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14. ABSTRACT: The aryl hydrocarbon receptor (AhR) is an environmental carcinogen-activated transcription factor associated with tumorigenesis. Little is known of the transcriptional consequences of constitutive AhR activation. The effects of constitutively active and environmental ligand-induced AhR on c-myc, an oncogene, were investigated. Results indicate that: (1) the AhR constitutively binds the c-myc promoter; (2) there is a low but significant baseline level of c-myc promoter activity, which is not regulated by NF-kappaB and is not affected by an environmental AhR ligand; (3) deletion of any one of the AhREs has no effect on constitutive reporter activity, while deletion of all six increases reporter activity approximately fivefold; (4) a similar increase in reporter activity occurs when constitutively active AhR is suppressed by transfection with an AhR repressor plasmid (AhRR); (5) AhRR transfection significantly increases background levels of endogenous c-myc mRNA and c-Myc protein. These results suggest that the AhR influences the expression of c-Myc, a protein critical to malignant transformation.					
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DOD Training Grant Annual Summary-2006

Introduction

It has long been suggested that ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), contribute to human breast cancer. The preferential targeting of breast tissue by orally administered PAH in rodent breast cancer models supports this contention. Most of the biologic activity of PAH and related dioxins is mediated by the AhR. AhR activation can induce cytochrome P-450 enzymes, proto-oncogenes (e.g. *c-myc*, *Ha-ras*, *c-erb-2*) and transcription factors (e.g. NF- κ B). Therefore, it is possible that AhR activation plays an important role in the initiation and progression of carcinogenesis by regulating a cascade of intracellular events involving NF- κ B, *c-Myc*, and/or other proto-oncogenes. To investigate the role of the AhR in breast cancer development, we originally proposed three aims: Aim 1 is to map the contact domains between AhR and Rel A, aim 2 is to assess AhR regulation of NF- κ B activity and *c-myc* transcription in mammary tumor cells, aim 3 is to characterize AhR regulation of cell cycle components in mammary tumor lines.

Our previous studies demonstrated that, in other cell types, galangin is a potent inhibitor of the aryl hydrocarbon receptor (AhR), an environmental carcinogen-responsive transcription factor implicated in mammary tumor initiation and growth control. Our previous results indicated that breast cancer Hs578T cells expressed high levels of constitutively active AhR. Constitutive and environmental chemical-inducible AhR activity was profoundly suppressed by galangin as was cell growth. However, the failure of a-naphthoflavone or FhAhRR transfection to block growth indicated that galangin-mediated AhR inhibition was either insufficient or unrelated to its ability to significantly block cell growth at therapeutically relevant doses. Galangin inhibited transition of cells from the G0/G1 to the S phases of cell growth, likely through the nearly total elimination of cyclin D3. The results suggest that this non-toxic bioflavonoid may be useful as a chemotherapeutic, particularly in combination with agents which target other components of tumor cell cycle and in situations where estrogen receptor-specific therapeutics are ineffective. This previous study was to complete aim 3.

This year we continued to work under aim #2 and finish some work left under aim #3. Our work resulted in two papers published during this report period. First, our study, the aryl hydrocarbon receptor constitutively represses *c-myc* transcription in human mammary tumor cells, was published on *Oncogene*. Then, another manuscript, growth of a human mammary tumor cell line is blocked by galangin, a naturally occurring bioflavonoid, and is accompanied by down-regulation of cyclins D3, E, and A, was published on *Breast Cancer Research* in early 2006. Here we share our new findings in our study of the relationships between AhR, *c-myc* and NF- κ B.

Body

Materials and methods

Cell culture

The estrogen receptor-negative Hs578T tumor cell line (ATCC, Manassas, VA, USA) was derived from a human mammary carcinoma and is epithelial in origin (Hackett et al., 1977). The nontransformed Hs578Bst myoepithelial cell line was derived from normal tissue adjacent to the tumor from which Hs578T cells were derived (Hackett et al., 1977). Both lines were grown in a humidified, 5% CO₂ atmosphere at 37°C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax-1 (L-alanyl-L-glutamine), supplemented with 10% fetal calf serum, penicillin (500 IU/ml), streptomycin (5 mg/ml), and L-glutamine (2 nM). All media components were purchased from Invitrogen (Carlsbad, CA, USA).

Protein extraction and Western immunoblotting

Total cell lysates were prepared from Hs578T and Hs578Bst cells by incubating washed cell pellets for 10 min in lysis buffer (50 mM KHPO₄, pH 7.4, 5 mM DTT) and 10 l/ml protease inhibitor cocktail (Sigma, St Louis, MO, USA) on ice. Following a 10 min centrifugation, protein concentrations of total cell lysates were quantified using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Nuclear and cytoplasmic fractions were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of protein (typically 40 g for Hs578T cells and up to 120 g for Hs578Bst cells) were boiled for 5 min in 1 SDS–PAGE sample buffer (50 mM Tris buffer, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue and 1% -mercapthoethanol) before SDS–PAGE electrophoresis through a 6.8% polyacrylamide gel and overnight transfer onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Following transfer, membranes were blocked with 5% skim milk powder in 1 TBS plus 0.05% Tween-20 (TBST). The primary antibody was either polyclonal rabbit anti-human AhR antibody (Santa Cruz, CA, USA) or mouse monoclonal anti-human c-Myc antibody (Santa Cruz, CA, USA) and the secondary antibody was HRP-linked rabbit Ig-specific goat antibody (Pierce, Rockford, IL, USA). Bands were detected using enhanced chemiluminescence substrate (Sigma, St Louis, MO, USA) and exposure to X-ray film (Fuji, Japan). Membranes were stripped with the Re-Blot Western Blot Recycling Kit (Chemicon, Temecula, CA, USA) and reblotted with -actin-specific mAb (Sigma, St Louis, MO, USA) to confirm equal loading. Where indicated, blots were reprobbed with -tubulin- (Oncogene Research, Boston, MA, USA) or lamin A/C- (Novocastra Laboratories, UK) specific mAbs to confirm the purity of cytoplasmic and nuclear protein extracts, respectively.

