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A dioxin response element in the multiple cloning site of the pGL3 luciferase reporter influences transcriptional activity¹

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Abstract

Luciferase reporter plasmids (pGL3 backbone, Promega) have been utilized to characterize the transcriptional effects of the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other aryl hydrocarbon receptor (AhR) ligands. Following ligand activation, the AhR and its dimerization partner AhR nuclear translocator (ARNT) regulate transcription by binding dioxin response elements (DREs) in regulatory regions of dioxin-sensitive genes. Upon sequencing of our luciferase reporters, we unexpectedly identified a DRE core motif within the multiple cloning site (mcsDRE) of the pGL3 luciferase plasmid backbone in a subset of our reporters. Therefore, the objective of this study was to determine if the mcsDRE inadvertently influences reporter activity. Utilizing deletional analysis we determined that the mcsDRE did significantly alter the transcriptional effect induced by TCDD. Since many chemicals have been shown to interact with the AhR and influence transcription through the DRE, the presence of the mcsDRE in the pGL3 luciferase plasmid may inappropriately influence promoter and enhancer analysis. As such, insertion of regulatory elements into pGL3 reporters should be designed to avoid retaining the mcsDRE core motif (GCGTG) and currently utilized pGL3 reporters should be evaluated for the presence of the mcsDRE.

Keywords

Aryl hydrocarbon receptor; dioxin response element; luciferase reporter; pGL3 plasmid; transcriptional activity

1. Introduction

The aryl hydrocarbon receptor (AhR) is expressed in most tissues and there is an increasing awareness that the AhR likely plays a significant role in many physiological processes

¹Nonstandard abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; AP-1, activator protein 1; DMSO, dimethyl sulfoxide; DRE, dioxin response element; Ig, immunoglobulin; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; Oct, octamer-binding factor; PBS, phosphate buffered saline; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; V_H, variable heavy chain.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

(McMillan and Bradfield, 2007). The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) represents a broad class of compounds commonly referred to as dioxins. TCDD is one of the most potent AhR ligands and induces a plethora of biological effects thought to be mediated by the AhR through altered gene transcription (Birnbaum and Tuomisto, 2000). One of the most sensitive effects of TCDD exposure is the upregulation of enzymes involved in xenobiotic metabolism such as the cytochrome P450 oxidases (e.g. CYP1A1). Extensive characterization of this response led to the elucidation of the AhR signaling pathway. In the absence of ligand, the AhR is sequestered in the cytosol and bound by other proteins that maintain an inactive state. Once bound by ligand, the AhR undergoes a conformational change resulting in protein dissociation, nuclear translocation, and dimerization with the AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer regulates transcription by binding dioxin response elements (DREs), which are abundant in genes encoding phase I and phase II metabolic enzymes (Swanson, 2002). However, the exact role of this signaling pathway is less well characterized in the physiological effects of TCDD that do not involve altered expression of enzymes mediating xenobiotic metabolism.

To elucidate the function of the AhR, DREs, and other transcriptional elements in the non-metabolic effects induced by dioxin and dioxin-like compounds, reporter plasmids are often employed. Likewise, we have utilized a variety of luciferase reporter plasmids (pGL3 backbone, Promega) to characterize the transcriptional effects of TCDD and other AhR ligands on a specific transcriptional regulatory region important to antibody expression, a physiological endpoint significantly targeted by TCDD and dioxin-like compounds (Sulentic and Kaminski, 2011). Since we have identified functional DRE motifs (i.e. AhR/ARNT bind to these DREs) within our transcriptional regulatory region of interest, we hypothesized that TCDD would modulate transcription through an AhR/DRE-mediated mechanism. However, sequencing of our luciferase reporters identified an unexpected DRE core motif (GCGTG) within the multiple cloning site (mcsDRE) of the pGL3 luciferase plasmid backbone (Promega) in a subset of our reporters. This inadvertent sequence may have enhancer activity thus further influencing the transcriptional regulation of reporter plasmids that retain this site after cloning. Therefore, the objective of this study was to determine the transcriptional effect of the mcsDRE.

