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Elucidating Transcription Factor Regulation by TCDD within the HS1,2 Enhancer

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ELUCIDATING TRANSCRIPTION FACTOR REGULATION
BY TCDD WITHIN THE HS1,2 ENHANCER

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

SHARON D. OCHS
B.S., Wright State University, 2010

2012
Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

April 6, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sharon D. Ochs ENTITLED Elucidating transcription factor regulation by TCDD within the hs1,2 enhancer BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Ochs, Sharon D. M.S., Department of Pharmacology and Toxicology, Wright State University, 2011. Elucidating transcription factor regulation by TCDD within the hs1,2 enhancer.

Immunoglobulin heavy chain (*IgH*) expression and Ig secretion is inhibited by the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Within the *IgH* gene, the 3' *IgH* regulatory region (3'*IgH* RR) has been identified as a transcriptional target of TCDD. The 3'*IgH* RR, which in part regulates transcription of the *IgH* gene, is composed of four enhancers in the mouse: hs3a; hs1,2; h3b; hs4 and three enhancers in the human: hs3a; hs1,2; hs4. In humans the hs1,2 enhancer has an invariant sequence (IS) containing a DRE, NF-κB, NF1 and AP-1 binding site. Also, the enhancer has an AP1.ETS and Oct site located 5' to the IS. The human hs1,2 enhancer is sensitive to TCDD-induced modulation but in contrast to the mouse hs1,2 and 3'*IgH* RR, TCDD activates the human hs1,2 enhancer. The current study demonstrates the complexity of how TCDD differentially induces modulation between mouse and human and what role these binding sites may have.

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ACKNOWLEDGMENTS

I distinctly remember my first day of work as an Undergraduate Research Assistant in the Sulentic Lab. It was February 1, 2008 and I was terrified. With the exception of the pipettes, everything was completely foreign to me: the people, the surroundings, the science. Of course I'm amazed by how far I've come and what I've been able to accomplish over these past few years, but I couldn't have done it alone. I deeply appreciate the guidance of my advisor, Dr. Sulentic, and the service of my committee members, Dr. Bigley and Dr. Kozak. I'm also thankful for all the lab members who taught and assisted me with the research and provided me with hours of laughter and amusement. Last but not least, I'm grateful for the never ending love and support of my friends and family.

I. INTRODUCTION

2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

Dioxins are composed of a polyhalogenated aromatic hydrocarbon and share similar biological mechanisms of action. Based on their hydrophobic properties, resistance towards metabolism and long half-life, dioxins tend to bioaccumulate and persist as environmental contaminants (Van den Berg et al., 1998). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, also known as TCDD, is the prototypical dioxin (Fig. 1). Much research has been dedicated to TCDD, because of its extreme potency as a toxin and its inadvertent exposure to humans through a number of well-publicized events.

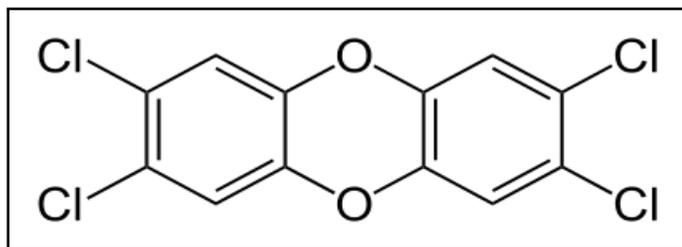


Figure 1. Chemical structure of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD).

TCDD is not intentionally produced, except on small scale for research purposes. Rather, it is synthesized from chemical reactions or incomplete combustion processes involving the presence of chlorine (paper bleaching, metal production, waste incineration, fossil fuel and wood combustion, volcanic activity) (ATSDR, 1998). In the 1960s and 1970s TCDD contaminated chlorophenoxy herbicides, such as 2,4,5-trichlorophenoxyacetic acid. This acid was a component of Agent Orange, a defoliant used during the Vietnam War (Schechter et al., 2006). Additionally, industrial accidents or environmental application of substances contaminated with TCDD led to evacuations of areas around Love Canal in Niagara Falls, New York, Times Beach, Missouri, and Seveso, Italy (Friedman et al., 1999; Reggiano, 1979). More recently, a large number of pig and poultry farms in Europe were closed after dioxin contamination was discovered in the animal feed.