Chromosome immunoprecipitation

ChIP and DNA quantification were performed as described previously (Hestermann and Brown, 2003). For gel electrophoresis, the region of the c-myc promoter 95–245 bp upstream of the transcription start site was amplified using the primers 5'-AGGCGCGCGTAGTTAATTCAT-3' and 5'-CGCCCTCTGCTTTGGGA-3' for 43 cycles (95°C for 15 s, 58°C for 60 s). The region of the CYP1A1 promoter 784–1156 bp upstream of the start site was amplified using the primers 5'-CACCCGCCACCCTTCGACAGTTCT-3' and 5'-

CTCCCGGGGTGGCTAGTGCTTTGA-3' for 40 cycles (95°C for 45 s, 58°C for 45 s, and 70°C for 60 s) as we described previously (Hestermann and Brown, 2003). In our hands, DNA is sheared within approximately 1 kb of the amplified fragment. Therefore, ChIP detects protein-bound DNA within approximately 1 kb of the region spanned by the primers. For quantification, equal amounts of DNA from each sample were amplified by real-time PCR using the same primers with SYBR Green master mix (Applied Biosystems, Bedford, MA, USA) in a DNA Engine Opticon 2 (MJ Research, Waltham, MA, USA).

Reporter plasmids and site-directed mutagenesis

The pGL3-c-myc reporter was constructed by cloning the HindIII (Blunted)–SacI human c-myc promoter fragment of pVCAT (kindly provided by Dr DL Levens; NCI) into the KpnI (Blunted)–SacI sites of the pGL-basic luciferase reporter plasmid (Promega, Madison, WI, USA). Deletion of two NF- κ B-binding sites in this plasmid was achieved by site-directed mutagenesis using two deoxyoligonucleotide primers: 5'-/5Phos/GAG TTA ACG GTT TTT TTC ACA ATG ACT CCC CCG GCT CGG-3' and 5'-/5Phos/GGC TAT TCT GCC CAT TTG CCC GCC GCT GCC AGG-3'. The resulting plasmid was designated 'pGL3-NF-BKO'. This plasmid was used as the template for further site-directed mutagenesis of AhREs as described (Matikainen et al., 2001). Plasmids in which each individual AhRE or pairs of AhREs (i.e. AhRE 3 and 4) were mutated (pGL3-AhRE1Mut, pGL3-AhRE2Mut, pGL3-AhRE3,4Mut, pGL3-AhRE5Mut, and pGL3-AhRE6Mut) were generated with the following primers: 5'-/5Phos/CCG TGT GGG AGG AAT GGG GGT GGG ACG-3' (pGL3-AhRE1Mut), 5'-/5Phos/CCC TAT CTA CAC TAA CAT CCC ATT CTC TGA ACG CGC GCC-3' (pGL3-AhRE2Mut), 5'-/5Phos/GCA GCC TGG TAC GCG AAT GGA ATG GCG GTG GGC GCG C-3' (pGL3-AhRE3,4Mut), 5'-/5Phos/GGG TTC CCA AAG CAG AGG GAA TGG GCG AAA AGA AAA AAG ATC C-3' (pGL3-AhRE5Mut); 5'-/5Phos/CTG CCT TAT GAA TAT ATT CAT TCT GAC TCC CGG CCG GTC GG-3' (pGL3-AhRE6Mut). The positions of the mismatches are underlined. A plasmid in which all six AhREs were mutated (pGL3-AhRE1-6Mut) was produced by sequential mutation of each of the AhREs. All of the site-directed mutagenesis was conducted using the QuickChange multisite-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed on each plasmid to verify the deletions/mutations and to confirm that no other sequence changes had occurred.

AhR-dependent expression of the pGudLuc6.1-firefly luciferase reporter construct (pGudLuc) is driven by four AhREs derived from the CYP1A1 promoter (Han et al., 2004). This construct has been optimized for TCDD responsiveness and was kindly provided by Dr M Denison (UC Davis).

Cloning and characterization of the FhAhRR expression vector has been described previously (Karchner et al., 2002). The product of this vector efficiently suppresses mammalian AhR activity induced by TCDD (Karchner et al., 2002).

Transient transfections, TCDD treatment, and luciferase assays

Hs578T cells (3 × 10⁴/well) were plated in 12-well culture plates and cultured to 80% confluence. Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's instructions to transfect cells. The Renilla luciferase vector pRL-TK (0.05 g) was cotransfected with Firefly luciferase reporter constructs (0.1 g pGudLuc, 0.5–1.0 g wild-type pGL3-c-Myc or mutant constructs). Where indicated, 0.5 g of pcDNA-FhAhRR or control pcDNA3.1 (Invitrogen) was added to the transfection mixture. For each experiment, the amount of total DNA transfected was equilibrated with parental expression vectors. Cells were incubated overnight, washed twice with phosphate-buffered saline (PBS) (pH 7.2), and resuspended in 75 l RPMI prior to luciferase analysis. Luciferase activity was determined with the Dual Glo Luciferase system (Promega, Madison, WI, USA), which allowed sequential reading of the Firefly and Renilla signals. Cells were lysed according to the manufacturer's directions (Promega, Madison, WI, USA), transferred to 96-well white wall plates, and analysed using a Reporter Luminometer (Promega, Madison, WI, USA). The Renilla signal was

read after quenching the Firefly output, thus allowing normalization between sample wells. The normalized Firefly luciferase signal is expressed relative to the Renilla signal.

TCDD was obtained from Cambridge Isotopes Laboratories (Andover, MD, USA) at >99% purity and was maintained as a 1000 stock solution in anhydrous tissue culture grade dimethylsulfoxide (DMSO). TCDD (1.0 nM) in DMSO or DMSO alone (final volume 0.1%) was added to cultures 2 h after transfections and cells were incubated for an additional 24 h. Cells then were washed twice with PBS (pH 7.2) and resuspended in 75 l RPMI for luciferase analysis.

