and 10 mL of the charcoal-stripped DME/F12 medium was added. Two days later, the cells were washed once with 5 mL of PBS and harvested by scraping. Cells were lysed in 200 μL of 0.25 M Tris-HCl (pH 7.6) via four repeated cycles of freezing in liquid nitrogen for 1.5 min, thawing at 37 °C for 1.5 min, sonication for 1.5 min, and vortexing for 30 s. Cell debris was pelleted, and protein concentrations were measured by the method of Bradford (*43*) using BSA as a standard. An aliquot of cell lysate was brought to 120 μL with 0.25 M Tris-HCl (pH 7.6) and incubated with 1 μL of [^{14}C]chloramphenicol (53 mCi/mol) and 42 μL of 4 mM acetyl CoA for 4 h at 37 °C. The reaction was stopped by vortexing with 700 μL of ethyl acetate. After vortexing for 30 s and centrifuging at 16000*g* for 1 min at room temperature, 600 μL of the ethyl acetate was spotted on a thin-layer chromatography (TLC) plate and developed using a 95:5 chloroform/methanol solvent mixture to separate the acetylated products. The percent protein conversion into acetylated chloramphenicol was quantitated using the number of counts per minute obtained from the Betagen Betascope 603 blot analyzer.

Electrophoretic Mobility Shift Assays. pcDNA 3.1 plasmids containing the AhR and Arnt were used to transcribe and translate in vitro the corresponding proteins in the rabbit reticulocyte lysate kit (TNT Coupled Reticulocyte Lysate System, Promega). Parallel reactions with [^{35}S]methionine were also performed to monitor translational efficiency which was comparable for both expressed proteins. Equal amounts of AhR and Arnt were mixed and incubated on ice for 3 h in the absence of any exogenous ligand prior to electrophoretic mobility shift assays. In vitro-transcribed and -translated proteins were aliquoted and stored at −80 °C. Gel electrophoretic mobility shift assays were performed by incubating 0, 5, 10, and 20 ng of pure Sp1 protein (Promega), in the presence or absence of appropriate cold oligonucleotides, in 10 μL of 1× binding buffer {6% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.1 mg/mL BSA, and 0.125 mg/mL poly[d(I-C)]}. After incubation for 20 min at 20 °C, ^{32}P-labeled oligonucleotides (50 000 cpm) were added to the reaction mixture and incubated for 20 min at 20 °C. For AhR−Arnt-enhanced DNA binding of Sp1 protein, 1 μL of expressed AhR−Arnt or unprogrammed lysate (UPL) was incubated with 5−20 ng of Sp1 protein in 1× binding buffer (no BSA) for 15 min at 20 °C before radiolabeled oligonucleotides were added. Approximately equal amounts of in vitro-translated AhR and Arnt proteins were used in these assays as previously described (*32*). Samples were loaded onto a 4% polyacrylamide gel (acrylamide:bisacrylamide ratio of 30:0.8) and run in 1× TBE buffer [0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA (pH 8.3)] at 110 V. Protein−DNA binding was visualized by autoradiography, quantitated using the Betagen Betascope 603 blot analyzer, and subjected to autoradiography using Kodak X-Omat film for about 2 h at −80 °C.

Co-Immunoprecipitation. ^{35}S-labeled Arnt was synthesized and incubated with equal amounts of AhR on ice for 3 h in the binding buffer [12 mM Hepes/NaOH (pH 7.9), 12% (w/v) glycerol, 30 mM KCl, 0.12 mM EDTA, 0.3 mM dithiothreitol, 0.1 mg/mL phenylmethanesufonyl fluoride (PMSF), 57 μg/mL aprotinin, and 1 mM sodium orthovanadate]. After 10 ng of Sp1 protein was added and the mixture incubated for 1 h at 0 °C, 1 μg of AhR, Sp1, or nonspecific (IgG) antibodies was added. After incubation on ice for an additional 1 h, 20 μL of Agarose A/G PLUS (Santa Cruz Biotechnology) was added and the mixture incubated (gently rocking) for 3 h at 4 °C. The bound complex was then washed four times with RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL PMSF, 57 μg/mL aprotinin, and 1 mM sodium orthovanadate). The target proteins were resolved via 8.5% SDS−PAGE, dried, and visualized by autoradiography. ^{14}C-labeled protein molecular weight standards (Amersham Corp., Arlington, IL) were used to determine the molecular weight of the precipitated ^{35}S-labeled ER.

Statistical Analysis. Statistical significance was determined by ANOVA and Scheffe's test, and the levels of probability are noted. Results are expressed as means ± the standard error (SE) for at least three replicate experiments.

RESULTS

Role of both GC-Rich and DRE Sequences in the Basal Activity of pCD/L. The CD/L region (residues −145 to −119)