Using deletional analysis we determined that the mcsDRE contributes to transcriptional activity. The mcsDRE was necessary for TCDD-induced activation of a minimal *Cyp1a1* promoter. It also significantly altered the transcriptional effect induced by TCDD in reporters regulated by a promoter and enhancer specific to antibody expression. Additionally, the transcriptional activity of the mcsDRE appeared to be specific to regulatory regions modulated by TCDD since the mcsDRE had no effect on SV40 promoter and/or enhancer activity which was not significantly affected by TCDD treatment. Therefore, the presence of the mcsDRE in the pGL3 luciferase plasmid may inappropriately influence promoter and enhancer analysis. Furthermore, the surprising and ever expanding myriad of AhR ligands which include substances from environmental, endogenous, dietary or pharmaceutical sources (Denison and Nagy, 2003) and the increasing evidence for a role of the AhR in many physiological processes (McMillan and Bradfield, 2007) underscore the potential for confounding results in a broad range of promoter and enhancer reporter studies that employ the pGL3 backbone (i.e. even those not intended to evaluate the AhR or dioxin-like compounds). To avoid the potential for confounding results, reporters with a pGL3 backbone should be evaluated for the presence of the mcsDRE and current cloning with the pGL3 luciferase constructs should be designed to avoid retaining the mcsDRE.

2. Materials and methods

2.1. Chemicals and Reagents

TCDD in 100% dimethyl sulfoxide (DMSO) was purchased from AccuStandard Inc. (New Haven, CT). The certificate of product analysis stated the purity of TCDD to be 99.1%, as determined by AccuStandard using gas chromatography/mass spectrometry. Lipopolysaccharide (LPS, *Escherichia coli*) and DMSO were purchased from Sigma Aldrich (St. Louis, MO). LPS was dissolved in 1× PBS.

2.2. Cell Lines

The CH12.LX B-cell line derived from the murine CH12 B cell lymphoma (Arnold et al., 1983), which arose in B10.H-2^aH-4^bp/Wts mice (B10.A × B10.129), has been previously characterized by Bishop and Haughton (Bishop and Haughton, 1986) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). Cells were grown as previously described (Henseler et al., 2009).

2.3. Plasmids

All luciferase reporter plasmids were constructed using a pGL3-Basic Vector (Promega, Madison, WI) and all retained the mcsDRE as determined by sequence analysis (Retrogen Inc., San Diego, CA) (Fig. 1). For determining the role of the mcsDRE in reporter activity, the mcsDRE was deleted and/or mutated in each plasmid by site-directed mutagenesis (see below). Additionally, as an added control, a 5 bp sequence upstream of the mcsDRE was deleted to demonstrate specificity of the mcsDRE deletion. Luciferase reporter plasmids under the regulation of promoter and enhancer elements from the human immunoglobulin heavy chain (*Igh*) locus were generously provided by Dr. Michel Cogné (Laboratoire d'Immunologie, Limoges, France) and included the enhancerless variable heavy chain promoter (V_H) and the polymorphic hs1,2 enhancer of the 3' *Igh* regulatory region (3' *IghRR*). The human hs1,2 reporters contained the V_H promoter 5' of the luciferase gene and either the α_{1A} or α_{1B} hs1,2 enhancer 3' of the luciferase gene (Denizot et al., 2001). Within the human hs1,2 alleles, we identified one DRE-like motif in α_{1A} and two in α_{1B}. The *Cyp1a1* minimal promoter contains only the proximal promoter region (Jones and Whitlock, 1990) extending -392 upstream of the transcriptional start site of the mouse *Cyp1a1* gene (EMBL AF210905.1) and lacks the upstream DRE enhancers. The Basic (no promoter or enhancer), Promoter (regulated by the SV40 promoter), Enhancer (regulated by the SV40 enhancer), and Control (regulated by SV40 promoter and enhancer) pGL3 Vectors were obtained from Promega.