Quantitative c-myc-specific real-time PCR

Hs578T cells were plated onto two 10 cm cell culture dishes (106 cell/well) and cultured to 80% confluence. Equal amounts of pcDNA-FhAhRR or parental vector pcDNA3.1 were added to separate plates and cells transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, adherent Hs578T cells were washed twice with PBS (pH 7.2) and harvested. Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA, USA). RNA samples were treated with RNase-free DNase according to the manufacturer's instructions. Total RNA was eluted from the columns with 60 l RNase-free water and quantified by UV absorbance. First-strand cDNA was synthesized using 2 g of each total RNA, random hexamers, and SuperscriptII reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real-time PCR amplification mixtures (25 l) contained 1 l template cDNA, 2 SYBR Green I Master Mix buffer (12.5 l) (Applied Biosystems, Foster City, CA, USA), and 300 nM of a forward and reverse primer pair. The sequences for c-myc amplification in real-time PCR were described previously (Latil et al., 2000) and are as follows: sense, 5'-ACC ACC AGC AGC GAC TCT GA-3'; antisense, 5'-TCC AGC AGA AGG TGA TCC AGA CT-3'. Human ribosomal RNA, amplified with previously published primer sequences (Nazarenko et al., 2002), was used for RNA normalization of the c-myc signal as described previously (Saez et al., 2003). The ribosomal RNA primers were as follows: sense, 5'-GAC TCA TTC GCC CTG TAA TTG GAA TGA GTC-3'; antisense, 5'-CCA AGA TCC AAC TAC GAG CTT-3'. Reactions were run in an ABI PRISM 5700 Sequence Detector (Applied Biosystems) using the following standard cycling conditions: 10 min polymerase activation at 95°C, 40 cycles for 15 s at 95°C, and 60°C for 60 s. Results for each experiment are calculated from three replicate PCR reactions. Threshold cycle (CT) values were collected at linearity. Relative mRNA expression was normalized against internal ribosomal controls. The parameter 2-CT, where CT equals CT of the c-myc signal minus the CT of the endogenous ribosomal RNA control, was used to describe the relative levels of c-myc mRNA normalized to 18S rRNA.

Data analyses

Statistical analyses were performed with Statview (SAS Institute, Cary, NC, USA) or Excel. Data from a minimum of three experiments are presented as means standard errors (s.e.). One-factor ANOVAs and a Fisher PLSD post hoc comparisons test or the Student's t-test were used to determine significant differences.

Results

Constitutive nuclear AhR localization and binding to the c-myc promoter in Hs578T mammary tumor cells but not in a syngeneic normal mammary cell line

A number of studies demonstrate AhR expression, usually at notably high levels, in rodent and human tumors (Ma and Whitlock, 1996; Weiss et al., 1996; Ge and Elferink, 1998; Puga et al., 2000, 2002; Trombino et al., 2000; Safe, 2001; Koliopanos et al., 2002; Abdelrahim et al., 2003; Hayashibara et al., 2003; Zhang et al.,

2003; Thomsen et al., 2004). Furthermore, we have shown in a primary mammary tumor model that much of that AhR resides in the tumor cell nucleus, a result suggestive of constitutive AhR activation in the absence of exogenous ligands (Wang et al., 1995; Trombino et al., 2000). To develop a model system in which the effects of constitutively active human AhR on gene transcription in mammary tumors can be evaluated, AhR expression, localization, and/or function were evaluated in the malignant human mammary tumor cell line, Hs578T, and in a syngeneic normal myoepithelial cell line, Hs578Bst.

Nontransformed Hs578Bst cells expressed low but significant levels of AhR protein, while malignant Hs578T cells expressed significantly higher levels of AhR (Figure 1a). Furthermore, approximately one-third of the AhR in Hs578T cells was located in the nucleus (Figure 1b), a result consistent with constitutive AhR activation. No nuclear AhR was detected in normal Hs578Bst cells, even when loading 120 g nuclear protein extract/well and after long exposures (>5 s) of the film to the Western blot (Figure 1c).

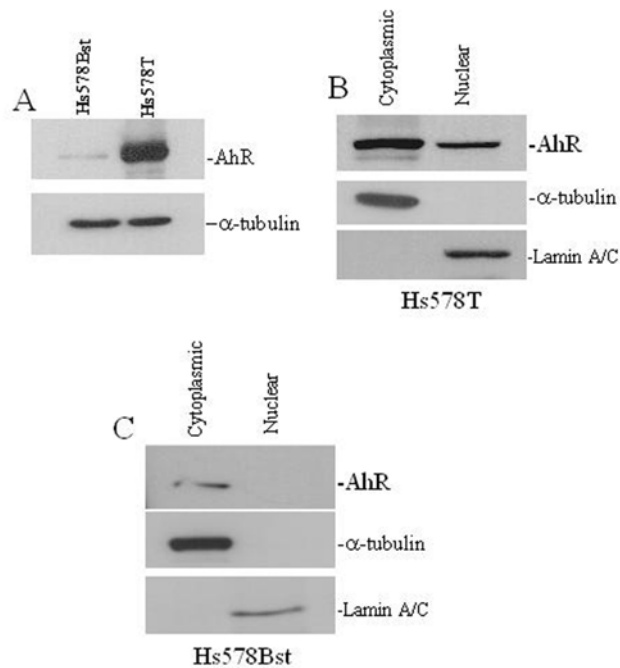


Fig 1 Hs578T human mammary tumor cells express high levels of cytoplasmic and nuclear AhR. **(a)** Total AhR protein was extracted from nonmalignant Hs578Bst and from malignant Hs578T cells and 40 μ g of extracts analysed by Western blotting. Blots were stripped and reprobed for α -tubulin to confirm equal loading of wells. Representative data from a total of three experiments are presented. **(b)** Cytoplasmic and nuclear cell extracts prepared from subconfluent monolayers of malignant Hs578T cells were analysed by Western immunoblotting with AhR-specific antibody following SDS-PAGE. Blots were stripped and reprobed for lamin A/C and α -tubulin to confirm purity of the nuclear and cytoplasmic cell fractions, respectively. Representative data from a total of three experiments are shown. **(c)** Cytoplasmic and nuclear cell extracts (120 μ g) prepared from subconfluent monolayers of Hs578Bst cells were analysed by Western immunoblotting with AhR-specific antibody following SDS-PAGE. Blots were stripped and reprobed for lamin A/C and α -tubulin to confirm purity of the nuclear and cytoplasmic cell fractions, respectively. Extended exposure times (3–5 s) were used to detect extremely low AhR levels in Hs578Bst cells. Representative data from a total of three experiments are shown