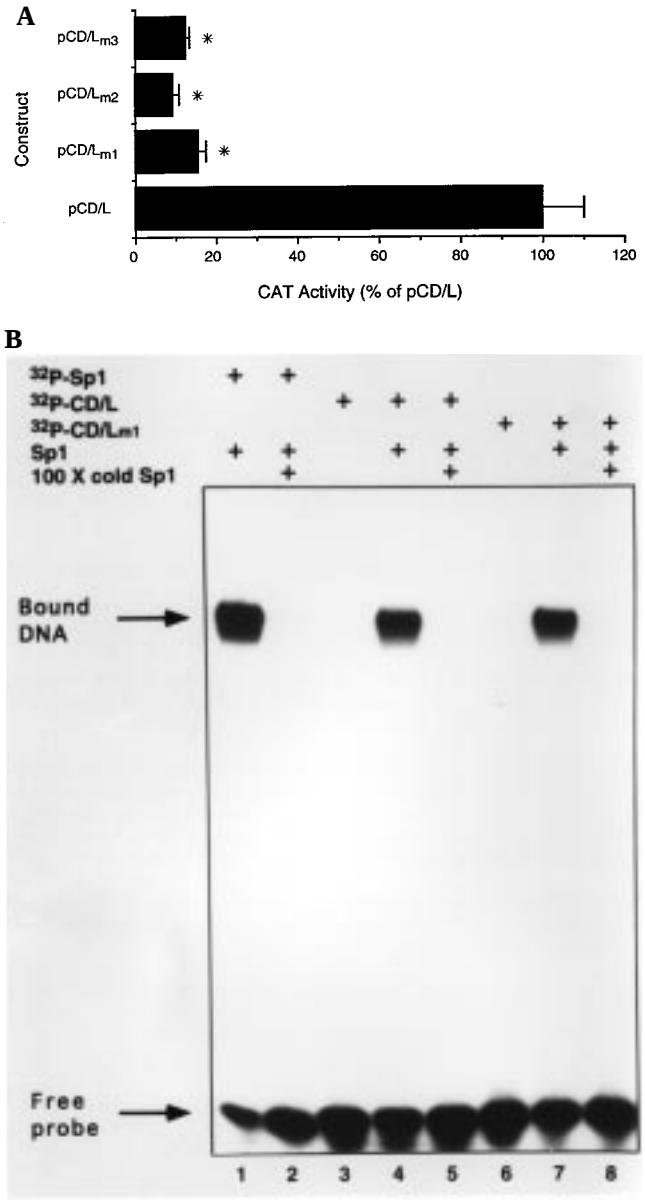

FIGURE 1: Wild-type and variant pCD/L transactivation (A) and Sp1 protein binding (B). (A) MCF-7 cells were transfected with pCD/L, pCD/L$_{m1}$, pCD/L$_{m2}$, and pCD/L$_{m3}$, and CAT activity relative to a β-Gal standard was determined as described in Materials and Methods. CAT activity in cells transfected with pCD/L$_{m1}$, pCD/L$_{m2}$, and pCD/L$_{m3}$ was 16, 9, and 13% of the activity observed for wild-type pCD/L. (B) ^{32}P-labeled Sp1−CD/L or −CD/L$_{m1}$ oligonucleotides were incubated with 10 ng of Sp1 protein and analyzed in a gel mobility shift assay as described in Materials and Methods. Specifically bound Sp1−DNA-retarded bands were observed for all three oligonucleotides (lanes 1, 4, and 7), and the intensities of bands were competitively decreased after coincubation with a 100-fold excess of unlabeled Sp1 oligonucleotides (lanes 2, 5, and 8).

of the cathepsin D gene promoter contains the Sp1(N)$_4$DRE motif (residues −145 to −126), and mutation of the GC-rich element to give CD/L$_{m2}$ results in the loss of Sp1−DNA binding in a gel mobility shift assay (*32*) and a 91% decrease in basal CAT activity in MCF-7 cells transiently transfected with pCD/L$_{m2}$ (Figure 1A). [^{32}P]CD/L$_{m1}$ is mutated in the core DRE sequence and in gel mobility shift assays forms a specifically bound Sp1−DNA complex (Figure 1B). However, in MCF-7 cells transiently transfected with pCD/L$_{m1}$, there was an 84% decrease in basal CAT activity compared to that of wild-type pCD/L (Figure 1A). [^{32}P]CD/L$_{m3}$ contained mutations in both GC-rich and core DRE motifs; this oligonucleotide did not bind Sp1 protein (*32*), and basal CAT activity was decreased by 87% (compared to that of wild-type pCD/L) in MCF-7 cells transiently transfected with pCD/L$_{m3}$ (Figure 1A). Thus, the core DRE is required for maximal basal activity of pCD/L. Direct binding of AhR−Arnt to CD/L cannot be detected in gel mobility shift assays; however, excess unlabeled CD/L but not CD/L$_{m1}$ competitively decreased the level of AhR−Arnt binding to a ^{32}P-labeled consensus dioxin responsive element (*32*). The unbound AhR is primarily nuclear in MCF-7 cells (*32*), and previous gel mobility shift and UV cross-linking assays using nuclear extracts have shown that AhR−Arnt from DMSO-treated cells (control) interacts with the core DRE in CD/L (*32*). Cross-linking studies using bromodeoxyuridine-substituted CD/L and nuclear extracts from untreated MCF-7 cells were repeated for this study (data not shown) and confirmed cross-linking of AhR−Arnt to this sequence as previously described (*32*).

DNA-Dependent and -Independent Interactions of Sp1 with AhR−Arnt. Direct interactions between Sp1 and AhR−Arnt proteins were investigated in both gel mobility shift and co-immunoprecipitation assays (Figure 2). Incubation of [^{32}P]-CD/L with 5, 10, and 20 ng of Sp1 protein (Figure 2A, lanes 3−5) resulted in a concentration-dependent increase in the extent of retarded band formation that was competitively decreased after coincubation with consensus unlabeled wild-type Sp1 (lane 10) but not mutant Sp1 (lane 11) oligonucleotides. Coincubation of [^{32}P]CD/L with the same concentration gradient of Sp1 protein and AhR−Arnt resulted in a 3.3−4-fold increase in the extent of formation of the Sp1-retarded band (lanes 7−9); no supershifted band was detected in this assay. The requirement of an intact core DRE site for Sp1−AhR−Arnt interactions was confirmed in gel mobility shift assays using [^{32}P]CD/L$_{m1}$ (Figure 2B). Incubation of 5 ng of Sp1 and wild-type [^{32}P]CD/L gave a retarded band (lane 1) that was enhanced 3-fold (lane 2) after coincubation with AhR−Arnt. In contrast, incubation of [^{32}P]-CD/L$_{m1}$ with Sp1 protein gave a retarded band (lane 4), and coincubation with AhR−Arnt did not affect the intensity of the retarded band (lane 5). Minimal differences were observed in binding of Sp1 protein alone to [^{32}P]CD/L and [^{32}P]CD/L$_{m1}$ since K_D values were 31 and 26 nM, respectively (data not shown). [^{35}S]Arnt, unlabeled AhR, and Sp1 proteins were coincubated with AhR or Sp1 antibodies (Figure 2C), and [^{35}S]Arnt was co-immunoprecipitated with both antibodies (lanes 2 and 3), but not with nonspecific IgG (lane 4). This confirmed the results of previous studies showing direct physical interactions between Sp1 and AhR−Arnt (*44*).