TCDD exposure occurs by ingestion, inhalation or dermal contact when it is incorporated into food, water, dust, smoke or air (Mandal, 2005). Toxic responses to TCDD are broad and depend upon many factors, like exposure dose, duration of exposure and type of species. *In vivo* studies have correlated TCDD to a multitude of pathophysiological abnormalities related to brain function, reproduction, hormone signaling and immunity (Mandal, 2005). Moreover, TCDD has been shown to promote tumor growth and is classified as a human carcinogen (IARC, 1997). In all mammalian species tested, lethal doses of TCDD lead to excessive loss in body weight preceding death (wasting syndrome) (IARC, 1997). Also, short-term exposure to high levels of TCDD in humans can disturb liver function and cause chloracne, a severe acne-like condition (Marinkovic et al., 2010). The mechanism involved in much of TCDD's

toxicity is yet to be determined, but many of its toxic effects are thought to be mediated via the aryl hydrocarbon receptor (AhR) signaling pathway.

Aryl Hydrocarbon Receptor Signaling

The aryl hydrocarbon receptor (AhR) is a constitutively expressed transcription factor belonging to the basic-helix-loop-helix/Per-ARNT-Sim family. In its inactivated state, the AhR is found in the cytosol coupled with several proteins: two heat-shock protein 90 molecules, p23, and XAP2. To date, a variety of chemicals, pharmaceuticals, and dietary constituents have been identified as AhR ligands, which can be categorized as either man-made or natural in origin (e.g. aromatic hydrocarbons, omeprazole, flavonoids) (Abel and Haarmann-Steeman, 2010). When AhR binds a ligand, such as TCDD, it undergoes a conformational change and dissociates from the cytosolic proteins. The AhR then translocates into the nucleus to form a complex with the aryl hydrocarbon receptor nuclear translocator (ARNT). The TCDD/AhR/ARNT complex binds a dioxin responsive element (DRE; 5'-TNGCGTG-3') within the promoter or enhancer regions of sensitive genes and consequently alters gene expression (Fig. 2). When the AhR ligand is no longer present, receptor signaling is seemingly terminated by either nuclear export of the receptor, followed by ubiquitination and proteosomal degradation or negative feedback-inhibition by the AhR repressor, an inhibitor that binds DREs leading to transcriptional repression (Abel and Haarmann-Steeman, 2010).

The AhR plays an important role in xenobiotic metabolism and therefore the upregulation of a number of drug metabolizing enzymes. Induction of CYP1A1, a member of the cytochrome P450 superfamily of enzymes, is one of the most well-

characterized gene responses targeted by AhR activation (Fig.2). CYP1A1 is involved in the biotransformation of substrates in phase I reactions and is highly induced by TCDD (Whitlock, 1999). In addition to drug metabolism, many studies have shown that the AhR possesses a multitude of diverse functions and capabilities. A ligand-activated AhR is directly involved in cross-talk pathways with androgen and estrogen receptor signaling by co-regulating transcription (Ohtake et al., 2008). Also, UVB irradiation of human skin can induce metabolites that activate the AhR (Jux et al., 2010). The AhR can further crosstalk by physically interacting with NF- κ B and SP1 and/or altering Nrf2 signaling as well (Tian et al., 1999; Kobayashi, 1996; Hayes, 2009).