2.4. Site-Directed Mutagenesis

Deletion of the 5 bp mcsDRE core sequence was generated using PCR-based site-directed mutagenesis according to the manufacturer's instructions (QuickChangeXL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, deletion of the mcsDRE within all of the pGL3 plasmids was created using the forward primer: 5'-GGTACCGAGCTCTTACCTAGCCCGGGC-3' and reverse primer: 5'-GCCCGGGCTAGGTAAGAGCTCGGTACC-3'. To ensure specificity of the mcsDRE deletion and that the effects on reporter activity were not a random result of the deletion, a 5 bp nucleotide sequence 17 nucleotides 5' of the mcsDRE was deleted from the *Cyp1a1* minimal promoter and the α_{1B} hs1,2 reporters using the forward primer: 5'-CAGAACATTTCTCTATGGTACCGAGCTCTTAC-3' and reverse primer: 5'-GTAAGAGCTCGGTACCATAGAGAAATGTTCTG-3'. The primer set for mutation of the mcsDRE from GCGTG to GATCG within the α_{1B} hs1,2 reporter included the forward primer: 5'-GATAGGTACCGAGCTCTTACGATCGCTAGCCCGGGCTCGAGATC-3'

and reverse primer: 5'-GATCTCGAGCCCCGGGCTAGCGATCGTAAGAGCTCGGTACCTA TC-3'. PCR reaction conditions were: 95°C for 1 min followed by 18 cycles at 95°C for 50 s, 60°C for 50 s, 68°C for 1 min/kb of plasmid length, then 68°C for 7 min. Following the PCR reaction, the parental plasmid was digested by incubating with Dpn I (1 µL/50 µL reaction) for 1 hr at 37°C. Clones of mutated plasmids were replicated in bacteria and screened for the correct mutation by DNA sequencing (Retrogen Inc.).

2.5. Transient Transfection and Luciferase Assay

For transient transfections, CH12.LX (1.0×10^7) cells were resuspended in 200 µL of culture media with 10 µg of luciferase reporter plasmid and transferred to a 2-mm gap electroporation cuvette (Molecular BioProducts, San Diego, CA). Cells were electroporated using an electro cell manipulator (ECM 630; BTX, San Diego, CA) with the following voltage, capacitance and resistance: 250 V, 150 µF, and 75 Ω. For each plasmid reporter, multiple transfections were pooled and cells were aliquoted in triplicate into 12-well plates at 2.0×10^5 cells/mL. Immediately after transfection, cells were treated for 24 hr with 10 nM TCDD in the absence or presence of LPS (1.0 µg/mL) stimulation. The vehicle control for TCDD was 0.01% DMSO. After the incubation period, cells were lysed with 50 µL of 1× reporter lysis buffer (Promega) and immediately frozen at -80°C. Measurement of luciferase enzyme activity was performed on 20 µL of lysate cleared of cellular debris and the activity was normalized to transfection efficiency by real-time PCR analysis of the luciferase gene as previously described (Fernando et al., 2012; Henseler et al., 2009; Sulentic et al., 2004b). Briefly, DNA was isolated from 4.0×10^5 cells in triplicate between 2 and 4 hr following transfection using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich) and was diluted 10- or 100-fold then analyzed by real-time PCR using SYBR Green for the luciferase gene. Plasmid number per cell was calculated with the equation: [(ng of plasmid) (molecules/ng of plasmid)/cell number] 10 or 100 to account for the fold dilution of DNA used in the PCR reaction. Luciferase activity is represented as either relative light units (RLU) or fold-change relative to the appropriate DMSO vehicle control.