In considering possible gene targets for what appears to be active AhR in tumor cells, we noted the presence of six consensus AhREs within a 3.2 kb region of the human c-myc promoter (Figure 2). If the AhR affects c-myc gene transcription in the absence of exogenous ligands, then it would be predicted that the AhR would constitutively bind this promoter region. Chromosome immunoprecipitation (ChIP) experiments were performed to test this hypothesis. As seen in Figure 3a, a relatively strong c-myc-specific PCR signal was seen

following immunoprecipitation with AhR-specific antibody and DNA amplification with c-myc-specific primers. Indeed, immunoprecipitation of the AhR complex resulted in a significant increase in the c-myc signal relative to the input control (Figure 3b, $P < 0.02$).

```

-1155      NF-κB binding site 1      -1120 -919      AhRE1
AACGGTITTTTTCACAAAGGGTCTCTGCTGACTCCCC--//--TCCGTGTGGGAGGCGTG

-893      -522      AhRE2      -495 -409
GGGGTGGGAC--//--CACTAACATCCACAGCTCTGAACGCGCG--//--CAGCCTGTACGC

AhRE3 AhRE4      -372 -96      AhRE5
GCGTGGCGTGCGCGTGGGCGCGAG--//--TCCCAAAGCAGAGGCGGTGGGGAAAA

-62 -10      +1 (P1)      +16 +448      NF-κB binding site 2
GAAAAAAG--//--GACGGCTGAGGACCCCGAGCTGTGCT--//--CTGCCCATTTGGGGACAC

+476 +816      AhRE6      +841
TTCCCGCGCGC--//--ATGAATATATTACAGCTGACTCCCGG

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Fig 2. The human *c-myc* promoter contains six aryl hydrocarbon response elements (AhREs) and two NF- κ B-binding sites. The locations of six consensus AhR binding motifs and two NF- κ B-binding sites are indicated

To determine if the constitutively active AhR binds to all AhRE-containing promoters uniformly, ChIP assays were performed with Hs578T cells using both CYP1A1- and *c-myc*-specific primers. Interestingly, AhR from Hs578T cells did not bind to the endogenous CYP1A1 promoter region amplified by the primers to any appreciable extent unless the cells were pretreated with TCDD, a strong AhR activator (Figure 3c, left histograms). As in previous experiments (Figure 3b), the AhR constitutively bound to the *c-myc* promoter (Figure 3c, fifth histogram). However, no further increase in *c-myc* promoter binding was observed following TCDD treatment. These data suggest that the constitutively active AhR in this mammary tumor line preferentially binds to the endogenous *c-myc* promoter.

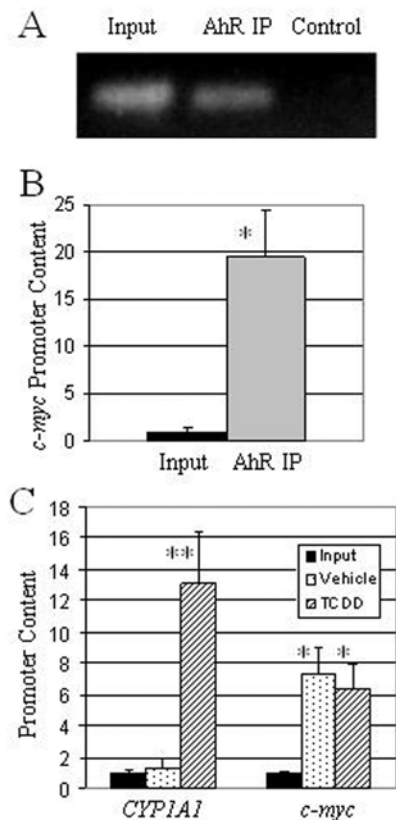


Fig 3. The AhR constitutively associates with the c-myc promoter. ChIP was performed on untreated Hs578T cells as described in Materials and methods. (a) PCR of DNA from the input fraction, AhR immunoprecipitation fraction (IP), and a no-antibody control. Representative data from a total of three ChIP experiments are presented. (b) Recovery of the c-myc promoter from equal quantities of DNA was measured by real-time PCR and normalized to the input fraction. Data are pooled from three experiments and expressed as mean $\pm$ s.e. An asterisk (*) indicates a significant level of promoter binding relative to the input control, $P < 0.02$. (c) Hs578T cells were treated for 20 min before ChIP analysis with AhR-specific antibody for immunoprecipitation and CYP1A1-specific and c-myc-specific primers. Data are pooled from three experiments and expressed as mean $\pm$ s.e. A single (*) or double (**) asterisk indicates a significant level of promoter binding relative to the input control, $P < 0.03$ or $P < 0.01$, respectively

AhRE-dependent regulation of the c-myc promoter

The AhR can both induce (Matikainen et al., 2001, 2002; Nebert et al., 1991) and suppress gene transcription (Sulentic et al., 1998; Wang et al., 1999, 2001). Therefore, a human c-myc promoter-reporter construct (pGL3-c-myc) was used to determine if constitutive AhR binding to the c-myc promoter effects an increase or decrease of c-myc levels. Since the 3.2 kb c-myc promoter region encompassing the six AhREs also contains two NF-B-binding sites, and since NF-B is well known for its ability to regulate c-myc (Duyao et al., 1990; Kessler et al., 1992), it also was important to evaluate the possible contribution of NF-B to background levels of c-myc transactivation. To address these issues, pGL3-basic, a control plasmid containing a minimal promoter sequence, wild-type pGL3-c-myc, and a series of reporter constructs mutated in the NF-B- and/or the AhR-binding sites were transfected into Hs578T cells and reporter activity assayed 24 h later.