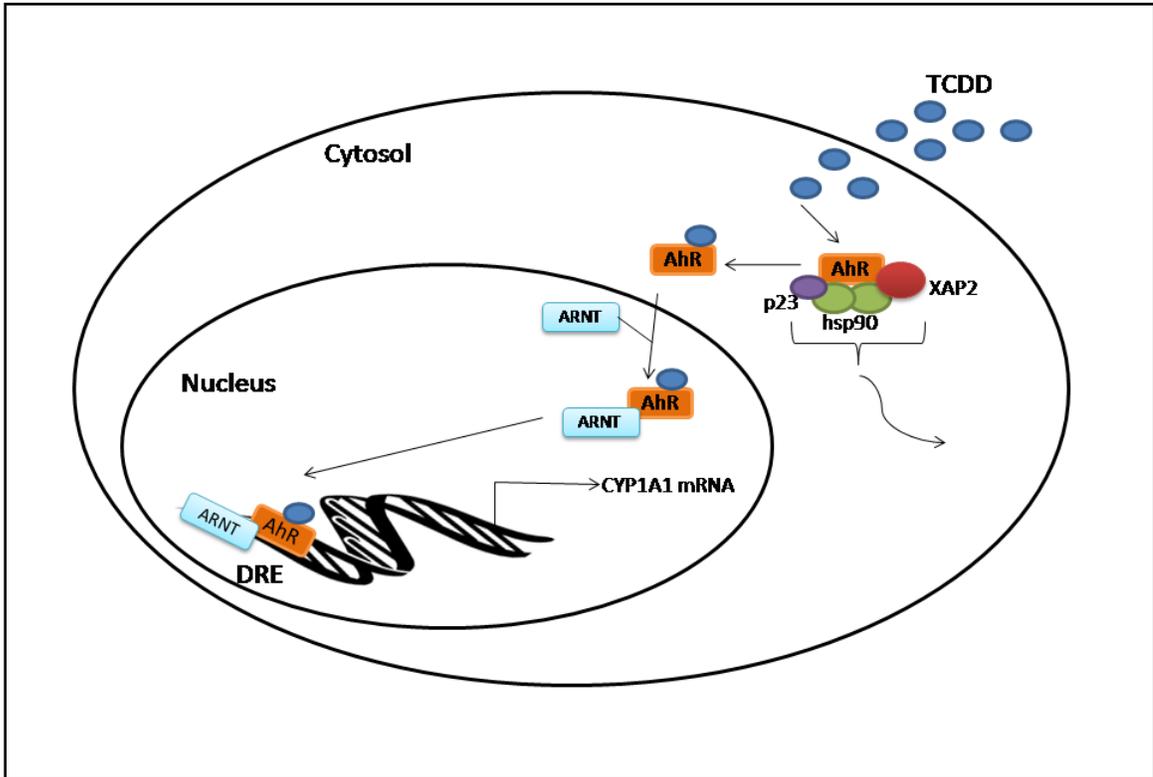


Figure 2. Aryl hydrocarbon receptor signaling pathway. TCDD binds to the AhR and cytoplasmic proteins dissociate. TCDD/AhR translocate to the nucleus and bind ARNT. The TCDD/AhR/ARNT complex modulates transcription by binding DREs.

The Immune System

The immune system is comprised of a variety of cells and mechanisms that work to clear the body of foreign antigens like bacteria, viruses, fungi, and/or parasites. There are two arms to immunity: innate and adaptive. Innate immunity provides an immediate, non-specific response towards pathogens and acts as the first line of defense (Medzhitov & Janeway, 2000). Physical barriers, mucosal surfaces and gut flora all resist infection. Additionally, phagocytes (macrophages, dendritic cells and neutrophils) recognize pathogen-associated molecular patterns and release inflammatory mediators. Adaptive immunity is subsequently activated by the innate system and eliminates specific antigens by mounting a strong, highly-specific cellular response (Medzhitov & Janeway, 2000). Ultimately, immunological memory of an antigen is retained so that a faster, more robust immune response can be achieved upon future exposure to the same antigen. In adaptive immunity there is cell-mediated immunity that is dominated by T cells and humoral immunity that is dominated by B cells. Naïve $CD4^+$ T_H cells recognize specific epitopes bound to major histocompatibility complex (MHC) II molecules found only on the surface of an antigen-presenting cell (B cells, dendritic cells, macrophages). When activated, these cells proliferate and differentiate into effector, memory or regulatory T_H cells and effector T_H cells will further differentiate into various subtypes. Alternatively, $CD8^+$ cytotoxic T cells respond to an epitope presented by MHC class I molecules, which are present on nearly every cell of the body. MHC II molecules present antigenic peptides from pathogens of extracellular origin (e.g. bacteria) whereas MHC I molecules present fragments from pathogens of intracellular origin (e.g. viruses). Both cell-

mediated and humoral immunity are interrelated and typically antigen-activated helper T cells are needed to effectively activate B cells (Cerutti, 2011).

B Cells

When a B cell is activated it eventually undergoes proliferation and differentiation into a plasma cell. Antibodies, the soluble form of immunoglobulin (Ig), are then secreted from the plasma cell to mark pathogens for destruction or elimination in an effort to reduce infection. There are five main isotypes of Ig (IgM, IgA, IgD, IgG, IgE) and each consists of two identical heavy chains and two identical smaller, light chains linked together by disulfide bonds (Fig. 3) (Woof & Burton, 2004). Both the heavy and light chain is composed of a variable region that forms the antigen binding site as well as a constant region, which is less diverse in its amino acid sequence (Fig. 3). Ig molecules are defined based on differences between the constant region of their heavy chains (C_H) and each class has distinct effector functions, because of this difference (Woof & Burton, 2004). IgM, IgD, IgG, IgE, and IgA are encoded by their respective C_H : C_μ , C_δ , C_γ , C_ϵ , C_α (Fig. 4). There are two isotypes for the light chain: lambda and kappa, but no functional differences have been found between the two. Gene segments that encode the variable region of the light chain are called variable (V) and joining (J) gene segments. The heavy chain variable region includes diversity (D) gene segments as an additional set (Fig. 4).