2.6. Statistical Analysis

Luciferase activity is represented as the mean ± SEM for each treatment group (n=3) from a representative experiment. For fold-change graphs (except Fig. 4 which is a representative experiment), the mean (n=3) was determined for each treatment group and the means generated from several separate experiments were then averaged and transformed to fold-change (mean ± SEM) with the DMSO vehicle control set to 1. As indicated in the figure legends, significant differences at $p < 0.05$ were determined either by a 2-way ANOVA with a Bonferroni post-hoc test, 1-way ANOVA with a Dunnett's post-hoc test, or an unpaired t-test.

3. Results and Discussion

Although much of the biological effects of TCDD and dioxin-like compounds are dependent on the AhR, the role of the AhR/DRE pathway (primarily elucidated with the induction of enzymes involved in xenobiotic metabolism) is less well characterized. The ever increasing evidence of protein-protein interactions between the AhR and other transcription factors (e.g. NF-κB) suggests the potential for AhR-dependent effects that are independent of binding to DREs. In an effort to understand the role of the AhR, DRE, and specific transcriptional regulatory elements in the effects of TCDD on antibody expression, we utilized several luciferase reporter plasmids (Denizot et al., 2001, Fig. 1B) regulated by specific transcriptional elements from the human immunoglobulin (Ig) heavy chain gene (encodes an essential subunit of the antibody polypeptide) including the variable heavy

chain (V_H) promoter and the hs1,2 (hypersensitive sites 1 and 2) enhancer, which is polymorphic in humans. The human hs1,2 polymorphism consists of an approximately 38 bp invariant sequence that is repeated in tandem up to three times and contains potential binding sites for several transcription factors (Denizot et al., 2001; Giambra et al., 2005) including a DRE-like site (Fig. 1B). TCDD significantly modulated reporter activity of the V_H promoter alone and the V_H promoter with different alleles of the human hs1,2 enhancer (e.g., α_{1A} and α_{1B}). LPS, a toll-like receptor 4 (TLR-4) ligand commonly used to stimulate B lymphocytes to make Ig and secrete antibody, enhanced the effect of TCDD on hs1,2 reporter activity (Fernando et al., 2012 and Fig. 2, left panel).

However, sequencing of the human hs1,2 luciferase reporters identified a DRE core motif within the multiple cloning site (mcsDRE) of the pGL3 vector backbone which was formed by the adjacent positioning of the MluI and NheI restriction sites (Promega) (Fig. 1A). The mcsDRE is upstream of the V_H promoter and may significantly contribute to the transcriptional effect of TCDD on the human hs1,2 reporters. To determine the role of the mcsDRE in the activity of the human hs1,2 reporters (i.e., V_H promoter alone and α_{1A} , α_{1B} hs1,2 reporters), the mcsDRE was deleted or mutated by site-directed mutagenesis and verified by sequencing (Fig. 1 and data not shown). Deletion of the mcsDRE appeared to blunt the overall transcriptional activity induced by TCDD but this effect was primarily restricted to the V_H promoter alone and most prominently the α_{1B} hs1,2 reporter (Fig. 2A, compare left and right panels). Additionally, deletion of the mcsDRE had the greatest effect on TCDD-induced fold change in LPS-stimulated cells. The mcsDRE contributed significantly to the activity of the V_H promoter alone; whereas, the DRE and other transcription factor binding sites (i.e. AP-1, NF- κ B, Sp1, and Oct, see Fig. 1B) in the α_{1A} hs1,2 appeared sufficient to maintain a certain level of TCDD-induced hs1,2 activity that is not greatly impacted by the mcsDRE (Fig. 2B). Notably, one to two total DREs consistently demonstrated the same degree of TCDD-induced activation, whether the DRE was within the multiple cloning site or the hs1,2 enhancer (Fig. 2B). In contrast to the α_{1A} hs1,2 reporter, the mcsDRE, whether it was deleted or mutated, appeared to significantly contribute to TCDD-induced activity in LPS-stimulated cells of the α_{1B} hs1,2 enhancer (Fig. 2 and data not shown), which contains not only the same transcription factor binding sites as α_{1A} hs1,2 but a second 38 bp invariant sequence containing an additional DRE along with other transcription factor binding sites (i.e. AP-1 and NF- κ B) (Fig. 1B). Furthermore, deletion of an unrelated nucleotide sequence upstream of the multiple cloning site had no impact on α_{1B} hs1,2 reporter activity, demonstrating specificity of the mcsDRE transcriptional effects (data not shown). Perhaps binding of the AhR to the mcsDRE (just upstream of the promoter) and to two DRE motifs (as opposed to one) within the α_{1B} hs1,2 enhancer allows for a greater interaction of the enhancer with the promoter and transcriptional machinery (Fig. 1). The AhR has been shown to physically interact with NF- κ B proteins and TCDD modulates both the AP-1 and NF- κ B signaling pathways (Hoffer et al., 1996; Puga et al., 2000; Suh et al., 2002; Sulentic et al., 2000; Sulentic et al., 2004a; Tian, 2009). Therefore, an additional AP-1 and NF- κ B binding site within the α_{1B} hs1,2 enhancer may be at least partially responsible for the enhanced transcriptional activity when the mcsDRE is present.