Transfection of wild-type pGL3-c-myc into Hs578T cells resulted in approximately a 50% increase in the background level of c-myc promoter activity as compared with cells transfected with the parental pGL3-basic reporter plasmid (Figure 4a, first and second bars, $P < 0.001$). Since mammary tumors, including Hs578T cells, frequently have elevated levels of transcriptionally active NF-B (Kim et al., 2000a, 2000b; Cao and Karin, 2003; Helbig et al., 2003), it was anticipated that deletion of both NF-B-binding sites would result in a decrease in constitutive c-myc reporter activity. However, deletion of both NF-B-binding sites (pGL3-NF-BKO) had no effect on reporter transactivation (Figure 4, third bar).

Mutation of individual AhREs (pGL3-AhRE1Mut, pGL3-AhRE2Mut, pGL3-AhRE5Mut, pGL3-AhRE6Mut) or a pair of adjacent AhREs (pGL3-AhRE3,4Mut) following deletion of the two NF-B-binding sites had no effect on constitutive c-myc reporter activity (Figure 4, bars 5–9). However, deletion of all six AhREs (pGL3-AhR1-6Mut) resulted in a significant, 4–5-fold increase in reporter activity (fourth bar, $P < 0.001$). These data suggest that constitutively active AhR represses baseline levels of c-myc transcription and that not all of the AhREs are required for AhR-mediated repression.

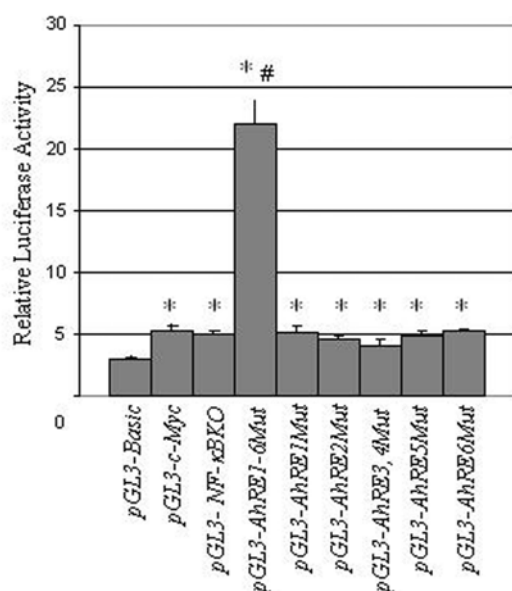


Fig 4. Deletion of all six AhREs increases constitutive c-myc promoter activity. Hs578T cells were transfected with 0.05 μ g *phRL-TK* and with 1.0 μ g *pGL3-basic*, wild-type *pGL3-c-Myc*, a reporter construct in which the two NF- κ B-binding sites were deleted (*pGL3-NF- κ B_{KO}*) or constructs in which the two NF- κ B sites were deleted, and individual AhREs (*pGL3-AhRE₁Mut*, *pGL3-AhRE₂Mut*, *pGL3-AhRE₅Mut*, *pGL3-AhRE₆Mut*), a pair of AhREs (*pGL3-AhRE_{3,4}Mut*) or all six AhREs (*pGL3-AhRE₁₋₆Mut*) were mutated. After 24 h, cells were harvested and lysates assayed for Firefly and Renilla luciferase activity. Data pooled from seven experiments are presented as the average normalized Firefly luciferase activity $\pm$ s.e. An asterisk (*) indicates a significant increase in normalized reporter activity relative to background activity observed following transfection with *pGL3-basic*, $P < 0.001$. A hash sign (#) indicates a significant increase in reporter activity relative to that seen in cells transfected with wild-type *pGL3-c-Myc*, $P < 0.001$.

Effect of a potent environmental AhR ligand, TCDD, on AhR-mediated repression of c-myc transactivation

A number of AhR ligands are carcinogenic. Indeed, some AhR ligands are used in animal models of mammary gland tumorigenesis (Daniel and Joyce, 1984; Russo and Russo, 1987; Das et al., 1989; McDougal et al., 1997; Trombino et al., 2000). Although a principle mechanism for AhR ligand carcinogenicity is the oxidation of parent compounds into mutagenic metabolites (Christou et al., 1987; Nebert et al., 1991; Buters et al., 1999), a process mediated by AhR-regulated CYP1 enzymes, other mechanisms, including the dysregulation of oncogenes, have been considered (Puga et al., 1992). Therefore, it is conceivable that binding of tumor cell AhR by exogenous environmental pollutants would release transcriptionally repressive AhR from the c-myc promoter, thereby augmenting the contribution of c-myc to the transformation process. Alternatively, hyperactivation of the AhR with environmental ligands could stabilize the AhR-c-myc complex, further inhibiting c-myc transactivation. To test these possibilities, the effect of a potent AhR ligand, TCDD, on AhR-mediated repression of c-myc reporter activity was evaluated. The effect of TCDD on a reporter construct known to be positively regulated by the AhR (i.e. *pGudLuc*) was evaluated first as a positive control.

Transfection of *pGudLuc* into Hs578T cells resulted in a significant 10-fold increase in reporter activity relative to that observed in cells transfected with the parental *pGL3-basic* plasmid (Figure 5a, second bar, $P < 0.04$). The addition of 1 nM TCDD further enhanced promoter activity (third bar; $P < 0.002$). The significant level of constitutive reporter activity is not inconsistent with data obtained by ChIP analysis and demonstrating