Results depicted in panels A and B of Figure 2 show that incubation of AhR−Arnt enhanced Sp1−DNA binding but did not form a quaternary supershifted complex. Figure 3A summarizes the time course formation of the Sp1-CD/L complex using 5 ng of Sp1 protein and [^{32}P]CD/L with or without AhR−Arnt (1 μL from in vitro-translated proteins). The results show that AhR−Arnt enhances the rate of retarded band formation, and the $t_{1/2}$ value is decreased from approximately 2.2 to <1 min after coincubation with AhR−Arnt. In contrast, the off-rates or the rates of decay of the retarded band (Sp1−CD/L complex) were similar in the presence or absence of AhR−Arnt. These data demonstrate

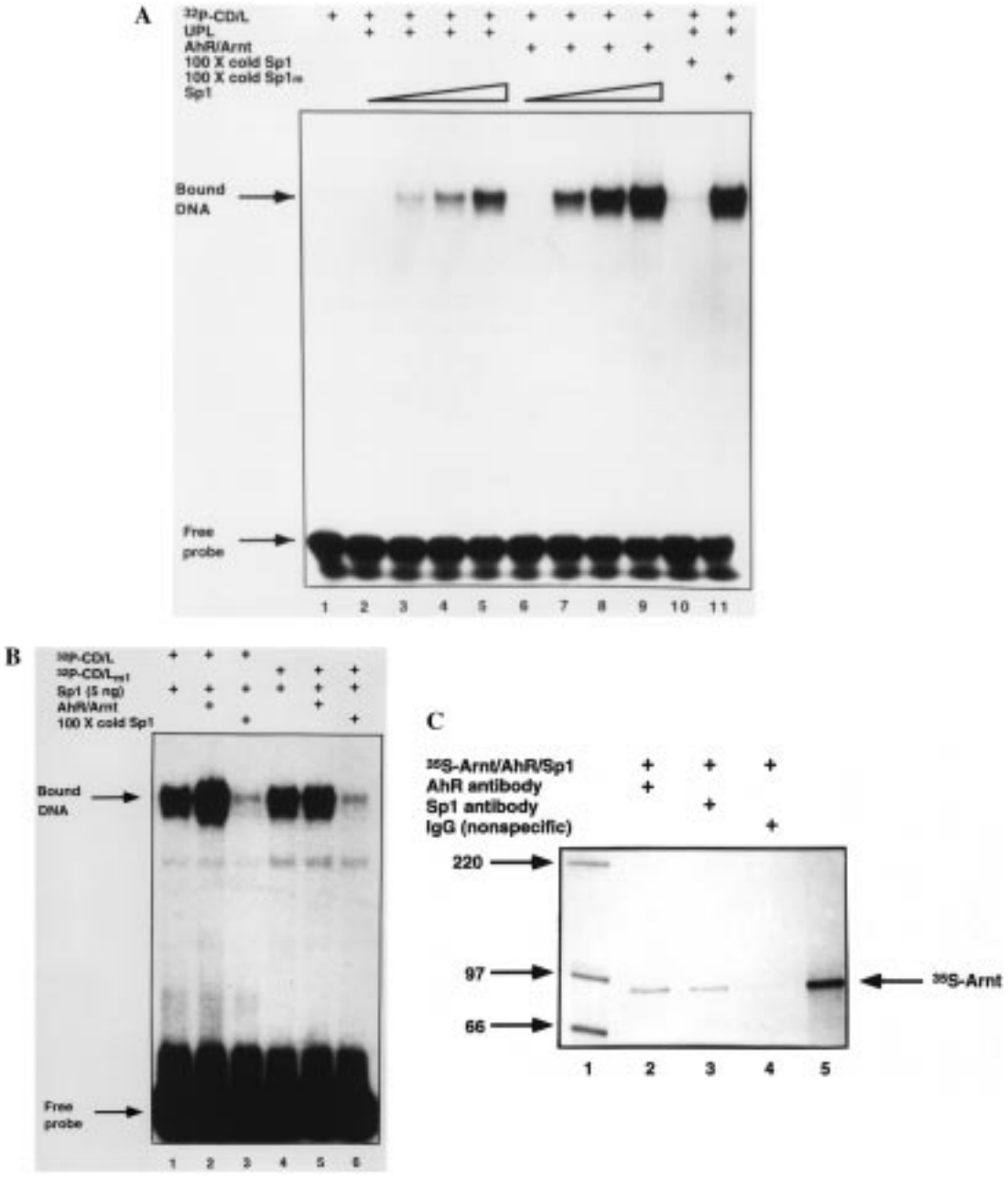

FIGURE 2: Interactions between AhR−Arnt and Sp1. (A) AhR−Arnt enhances Sp1−CD/L binding. [^{32}P]CD/L was incubated with Sp1 protein in the presence or absence of AhR−Arnt and competing unlabeled oligonucleotides, and analyzed by gel mobility shift assay as described in Materials and Methods. Incubation of [^{32}P]CD/L with 5, 10, and 20 ng of Sp1 protein gave retarded bands in lanes 3−5 with intensities (relative to lane 3) of 100 ± 8, 253 ± 12, and 503 ± 18, respectively; coincubation with the same Sp1 gradient and in vitro-translated AhR−Arnt (lanes 7−9) gave retarded band intensities (relative to lane 3) of 441 ± 21, 980 ± 69, and 1665 ± 95, respectively. Competition with a 100-fold excess of unlabeled Sp1 (lane 10) but not mutant Sp1$_m$ (lane 11) oligonucleotides decreased the retarded band intensity. Addition of lysate alone did not affect Sp1−DNA binding (data not shown). (B) Role of core DRE in Sp1−DNA binding. Using the same conditions described for panel A, [^{32}P]CD/L formed a retarded band with 5 ng of Sp1 protein (lane 1, relative intensity of 100 ± 7) which exhibited enhanced intensity (299 ± 20) after coincubation with AhR−Arnt (lane 2). In contrast, the intensity of the [^{32}P]CD/L$_{m1}$−Sp1-retarded band (lane 4) (100 ± 10) was not significantly increased (131 ± 16) after coincubation with AhR−Arnt (lane 5). The effects of the core DRE mutation on Sp1 binding were determined by incubation of 5 ng of Sp1 with variable concentrations of radiolabeled CD/L and CD/L$_{m1}$ (0.1−1.0 nM). K_D values for Sp1 binding to CD/L and CD/L$_{m1}$ were 31 and 26 nM, respectively, indicating minimal differences in binding of Sp1 to both oligonucleotides. In control experiments, reticulocyte lysate alone did not bind ^{32}P-labeled CD/L or CD/L$_{m1}$; in combination with Sp1 protein, lysate slightly decreased the intensity of the Sp1−CD/L- and Sp1−CD/L$_{m1}$-retarded bands. (C) Co-immunoprecipitation of [^{35}S]Arnt−AhR and Sp1. [^{35}S]Arnt and unlabeled AhR and Sp1 proteins were incubated with AhR, Sp1, or nonspecific IgG antibodies (lanes 2−4) and analyzed by electrophoresis. Both AhR and Sp1 but not IgG antibodies immunoprecipitated [^{35}S]Arnt (lanes 2−4). Molecular weight markers and [^{35}S]Arnt alone are shown in lanes 1 and 5, respectively.

that AhR−Arnt increases the rate of Sp1−CD/L formation, and similar results were previously reported for ER enhancement of Sp1−DNA binding *(26)*.