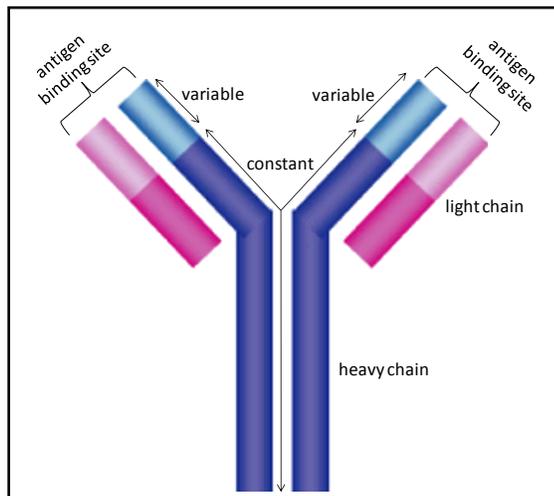


Figure 3. Immunoglobulin (Ig) structure. Secreted Ig (antibody) is composed of a heavy chain and light chain that binds a specific antigen.

Diversity of the antigen binding site is increased by the random combination of the gene segments through a process termed, somatic recombination. During B cell development gene segments are cut and spliced so that a V and J gene segment is selected and joined for the light chain variable region and likewise a V, D and J segment for the heavy chain variable region. Following successful rearrangement of the light chain and heavy chain, an immature B cell will express surface IgM to ensure that there is no reaction to self-antigen. The B cells that pass this test become mature B cells and use alternative mRNA splicing to simultaneously produce membrane-bound IgM and IgD. Before a B cell differentiates and proliferates in response to an antigen it must be activated either with or without T-cell help. In T-cell dependent activation a T cell recognizes an antigen that it is specific for and clonally expands and differentiates into effector cells. This same antigen delivers the first signal to a B cell when it binds the B-cell receptor (BCR), which is composed of a membrane-bound Ig complexed with Ig α and Ig β chains. The second signal is delivered to the B cell when a helper T-cell activated by the same antigen recognizes a peptide fragment of the antigen bound to the major histocompatibility complex (MHC) class II molecule on the B-cell surface. These two signals together are needed to drive B-cell proliferation and differentiation into plasma cells. In contrast, certain antigens can activate B cells independent of T-cell help. One such example is lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria. In addition to binding an LPS-specific BCR, LPS binds the CD14/TLR4/MD2 receptor complex, which produces activating signals for inflammatory cytokines. Whether or not T cells are involved, antibody production may proceed once the B cell is activated.

IgM is the first antibody produced, but isotype switching can take place to change which Ig class is produced by recombining the rearranged V-region DNA with a different heavy-chain C gene. Highly repetitive sequences, called switch regions, mediate recombination whereby the switch region that flanks the μ gene interacts with the switch region flanking one of the other C genes (Fig. 4). This interaction allows for excision of the previous C gene as a circular DNA molecule bringing the V region and new C gene together. Essentially, antigen specificity can be preserved while the functional role of the Ig can be altered. Somatic hypermutation can also take place, which involves point mutations to the rearranged V-region DNA. Ig with differing affinities are produced that allow for its receptor to possess an enhanced ability to bind a specific foreign antigen.

In the end, an activated B cell develops into a plasma cell or a long-lived memory B cell. The Ig secreted from a plasma cell helps clear the pathogen from the body through various ways. Antibodies can directly bind and neutralize a pathogen or toxin so that it cannot interact with human cells. Also, antibodies can coat pathogens, referred to as opsonization, to improve the effectiveness of ingestion by phagocytes. Alternatively, a memory B cell will persist long-term and retain the surface Ig specific for the foreign antigen. When exposed to the same antigen in the future, a quicker immune response can be achieved from the memory B cell, because the time required to get a high affinity, class switch Ab is reduced.

TCDD-induced Immunotoxicity

The immune system is an early and sensitive target to TCDD-induced toxicity. Rodents display a variety of innate and acquired immune-related disturbances following acute and chronic exposure to low levels of TCDD (Holsapple et al., 1991; Vos, 1997). Examples of responses to TCDD in rodents include immune-cell dysfunction, thymic atrophy, susceptibility to infectious diseases and prevention of transplant rejection (Luster, 1987; Kerkvliet, 2002). Although the exact molecular mechanisms responsible for the immunosuppressive effects of TCDD have not been clearly established, it is believed the AhR plays a vital role. One study, for example, showed AhR-deficient mice were able to mount normal immune responses when exposed to TCDD and challenged with different antigens (allogeneic P815 tumor cells, sheep red blood cells) (Vorderstrasse et al., 2001). It seems absence of the AhR does not affect immune system function, but the receptor is somehow necessary for immune-related effects of TCDD.