that constitutive AhR does not bind the endogenous human CYP1A1 promoter since the AhRE-containing pGudLuc reporter is derived from mice not humans, and since it has been modified and selected on the basis of maximal responsiveness to AhR ligands such as TCDD (Garrison et al., 1996; Ziccardi et al., 2000).

In contrast, TCDD treatment of Hs578T cells transfected with pGL3-c-myc had no effect on the low but significant ($P<0.002$) levels of reporter activity (Figure 5b, third and fourth bars). As in previous experiments, Hs578T cell transfection with pGL3-c-myc1-6Mut significantly increased the level of constitutive reporter activity (fifth bar, $P<0.001$). As expected, in the absence of AhREs in the reporter construct, TCDD had no further effect on reporter activity (sixth bar). These results suggest that hyperactivation of the AhR with environmental chemicals in Hs578T cells has no effect on c-myc transcription.

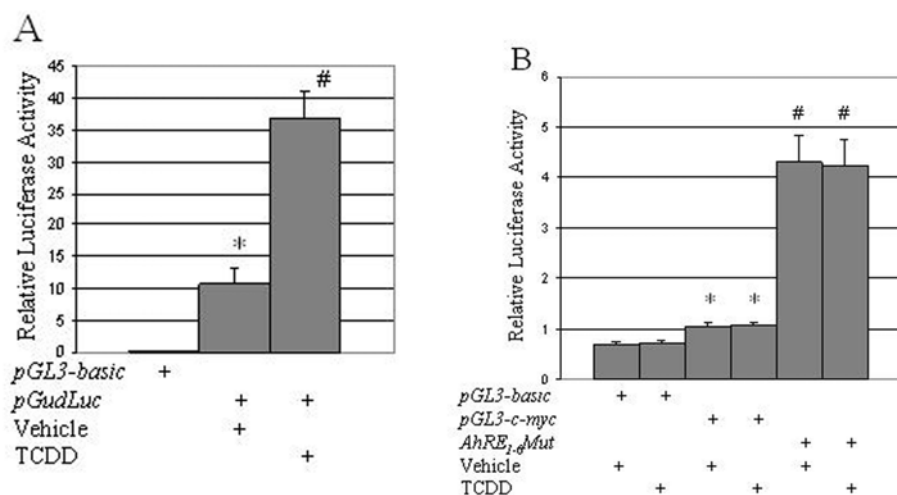


Fig 5. AhR activation with an exogenous ligand, TCDD, has no effect on c-myc reporter activity. (a) Subconfluent Hs578T cells were transfected with 0.1 μ g of a control vector (*pGL3-basic*) or with 0.1 μ g of a *CYP1A1* promoter-driven Firefly luciferase gene (*pGudLuc*) and 0.5 μ g of the Renilla luciferase vector *phRL-TK*. After 24 h, cultures were treated with vehicle (0.1% acetone) or with TCDD (10^{-9} M final concentration). Cells were harvested 24 h later and lysates assayed for Firefly and Renilla luciferase activity. Data pooled from four experiments are presented as the average normalized firefly luciferase activity $\pm$ s.e. An asterisk (*) indicates a significant increase in normalized reporter activity relative to *pGL3-basic*-transfected cells, $P<0.04$. A hash sign (#) indicates a significant increase relative to *pGudLuc*-transfected, vehicle-treated cells, $P<0.002$. **(b)** Cells were treated as above, except that 1.0 μ g wild-type *pGL3-c-myc* or AhRE mutant *pGL3-AhRE₁₋₆Mut* was substituted for the *pGudLuc* reporter. Data pooled from six experiments are presented as the average normalized Firefly luciferase activity $\pm$ s.e. An asterisk (*) indicates a significant increase in normalized reporter activity relative to the activity in *pGL3-basic*-transfected cells, $P<0.002$. A hash sign (#) indicates a significant increase relative to *pGL3-c-myc*-transfected cells, $P<0.001$

Inhibition of AhR activity derepresses c-myc promoter activity

Experiments with pGL3-c-myc mutants are consistent with a role for the AhR in constitutively repressing c-myc transactivation. However, the formal possibility that deletion of NF-B-binding sites and mutation of all six AhREs resulted in changes that could affect transcription factors other than NF-B and AhR could not be ruled out. Therefore, a second approach to evaluating putative AhR repression of c-myc transcription was taken. An evolutionarily conserved AhRR has been described in several species (Mimura et al., 1999; Baba et al., 2001; Watanabe et al., 2001; Karchner et al., 2002; Korkalainen et al., 2004). The AhRR potently inhibits AhR-dependent CYP1A1 transcription by competing for the AhR binding partner ARNT and by blocking AhR–AhRE binding (Mimura et al., 1999; Karchner et al., 2002). AhRR–ARNT complexes are transcriptionally inactive (Mimura et al., 1999). Notably, AhRR derived from killifish (*Fundulus heteroclitus*)

inhibits both human and mouse AhR-dependent transactivation in an AhR-specific manner (Karchner et al., 2002). In preliminary experiments, the *F. heteroclitus* AhRR (FhAhRR) was more effective at suppressing pGudLuc activity in Hs578T cells than a human AhRR expression construct (not shown). Therefore, the FhAhRR construct was chosen to test the prediction that inhibition of constitutive AhR activity in Hs578T cells would derepress AhR-dependent c-myc promoter transactivation.