AhR−Arnt-Dependent Basal Activity of pCD/L. Transactivation of pCD/L is dependent on both GC-rich and DRE sites and binding of Sp1 and AhR−Arnt proteins to these sites (Figures 1−3). Further confirmation of the role of AhR−Arnt was obtained with MCF-7 cells transiently transfected with pCD/L (5 μg) and different concentrations of antisense Arnt (10 and 30 μg) (lanes 1−3) (Figure 4A)

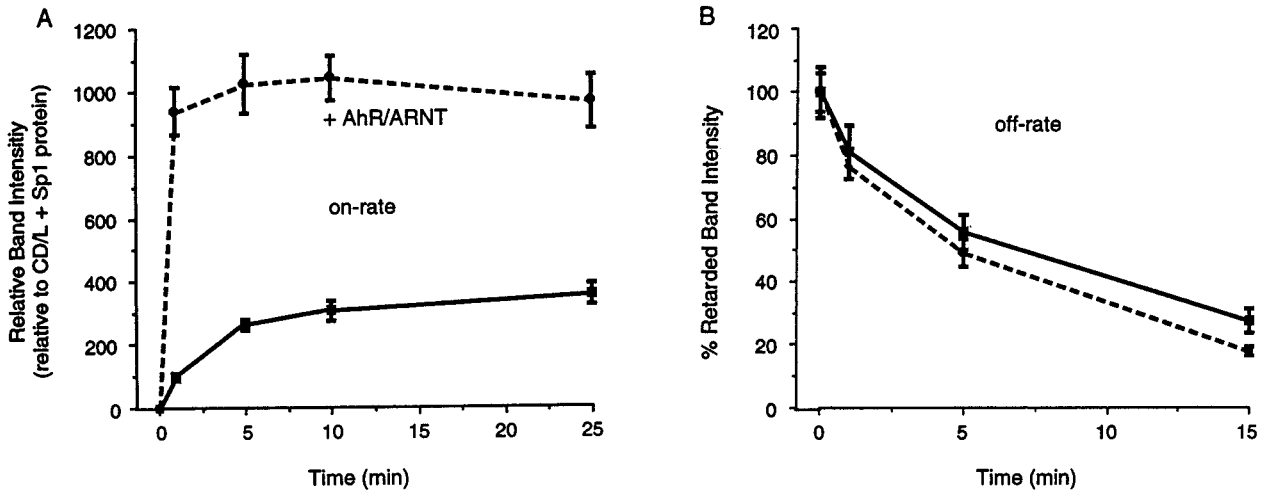

FIGURE 3: Rates of formation and decay of the [^{32}P]CD/L−Sp1 protein complex. Sp1 protein (10 ng) was incubated with [^{32}P]CD/L in the presence or absence of AhR−Arnt (1 μL of lysate), and the rate of formation of the [^{32}P]CD/L−Sp1-retarded band was determined as described previously (*30*). AhR−Arnt significantly increased the retarded band intensity at all time points, and the $t_{1/2}$ values for retarded band formation were 2.2 and <1 min in the absence and presence of AhR−Arnt, respectively. In contrast, the rates of [^{32}P]CD/L−Sp1 decay in the presence and absence of AhR−Arnt were not significantly different.

which resulted in a 47% decrease in CAT activity at the highest concentration of antisense Arnt. In contrast, cotransfection of MCF-7 cells with the antisense Arnt expression plasmid and a consensus pSp1 construct that does not contain a core DRE did not result in significant changes in CAT activity (lanes 4−6). The results depicted in Figure 4B show that the level of CAT expression in wild-type Ah-responsive Hepa 1c1c7 cells transfected with wild-type pCD/L was 4.9-fold higher than that observed in wild-type cells transfected with pCD/L_{m1} (lanes 1 and 2). In mutant Arnt-deficient cells transfected with pCD/L, CAT activity was approximately 4.6-fold lower than that observed in wild-type cells; however, cotransfection with the Arnt expression plasmid significantly increased CAT activity (lanes 3 and 4). In contrast, transfection of Arnt-deficient cells with pCD/L_{m1} (which contains a mutant core DRE) and Arnt expression did not increase CAT activity (lanes 5 and 6). These data also confirm that AhR−Arnt and an intact core DRE site within CD/L are required for maximal CAT activity in MCF-7 or Hepa 1c1c7 cells transfected with pCD/L.

Role of the Endogenous AhR Ligand in Activation of CD/L. Chang and Puga (*45*) recently reported that constitutive activation of AhR−Arnt through a consensus DRE may be due to a CYP1A1 substrate that is also an AhR ligand. The potential role of such an endogenous ligand in AhR−Arnt-mediated activation through the core DRE motif in CD/L was investigated by comparing CAT activity in wild-type mouse Hepa cells and MCF-7 cells transfected with pDRE_3 containing three tandem consensus DREs and pCD/L. In addition, pCD/L_{m1} (core DRE mutation) was used as a control construct. The results obtained with pDRE_3 in Hepa and MCF-7 cells (Figure 5) were consistent with the hypothesis of Chang and Puga (*45*); the CYP1A1 inhibitor caused a concentration-dependent increase in CAT activity as previously reported (*45*). In contrast, CAT activity was not significantly affected in Hepa or MCF-7 cells transfected with pCD/L and treated with 10 or 100 nM ellipticine. Moreover, similar results were also obtained with pCD/L_{m1}, a construct containing only a GC-rich Sp1 binding site. These results suggest that AhR−Arnt action at the core DRE motif does not involve an endogenous ligand−CYP1A1 substrate as proposed for promoters containing consensus DREs. However, this does not include a role for other unknown endogenous substrate(s) for the AhR.