To further characterize the transcriptional activity of the mcsDRE, we utilized a pGL3 plasmid in which the *Cyp1a1* minimal promoter was inserted into the multiple cloning site just downstream of the mcsDRE (Fig. 1B). The *Cyp1a1* minimal promoter consists of only the proximal promoter region (Jones and Whitlock, 1990) extending -392 upstream of the transcriptional start site of the mouse *Cyp1a1* gene (EMBL AF210905.1) and lacks the upstream DRE enhancers. TCDD-induced upregulation of *Cyp1a1* gene expression has been extensively characterized as an AhR/DRE-dependent mechanism and the at least five functional DRE binding sites within the *Cyp1a1* promoter likely account for the sensitivity

of this gene to TCDD (Ma, 2001). In our study, TCDD markedly induced transcriptional activity of the *Cyp1a1* minimal promoter but only in the presence of the mcsDRE, since deletion of the mcsDRE, but not the deletion of an unrelated nucleotide sequence upstream of the multiple cloning site, completely abrogated the TCDD-inducible effect on reporter activity (Fig. 3). These results correspond to earlier studies demonstrating that the *Cyp1a1* minimal promoter has very little to no TCDD-inducible activity when not linked to at least one DRE binding motif (Jones and Whitlock, 1990). Taken together the *Cyp1a1* minimal promoter and the hs1,2 reporters indicate that the mcsDRE has transcriptional activity and could inadvertently skew the results of reporter studies.

To determine the specificity of the transcriptional effects induced by the mcsDRE, we evaluated the control pGL3 vectors (Promega) which also contained the mcsDRE and consisted of the Basic (no promoter or enhancer), Promoter (regulated by the SV40 promoter), Enhancer (regulated by the SV40 enhancer), and Control (regulated by SV40 promoter and enhancer) vector (Fig. 1B). As predicted, the mcsDRE alone was not sufficient to alter the transcriptional activity of the Basic vector which corresponded with previous results demonstrating that a consensus DRE motif from the *Cyp1a1* gene was incapable of initiating transcription without inclusion of the minimal *Cyp1a1* promoter extending -392 upstream of the transcriptional start site (Jones and Whitlock, 1990). Additionally, TCDD did not significantly change the activity of the Promoter, Enhancer, and Control vectors and the mcsDRE appeared to have no transcriptional role in reporter activity since deletion of the mcsDRE did not alter the overall activity of these vectors (Fig. 4 and data not shown). These results demonstrate a transcriptional influence of the mcsDRE that appears to be specific to reporters that are sensitive to TCDD-induced modulation.