Significant levels of FhAhRR were detected in Hs578T cells 24 h after transfection with FhAhRR (Figure 6). As expected from previous experiments demonstrating AhRR specificity (Karchner et al., 2002), FhAhRR transfection had no effect on SV40 promoter-driven, pGL3-promoter reporter activity (Figure 7a). As in previous experiments, pGudLuc transfection resulted in a significant (7-fold) increase in luciferase activity (Figure 7b, second bar) that was further augmented by the addition of TCDD (seventh bar). Cotransfection of cells with pGudLuc and 0.1, 0.5, or 1.0 μ g FhAhRR ablated the background levels of reporter activity (bars 3–5, $P < 0.001$) and significantly reduced the TCDD-induced reporter activity (bars 8–10, $P < 0.008$). These data confirm that FhAhRR is a potent inhibitor of both the constitutive and inducible human AhR in these tumor cells

As in previous experiments, transfection of Hs578T cells with the wild-type pGL3-c-myc reporter resulted in a low level of reporter activity, which, in this series of experiments, was not significantly greater than the background activity in pGL-basic-transfected cells (Figure 7c, third bar). Importantly, cotransfection of pGL3-c-myc with 1.0 μ g FhAhRR significantly ($P < 0.007$) increased reporter activity 3–4-fold (fourth bar). As expected, transfection with a construct in which all AhREs were mutated increased reporter activity (fifth bar, $P < 0.007$) and cotransfection with the FhAhRR had no further effect on the pGL3-AhRE1-6Mut luciferase activity (sixth bar). These data are consistent with the conclusion that the AhR constitutively represses c-myc transactivation through its interaction with at least some of the AhREs.

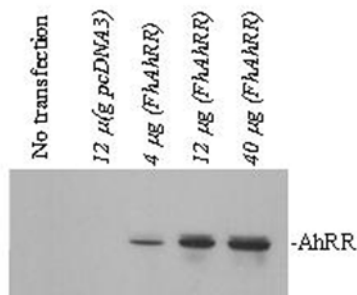


Fig 6. AhR repressor (AhRR) expression after Hs578T cell transfection. Hs578T cells were transfected with 0.5 μ g of *pcDNA3.1* or *pcDNA3-FhAhRR* (*FhAhRR*). After 24 h, cells were harvested, lysed, and 12–40 μ g of protein loaded into SDS–PAGE gels for Western immunoblotting with a purified species-specific AhRR-specific antibody. Immunoblotting of stripped gels with β -actin-specific antibody confirmed relative levels of protein loaded (not shown). Representative data from a total of three experiments are presented

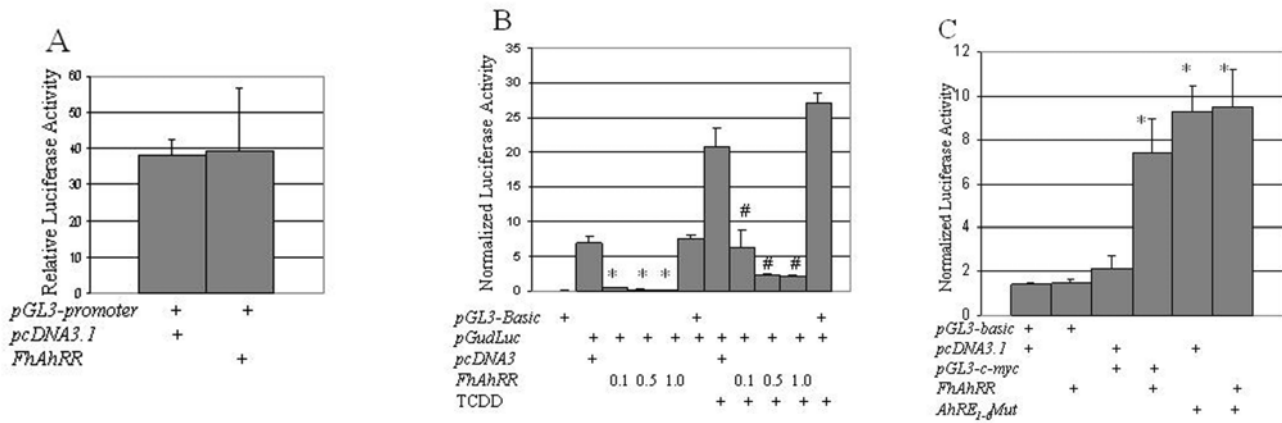


Fig 7. AhRR specifically inhibits constitutive *pGudLuc* and induces *pGL3-c-myc* promoter activity. (a) Hs578T cells were cotransfected with 1.0 μ g of a Firefly luciferase reporter construct driven by the SV40 promoter (*pGL3-promoter*), 0.5 μ g control vector (*pcDNA3.1*), or 0.5 μ g *pcDNA3-FhAhRR* (*FhAhRR*). Cells were harvested 24 h later and assayed for reporter activity as above. Data pooled from five experiments are presented as the average normalized Firefly luciferase activity $\pm$ s.e. (b) Hs578T cells were cotransfected in triplicate wells with control vectors *pGL3-basic* or *pcDNA3.1*, with 0.1 μ g *pGudLuc* and 0.05 μ g *phRL-TK*, and with titrated concentrations of *pcDNA3-FhAhRR* (*FhAhRR*) as indicated. After 24 h, cultures were treated with 10^{-9} M TCDD, where indicated. Cells were harvested 24 h later and lysates assayed for Firefly and Renilla luciferase activity. Data pooled from three experiments are presented as the average normalized Firefly luciferase activity $\pm$ s.e. An asterisk (*) indicates a significant decrease in normalized *pGudLuc* reporter activity in *FhAhRR*-transfected cells relative to cells transfected with the control vector, *pcDNA3.1*, $P < 0.001$. A hash sign (#) indicates a significant decrease in normalized *pGudLuc* reporter activity in TCDD-treated, *FhAhRR*-transfected cells relative to cells transfected with control vector, *pcDNA3.1*, and treated with TCDD, $P < 0.008$. (c) Hs578T cells were transfected with 1.0 μ g *pGL3-basic*, wild-type *pGL3-c-myc*, or AhRE mutant (*pGL3-AhRE₁₋₆Mut*) reporter plasmids, 0.05 μ g *phRL-TK*, and, where indicated, 1.0 μ g *pcDNA3-FhAhRR* (*FhAhRR*) or control plasmid *pcDNA3.1*. Cells were harvested 24 h later and lysates assayed for Firefly and Renilla luciferase activity. Data pooled from five experiments are presented as the average normalized Firefly luciferase activity $\pm$ s.e. An asterisk indicates a significant increase in normalized reporter activity relative to cells transfected with *pGL3-c-myc* and control vector *pcDNA3.1*, $P < 0.007$.

AhR-mediated repression of endogenous c-myc mRNA and c-Myc protein