Sp1 and AhR−Arnt Protein Interactions with Sp1-$(N)_x$DRE: Effects of Nucleotide Spacing between Binding Sites on DNA Binding and Functional Activity. Previous studies have demonstrated that Sp1 and E2F1 physically interact and synergistically induce reporter gene activity using constructs derived from several gene promoters containing Sp1 and E2F binding sites (*9*). It was also shown that reporter gene activity associated with Sp1−E2F1 interactions on the thymidine kinase promoter was dependent on the distance between the cognate binding sites. Figure 6A compares the CAT activities in MCF-7 cells transiently transfected with pCD/L and constructs containing 6 (pCD/L_6), 10 (pCD/L_{10}), and 20 (pCD/L_{20}) nucleotides separating the Sp1 and core DRE sites. There was a marked decrease in basal CAT activity with increasing distance between the Sp1 and DRE elements within CD/L. In contrast, ^{32}P-labeled CD/L, CD/L_6, CD/L_{10}, and CD/L_{20} all specifically bound Sp1 protein (10 ng) in gel mobility shift assays (Figure 6B) (lanes 1, 4, 7, and 10). The results demonstrate that Sp1 binding was similar using wild-type CD/L and the "spaced" CD/L oligonucleotides, indicating that the effects of a decreased level of transactivation (Figure 6B) must be related to the proximity of the Sp1−DRE motif in CD/L. However, coincubation of the ^{32}P-labeled oligonucleotides with Sp1 protein and AhR−Arnt resulted in a 3.3-fold enhancement in the level of Sp1 binding to CD/L (lane 2); however, the enhanced level of binding of Sp1 to CD/L_6 (lanes 4−6), CD/L_{10} (lanes 7−9), and CD/L_{20} (lanes 10−12) decreased with increasing distance between the Sp1 and DRE(core) elements. These effects of nucleotide spacing on AhR−Arnt-enhanced Sp1−DNA binding correlated with levels of CAT activity in cells transfected with pCD/L, pCD/L_6, pCD/L_{10}, and pCD/L_{20} (Figure 6A). The results summarized in Figure 6C compare basal activities in MCF-7 cells using wild-type pCD(−145/−101), pCD(−208/−161), and pCD(−365/−10), and the corresponding pCD(−145/−101)$_{m1}$, pCD(−208/−161)$_{m1}$, and pCD(−365/−10)$_{m1}$ constructs containing mutations in the downstream (residues −130 to −126), upstream