The current study suggests that the mcsDRE may influence the overall transcriptional effect of reporters regulated by promoters and/or enhancers that contain additional DRE motifs and/or binding sites for transcription factors (e.g. NF- κ B, AP-1) known to interact with the AhR. Additionally, the list of non-dioxin AhR ligands appears to be ever expanding with several studies identifying a surprising plethora of ligands that activate the AhR including environmental (e.g. polycyclic aromatic and halogenated hydrocarbons of which TCDD is the prototype), endogenous (e.g. bilirubin), dietary (e.g. indolo(3,2,b)carbazole) and pharmaceutical (e.g. omeprazole) compounds (Denison and Nagy, 2003). Furthermore, the emerging role of the AhR in physiological processes independent of xenobiotic metabolism (McMillan and Bradfield, 2007) suggests the possibility for confounding results in a broad range of promoter and enhancer reporter studies employing the pGL3 backbone regardless of whether these studies were designed to evaluate transcriptional effects mediated by the AhR or DRE. Therefore to prevent potential confounding results, care should be taken to avoid retaining the mcsDRE (GCGTG) in reporters with a pGL3 backbone. Notably, the mcsDRE is not present in the pGL2 and pGL4 luciferase constructs (Promega).

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Highlights

Dioxin response element identified in multiple cloning site of pGL3 luciferase vector. >
Multiple cloning site dioxin response element induces transcriptional activity. > Effects
are selective to dioxin sensitive promoter and enhancer elements. >

A. pGL3 Multiple Cloning Site

B. pGL3 Luciferase Reporter Constructs with mcsDRE

Figure 1. Schematic of luciferase reporters and the pGL3 multiple cloning site

A) A DRE core consensus sequence (GCGTG) is present in the pGL3 multiple cloning site (mcs) due to the adjacent positioning of the MluI and NheI restriction sites. The arrow denotes the transcription start site. B) The pGL3 luciferase reporters are schematically represented and include reporters regulated by either the variable heavy chain (V_H) promoter alone, the V_H promoter with different alleles of the human hs1,2 enhancer (e.g., α_{1A} and α_{1B}), the *Cyp1a1* minimal promoter (*CypMP*), or the control pGL3 vectors from Promega which include the Basic (no promoter or enhancer), Promoter (regulated by the SV40 promoter), Enhancer (regulated by the SV40 enhancer), and Control (regulated by SV40 promoter and enhancer) vectors. All pGL3 luciferase reporters originally contained the mcsDRE as indicated. The mcsDRE was deleted from all of the reporters or mutated in the α_{1B} by site-directed mutagenesis (not represented). The arrow denotes the transcription start site. The transcription factor binding sites within the human hs1,2 enhancer are identified and the star indicates the core DRE motif contained within the invariant sequence which is repeated in the α_{1B} hs1,2 allele. Schematics are not to scale.

Figure 2. Deletion of the mcsDRE decreases TCDD-induced transcriptional activation of the V_H promoter and the α_{1B} hs1,2 reporters

The CH12.LX cells were transiently transfected with V_H , α_{1A} hs1,2 or α_{1B} hs1,2 reporter plasmids that either contained the mcsDRE or a deletion by site-directed mutagenesis of the mcsDRE. The change in total number of DREs following deletion of the mcsDRE is indicated; the α_{1A} and α_{1B} hs1,2 alleles contain one and two DREs, respectively (see Fig. 1B). A) Transfected cells were cultured for 24 hr in the absence of further treatment (naïve, data not shown) or treated with 0.01% DMSO (vehicle control denoted by “-”) or 10 nM TCDD (denoted by “+”) in the absence (white bars) or presence (black bars) of 1 μ g/ml LPS stimulation. Luciferase enzyme activity is represented on the y-axis as relative light units normalized for transfection efficiency. Results (mean $\pm$ SEM; $n=3$ for each treatment group) are representative of separate experiments (five for V_H ; five for α_{1A} ; and seven for α_{1B}) that directly compared plasmid with the mcsDRE to the corresponding plasmid with deletion of