While the data described above support regulation of tumor cell c-myc transcription by the AhR, they do not address the potential regulation of steady-state c-myc levels by the AhR in situ. To determine if constitutively active AhR affects the steady-state levels of c-myc, Hs578T cells were transfected with *FhAhRR* or control vector (*pcDNA3.1*) and c-myc mRNA levels quantified 24 h later by real-time PCR. Indeed, c-myc mRNA levels almost doubled following transfection with *FhAhRR* (Figure 8, $P < 0.05$).

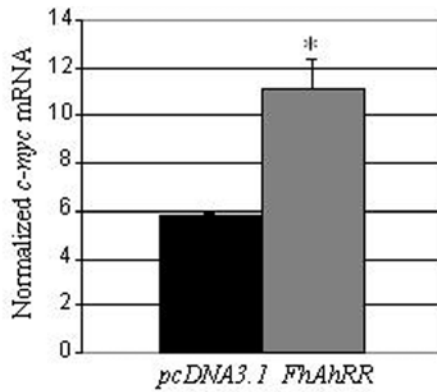


Fig 8. AhRR increases endogenous c-myc mRNA. Hs578T cells were transfected with 0.5 μ g control vector pcDNA3.1 or with 0.5 μ g pcDNA3-FhAhRR (FhAhRR). After 24 h, cells were harvested, RNA extracted, and c-myc RNA quantitated by real-time PCR. Data are pooled from three experiments and are presented as the average normalized c-myc levels $\pm$ s.e. An asterisk (*) indicates a significant increase in c-myc mRNA, $P < 0.05$

Similarly, FhAhRR transfection significantly increased c-Myc protein levels approximately two-fold (Figure 9a and b, $P < 0.004$). Although many factors contribute to maintenance of steady-state mRNA and protein levels, these data suggest that at least one contributor to the downregulation of c-myc mRNA and c-Myc protein is constitutively active AhR.

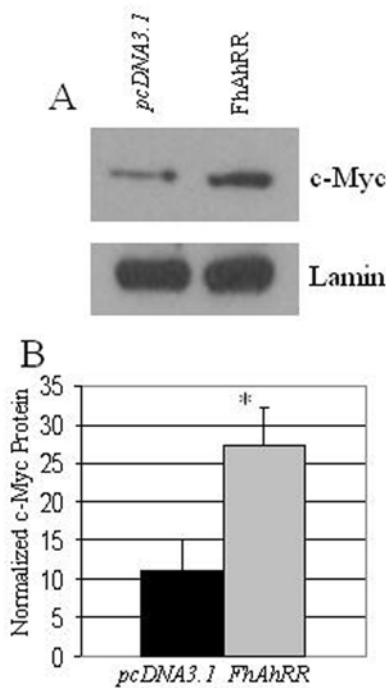


Fig 9. AhRR increases endogenous c-Myc protein. Hs578T cells were transfected with 0.5 μ g control vector pcDNA3.1 or with 0.5 μ g pcDNA3-FhAhRR (FhAhRR). After 24 h, cells were harvested, protein extracted, and c-Myc quantitated by Western blotting. (a) A representative Western blot (three experiments total). (b) c-Myc band densities were normalized to lamin band densities. Data are pooled from three experiments and are presented as the average normalized c-Myc levels $\pm$ s.e. An asterisk (*) indicates a significant increase in c-Myc protein, $P < 0.004$

Key Research Accomplishments

The aryl hydrocarbon receptor (AhR) is an environmental carcinogen-activated transcription factor associated with tumorigenesis. High levels of apparently active AhR characterize a variety of tumors, even in the absence of environmental ligands. Despite this association between transformation and AhR upregulation, little is known of the transcriptional consequences of constitutive AhR activation. Here, the effects of constitutively active and environmental ligand-induced AhR on c-myc, an oncogene whose promoter contains six AhR-binding sites (AhREs (aryl hydrocarbon response elements)), were investigated. A reporter containing the human c-myc promoter, with its six AhREs and two NF-B-binding sites, was constructed. This vector, and variants with deletions in the NF-B and/or AhR-binding sites, was transfected into a human breast cancer cell line, Hs578T, which expresses high levels of apparently active, nuclear AhR. Results indicate these key research accomplishments:

1. The AhR constitutively binds the c-myc promoter;.
2. There is a low but significant baseline level of c-myc promoter activity, which is not regulated by NF-B and is not affected by an environmental AhR ligand;
3. Deletion of any one of the AhREs has no effect on constitutive reporter activity, while deletion of all six increases reporter activity approximately fivefold
4. A similar increase in reporter activity occurs when constitutively active AhR is suppressed by transfection with an AhR repressor plasmid (AhRR)
5. AhRR transfection significantly increases background levels of endogenous c-myc mRNA and c-Myc protein.
6. These results suggest that the AhR influences the expression of c-Myc, a protein critical to malignant transformation

Reportable Outcomes

1. Yang X, Liu D, Murray TJ, Mitchell GC, Hesterman EV, Karchner SI, Merson RR, Hahn ME, Sherr DH. The aryl hydrocarbon receptor constitutively represses c-myc transcription in human mammary tumor cells. *Oncogene*. 2005 Nov 24;24(53):7869-81.
2. Murray TJ, Yang X, Sherr DH. Growth of a human mammary tumor cell line is blocked by galangin, a naturally occurring bioflavonoid, and is accompanied by down-regulation of cyclins D3, E, and A. *Breast Cancer Res*. 2006;8(2):R17. Epub 2006 Mar 27.