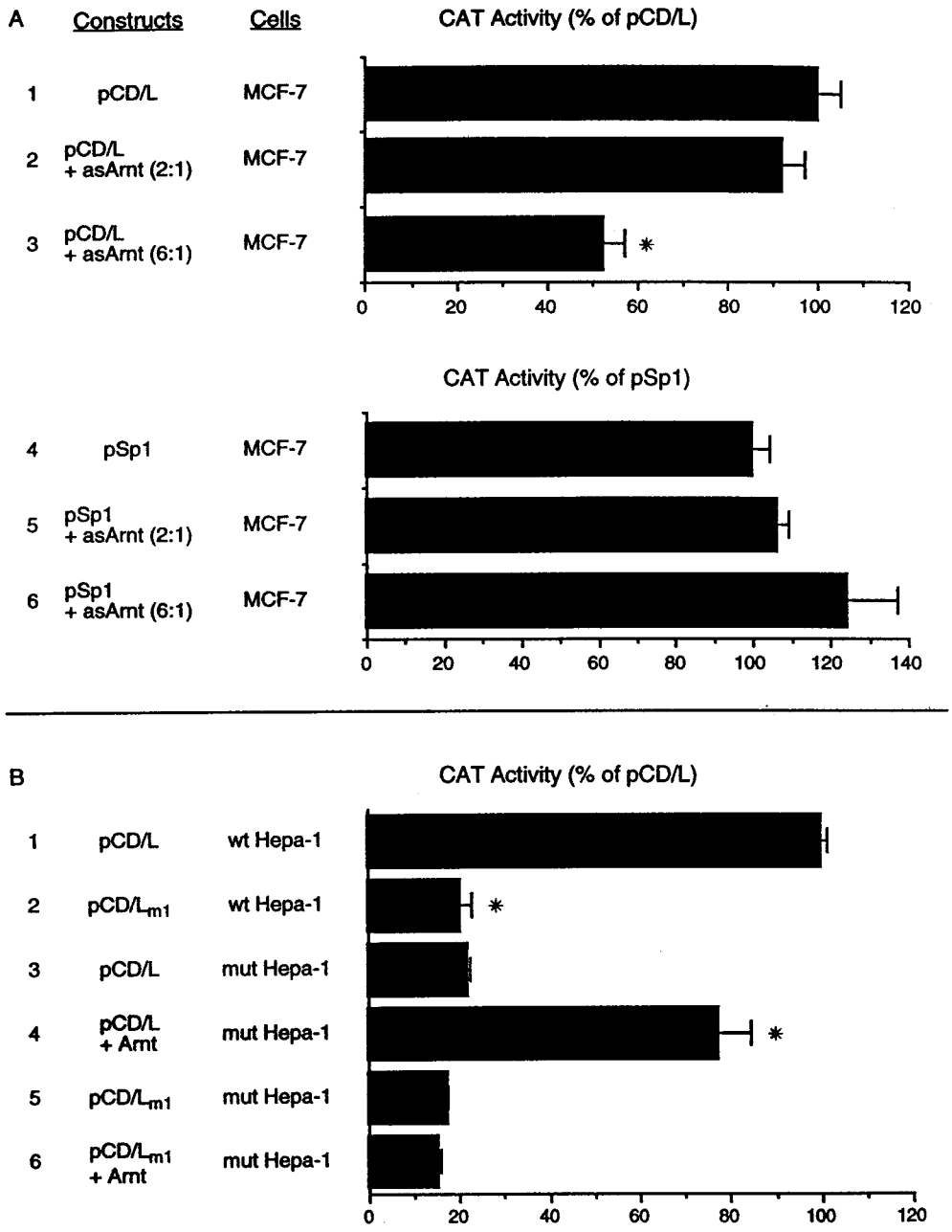

FIGURE 4: Requirement of AhR−Arnt for high basal activity of pCD/L. (A) Effects of antisense Arnt. MCF-7 cells were transiently cotransfected with 5 μg of pCD/L or pSp1 alone or in combination with antisense Arnt expression plasmid (10 or 30 μg), and CAT activities relative to a β-Gal control were determined as described in Materials and Methods. Antisense Arnt expression significantly (*$p < 0.05$) decreased CAT activity in cells transfected with pCD/L (lanes 1 and 3) but not pSp1 (lanes 4 and 6). (B) Effects of Arnt in wild-type and Arnt-deficient Hepa-1 cells. In wild-type Hepa-1 cells, CAT activity was significantly lower (*$p < 0.05$) in cells transfected with pCD/L_{m1} compared to those transfected with pCD/L (lanes 1 and 2); in Arnt-deficient mutant Hepa-1 cells, Arnt expression significantly increased CAT activity in cells transfected with pCD/L compared to pCD/L alone (lanes 3 and 4) (*$p < 0.05$), whereas Arnt expression did not affect CAT activity in cells transfected with pCD/L_{m1} (lanes 5 and 6).

(residues −183 to −179), and both upstream and downstream core DREs. Activities with the mutant constructs were decreased 41, 31, and 49%, respectively.

DISCUSSION

The Sp1 protein is widely expressed in eukaryotes, and interactions of this protein with GC-rich cellular and viral gene promoter sequences have been characterized as important determinants in the expression of diverse genes (*1*−*3*). The functional activity of Sp1 is also influenced by direct or indirect interactions with many other nuclear proteins, including steroid hormone receptors, resulting in the modulation of basal or inducible gene expression (*6*−*24*, *31*−*34*, *44*). Sp1 also physically interacts with the AhR complex and enhanced exogenous ligand-dependent activation of Ah-responsive constructs containing both consensus DRE motifs and adjacent GC-rich sites (*44*). We have recently characterized a unique physical and functional interaction between an ER−Sp1 protein complex binding to a GC-rich sequence within the CD/L region (residues −145 to −126) of the cathepsin D gene promoter and an AhR−Arnt complex binding to an adjacent core DRE motif (*32*). Both Sp1 and DRE core sites within the Sp1$(N)_4$DRE(core) sequence were required for estrogen responsiveness of pCD/L in MCF-7

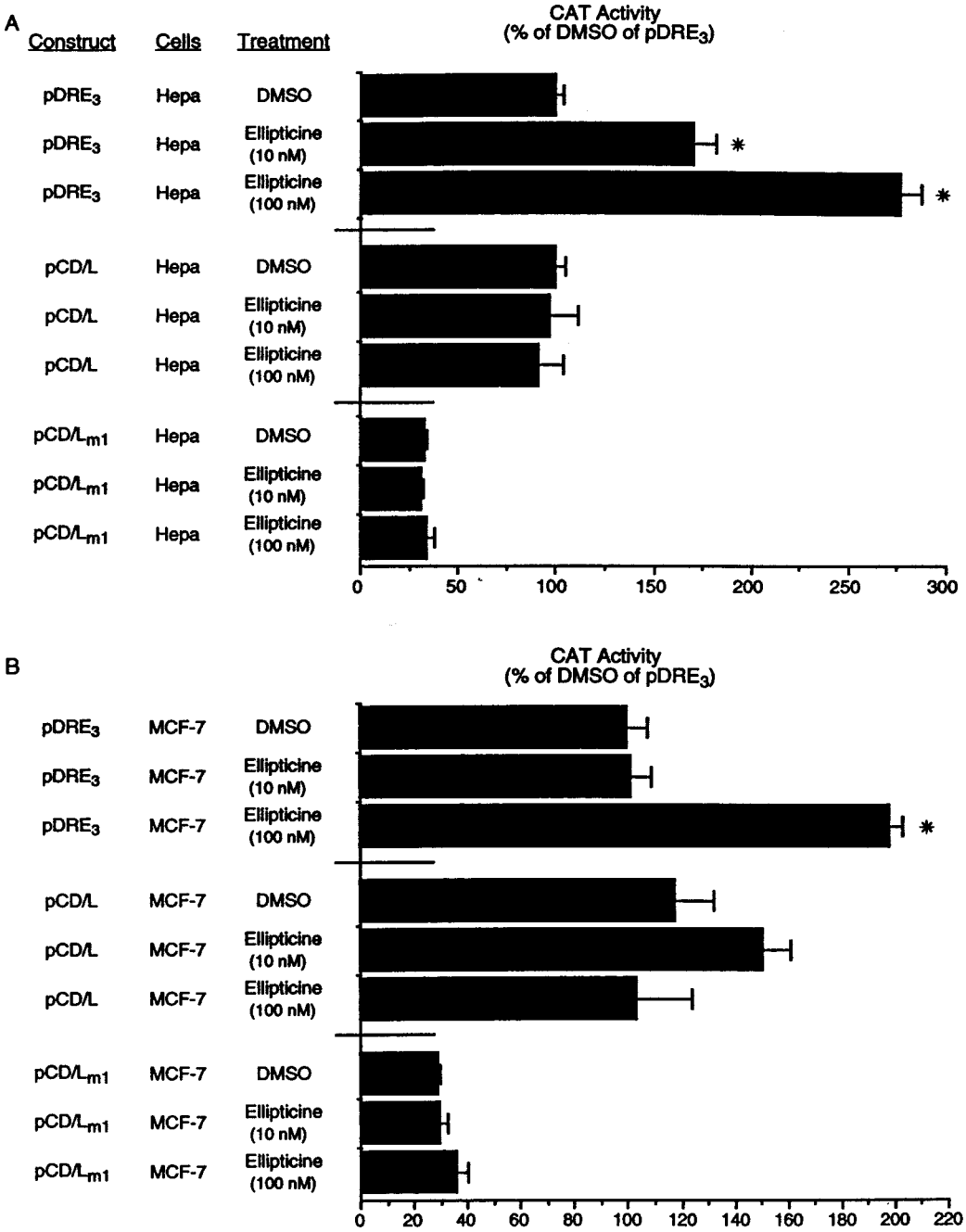

FIGURE 5: Role of an endogenous AhR ligand−CYP1A1 substrate. (A) CAT expression in Hepa-1 cells. Hepa-1 cells were transiently transfected with $pDRE_3$, pCD/L, and pCD/L_{m1} and treated with 10 or 100 nM ellipticine, and CAT activity was determined as described in Materials and Methods. Ellipticine significantly increased CAT activity ($*p < 0.05$) only in Hepa cells transfected with $pDRE_3$. (B) CAT expression in MCF-7 cells. Cells were transfected and treated as described for panel A, and CAT activity was significantly increased by ellipticine only in cells transfected with $pDRE_3$ ($*p < 0.05$). Results are expressed as means ± the standard deviation for three separate experiments for each treatment group, and the transfection efficiency was corrected from β-Gal activity.

cells, and this represented an endogenous role for the AhR complex in the absence of added ligand.