the mcsDRE. B) TCDD-induced activation is represented on the y-axis as fold-change relative to the DMSO vehicle control. The presence or deletion of the mcsDRE is denoted by “+” or “-”, respectively. Results (mean $\pm$ SEM) were generated by averaging the means (n=3 for each treatment group) from eight to 11 separate experiments per plasmid. Significance between treatment groups for a given reporter (A) or between corresponding reporters (B) was determined by a 1-way ANOVA followed by a Dunnett’s post-hoc test. “*”, “**” and “***”, denote significance compared to the corresponding DMSO vehicle control (A) or corresponding V_H promoter (B) at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. “^” denotes significance between α_{1A} and α_{1B} hs1,2 reporters (B). Significance due to LPS stimulation (B) or deletion of the mcsDRE (A and B) was determined by a 2-way ANOVA followed by a Bonferroni’s post-hoc test. “‡”, represents significance between unstimulated and stimulated groups at $p<0.05$. “††” and “†††”, denote significance between the mcsDRE positive plasmid and the corresponding mcsDRE deletion plasmid at $p<0.01$ and $p<0.001$, respectively. Horizontal lines are present to facilitate visual comparisons.

Figure 3. The mcsDRE is necessary for TCDD-induced transcriptional activation of the *Cyp1a1* minimal promoter

CH12.LX cells were transiently transfected with a luciferase reporter regulated by the *Cyp1a1* minimal promoter that either contained the mcsDRE (+mcsDRE) or deletion by site-directed mutagenesis of either the mcsDRE (mcsDRE deletion) or an unrelated 5 bp nucleotide sequence 17 nucleotides 5' of the mcsDRE (+mcsDRE 5bp deletion). A) Transfected cells were cultured for 24 hr in the absence of further treatment (naïve, data not shown) or treated with 0.01% DMSO (vehicle control denoted by “-”) or 10 nM TCDD (denoted by “+”). Luciferase enzyme activity is represented on the y-axis as relative light units normalized for transfection efficiency. Significance due to deletion of the mcsDRE or between treatment groups was determined by a 2-way ANOVA followed by a Bonferroni's post-hoc test. “***”, denotes significance compared to the DMSO vehicle control at $p < 0.001$. “†††” denotes significance at $p < 0.001$. Results (mean $\pm$ SEM; $n=3$ for each treatment group) are representative of four separate experiments. B) TCDD-induced activation is represented on the y-axis as fold-change relative to the DMSO vehicle control. The presence or deletion of the mcsDRE is denoted by “+” or “-”, respectively. Results (mean $\pm$ SEM) were generated by averaging the means ($n=3$ for each treatment group) from four separate experiments. Significance due to deletion of the mcsDRE was determined by a 1-way ANOVA and Dunnett's post-hoc test and “†” denotes significance at $p < 0.05$.

Figure 4. Transcriptional activity of the pGL3 control vectors are not influenced by TCDD or the mcsDRE

The CH12.LX cells were transiently transfected with the Basic, Promoter, Enhancer or Control pGL3 vectors that either contained the mcsDRE (denoted by “+”) or a deletion of the mcsDRE by site-directed mutagenesis (denoted by “-”). Transfected cells were cultured for 24 hr in the absence of further treatment (naïve, data not shown) or treated with 0.01% DMSO (vehicle control) or 10 nM TCDD. TCDD-induced activation is represented on the y-axis as fold-change relative to the DMSO vehicle control. TCDD treatment and deletion of the mcsDRE did not significantly alter reporter activity as determined by a 2-way ANOVA followed by a Bonferroni post-hoc test. Results (mean $\pm$ SEM) were generated by averaging the means ($n=3$ for each treatment group) from four separate experiments. Horizontal line is present to facilitate visual comparisons.