Results of preliminary studies with pCD/L suggested that protein interactions at the $Sp1(N)_4DRE$ motif within CD/L may also influence the basal activity of this promoter, and this was confirmed in MCF-7 cells transfected with wild-type pCD/L, pCD/L_{m1}, or pCD/L_{m2} containing mutations in the DRE and Sp1 binding sites (Figure 1). The decreased activity of pCD/L_{m2} (GC-rich mutation) was not unexpected since we have previously shown by DNA footprinting that Sp1 binds to this region of the cathepsin D gene promoter and undoubtedly plays a role in the constitutive expression of this gene (*42*). However, in transient transfection studies with pCD/L_{m1} (core DRE mutation), there was also an 84% decrease in the level of CAT expression, suggesting that Sp1 and AhR−Arnt interactions with the $Sp1(N)_4DRE$ (core) motif may be required for basal transactivation of pCD/L. These interactions were further investigated so a possible endogenous role for AhR−Arnt in the absence of exogenous ligand and the functional significance of $Sp1(N)_xDRE$ (core) ($x = 1-6$) motifs which are present in diverse gene promoters could be determined (*46*−*64*).

A series of experiments utilizing wild-type pCD/L and mutant pCD/L_{m1} and pCD/L_{m2} and their corresponding ^{32}P-

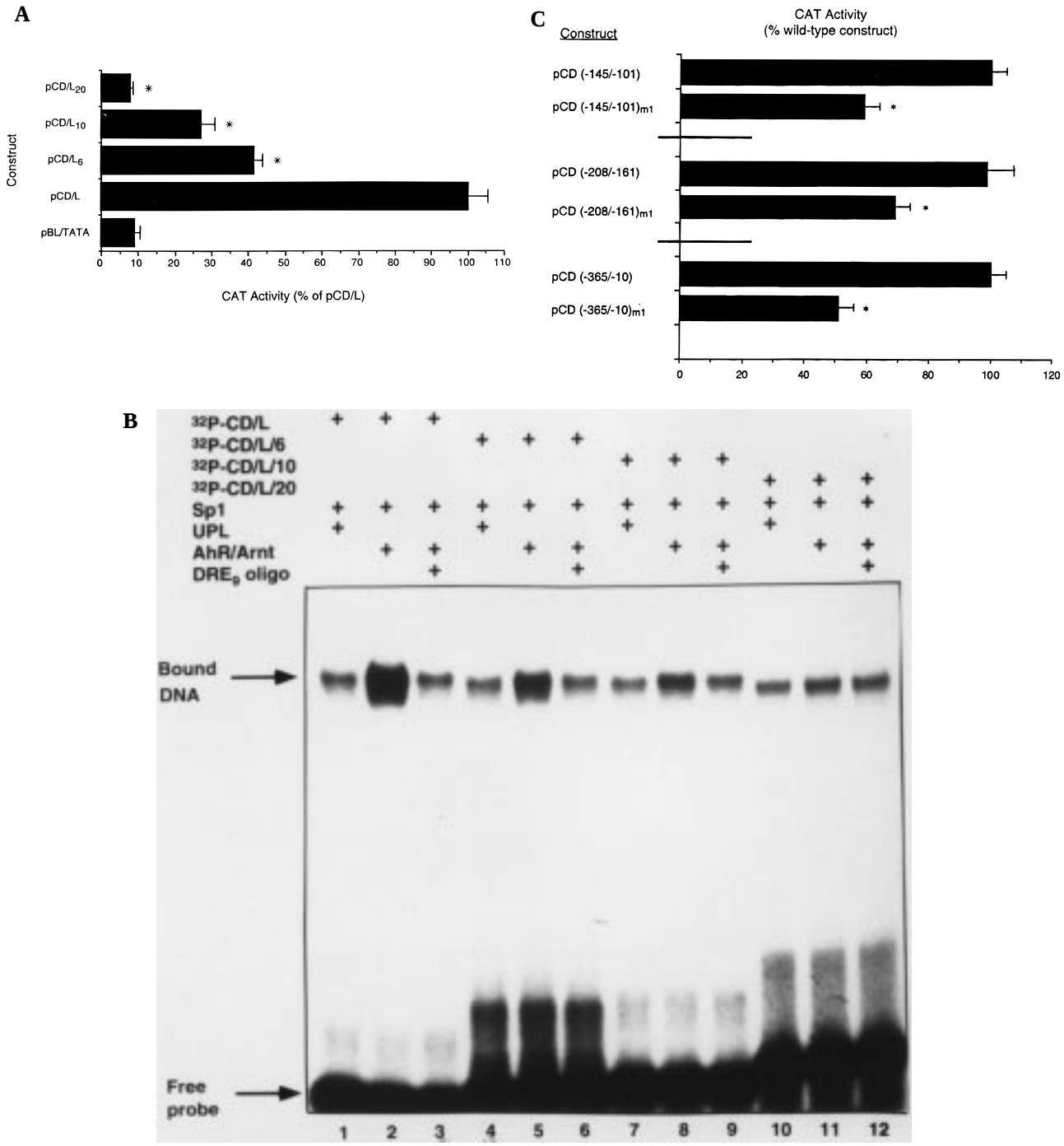

FIGURE 6: Effects of nucleotide spacing between Sp1 and DRE sites. Role of the core DRE motif in the activity of constructs containing other cathepsin D gene promoter inserts. (A) Basal CAT activities. MCF-7 cells were transfected with pCD/L, pCD/L_6, pCD/L_{10}, and pCD/L_{20}, and CAT activities were determined as described in Materials and Methods. Compared to that of wild-type pCD/L (set at 100 ± 5), CAT activities for pCD/L_6, pCD/L_{10}, pCD/L_{20}, and the empty vector pBL/TATA were significantly (*$p < 0.05$) lower. (B) Effects of AhR−Arnt on Sp1−DNA binding. ^{32}P-labeled oligonucleotides were incubated with Sp1 protein (10 ng) alone or in combination with AhR−Arnt and analyzed by gel mobility shift assay as described in Materials and Methods. ^{32}P-labeled CD/L, CD/L_6, CD/L_{10}, and CD/L_{20} bound Sp1 protein to form retarded bands (lanes 1, 4, 7, and 10, respectively); coincubation of these same oligonucleotides with Sp1 and AhR−Arnt resulted in significantly enhanced ($p < 0.05$) retarded band formation (compared to incubation with Sp1 protein alone) with CD/L (lane 2, 3.32-fold), CD/L_6 (lane 5, 1.76-fold), and CD/L_{10} (lane 8, 1.45-fold), but not with CD/L_{20} (lane 11, 1.12-fold). The levels of AhR−Arnt-mediated enhanced binding to ^{32}P-labeled CD/L, CD/L_6, and CD/L_{10} were significantly decreased by competition with a 100-fold excess of unlabeled $(DRE)_9$ oligonucleotide (lanes 3, 6, and 9, respectively). Unprogrammed lysate was used as a control for effects of in vitro-translated AhR−Arnt (lanes 1, 4, 7, and 10). (C) Comparative CAT activities using constructs containing wild-type and mutant core DREs. Basal CAT activities were determined in MCF-7 cells transfected with wild-type pCD(−145/−101), pCD(−208/−161), and pCD(−365/−10) and core DRE mutant pCD(−145/−101)$_{m1}$, pCD(−208/−161)$_{m1}$, and pCD(−365/−10)$_{m1}$ constructs; compared to those of wild-type plasmids, activities for the core DRE mutants were decreased 41, 31, and 49%, respectively.

labeled oligonucleotides (gel mobility shift assays) (Figures 1−3) confirmed that interactions of Sp1 and AhR−Arnt with the Sp1$(N)_4$DRE (core) were required for basal activity of pCD/L. The importance of the core DRE within CD/L and a second upstream core DRE (residues −183 to −179) to basal activity were also confirmed in transient transfection

assays using constructs containing more extensive regions of the cathepsin D gene promoter (Figure 6C). Moreover, utilizing antisense Arnt expression in MCF-7 cells and Arnt expression in Arnt-deficient Hepa 1c1c7 cells, it was further demonstrated that AhR−Arnt was required for maximal basal activity of pCD/L (Figure 4). Kobayshi and co-workers (*44*) previously reported that both AhR and Arnt antibodies immunoprecipitate [^{35}S]Sp1 protein after incubation with AhR or Arnt proteins, and complementary results were obtained in this study using [^{35}S]Arnt protein (Figure 2A). Indirect evidence for interaction of AhR−Arnt with Sp1 was also observed in kinetic studies showing the AhR−Arnt markedly enhanced the on-rate and B_{max} value (5-fold) for Sp1−CD/L complex formation. Similar results have been observed for ER−Sp1 proteins where ER enhanced the rate of Sp1−DNA complex formation using several different GC-rich oligonucleotides; however, supershifted complexes were not observed (*26*, *31*−*33*). Sterol regulatory element binding protein also enhanced Sp1−DNA complex formation (*21*); other examples of protein-enhanced binding of transcription factors to their cognate DNA elements include human T-cell leukemia virus-1 transcriptional activator Tax-enhanced DNA binding of bZip and cAMP response element binding protein (*65*, *66*) and cyclin D1-enhanced DNA binding of ER (*67*).

In the absence of added ligand, the level of consensus DRE-dependent transactivation is increased in Ah-responsive mouse Hepa-1 cells by addition of ellipticine, a CYP1A1 inhibitor, and it has been suggested that a CYP1A1 substrate may be an endogenous AhR ligand (*45*). In this study, ellipticine also increased reporter gene activity in Hepa-1 and MCF-7 cells transfected with $pDRE_3$ (containing three consensus DREs) (Figure 5), as previously reported (*45*). In contrast, basal activity associated with AhR−Arnt interactions with the Sp1(N)$_4$DRE(core) motif in CD/L was not affected by treatment with ellipticine (Figure 5). This does not exclude the possibility of a role for other endogenous AhR ligand(s) in modulating basal activity through the core DRE.

Previous studies (*11*) have shown that Sp1 physically interacts with E2F1; however, the importance of their respective cognate enhancer sequences and their proximity was promoter- and possibly cell-specific. Both E2F1 and Sp1 binding sites were required for transactivation of constructs derived from the thymidine kinase gene promoter in Swiss 3T3 cells, and as the distance between these sites was increased by 6, 10, and 20 bp, there was a corresponding decrease in basal activity (*9*). Using a similar approach, it was also shown that as the distance between the GC-rich and core DRE sites was increased by 6, 10, and 20 bp, the basal activity of the corresponding constructs also decreased. Moreover, the level of enhanced Sp1−DNA binding by AhR−Arnt proteins was also decreased using the same oligonucleotides. Thus, there was a correlation between the functional and physical interactions of Sp1 with AhR−Arnt. However, these results were somewhat paradoxical since the 10 bp insertion would maintain the DNA-bound proteins on the same face of DNA and should not significantly affect protein−protein interactions. Thus, ongoing studies are extensively investigating physical and functional interactions of Sp1 with AhR−Arnt, E2F, and other Sp1-binding proteins using variable oligonucleotides, mutant proteins, and variable cellular context.

In summary, results of this study demonstrate physical and functional interactions between the Sp1 and AhR−Arnt proteins that bind to the Sp1(N)$_4$DRE(core) motif in the CD/L gene promoter. These interactions were observed in the absence of exogenous AhR ligand and thus may represent an endogenous function for the AhR−Arnt complex. Results of other studies with AhR-knockout mice and AhR-deficient cells demonstrate that the AhR protein (in combination with Arnt) may influence multiple cell- and tissue-specific responses (*68*−*70*), and this could be related, in part, to regulation of basal gene expression as observed in this study with pCD/L. Current research is focused on other gene promoters that contain Sp1(N)$_x$DRE(core) motifs (*46*−*64*) and the role of interactions between AhR−Arnt and Sp1 protein in regulating constitutive expression of these genes.

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