TCDD-induced B Cell Dysregulation

TCDD affects B cell maturation, activation, differentiation and to a lesser extent proliferation (Sulentic and Kaminski, 2011). Cell separation/reconstitution studies of splenocytes determined that B cells are a direct, cellular target of TCDD-induced antibody suppression (Holsapple et al., 1986; Dooley and Holsapple, 1988). TCDD-cultured B cells activated by lipopolysaccharide (LPS) (T-independent), dinitrophenyl (T-independent) or sheep red blood cells (T-dependent) provided evidence that inhibition of antibody production can occur without T-helper function (Holsapple et al., 1986; Dooley

and Holsapple, 1988). Mitogenic activation increases AhR expression and therefore may increase a B cell's responsiveness to TCDD (Allan and Sherr, 2010). When activated by LPS, mouse B cells (CH12.LX) and purified splenic B cells induce AhR expression (Marcus, 1998; Sulentic, 1998). In LPS-stimulated CH12.LX cells IgM secretion is decreased when treated with TCDD while AhR-deficient mouse B cells (BCL-1) did not demonstrate an inhibition of IgM secretion when activated by LPS (Sulentic, 1998). This result indicates that antibody production is potentially inhibited by TCDD through an AhR-dependent pathway (Sulentic, 1998). AhR^{-/-} mice mount normal immune responses when treated with sheep red blood cells or allogeneic tumor cells and when treated with TCDD these responses are not suppressed (Vorderstrasse et al., 2001). This demonstrates that the AhR is not required for normal immune function, but is necessary for TCDD-induced immune suppression (Vorderstrasse et al., 2001).

The Immunoglobulin Heavy Chain Gene Locus

The immunoglobulin heavy chain (*IgH*) gene locus is comprised of the variable heavy chain promoter (V_H), VDJ region, E_μ intronic enhancer, heavy chain constant regions (C_H) with germline promoters and the 3' immunoglobulin heavy chain regulatory region (3'*IgH* RR) (Fig. 4). There is one 3'*IgH* RR present in mouse while there are two 3'*IgH* RRs present in human-likely the result of an evolutionary duplication event (Mills et al., 1997) (Fig. 4). 3' to the α₁ and α₂ heavy chain constant regions are the α₁ 3'*IgH* RR and α₂ 3'*IgH* RR, respectively. The 3'*IgH* RR plays an important role in modulating transcription of the *IgH* gene, class switch recombination and somatic hypermutation

(Cogne et al., 1994; Dunnick et al., 2005; Dunnick et al., 2009). Splenocytes from 3' *IgH* RR-deficient mice show a decrease in μ IgH transcripts, defective class switch recombination and Ig secretion (Vincent-Fabert et al., 2010). TCDD along with other AhR ligands of dietary, pharmaceutical, environmental and industrial origin inhibit transcriptional activity of the 3' *IgH* RR in LPS-stimulated CH12.LX cells (Sulentic et al., 2004a; Henseler, 2009). Additionally, several lymphomas have been correlated with chromosomal translocations between the 3' *IgH* RR and oncogenes. In Burkitt's lymphoma, translocation of the 3' *IgH* RR with the cellular oncogene MYC induces deregulated gene expression (Yan et al., 2007). Furthermore, most follicular lymphomas contain a chromosomal translocation between the bcl-2 gene and 3' *IgH* RR that also deregulates gene expression and increases resistance to cell death (Heckman et al., 2003).

There are four enhancers in the mouse 3' *IgH* RR (hs3a; hs1,2; hs3b; hs4) and three enhancers in the human 3' *IgH* RR (hs3a; hs1,2; hs4) that display DNase I hypersensitivity (Madisen and Groudine, 1994; Chauveau and Cogne, 1996; Mills et al., 1997) (Fig. 4). Enhancers of the murine 3' *IgH* RR display strong synergistic activity when in combination versus being independent of one another and individual enhancers show different profiles in transcriptional activity depending on the B cell stage (Madisen and Groudine, 1994; Saleque et al., 1997; Chauveau, 1998). The hs4 is active throughout B cell development, the hs1,2 is most active in mature B cells and plasma cells and the hs3 enhancers have slight activity in activated B cells (Madisen and Groudine, 1994; Saleque et al., 1997; Chauveau, 1998). As a whole, the 3' *IgH* RR shows less activity in pre-B cells compared to surface Ig⁺ B cells and plasma cells (Ong et al., 1998).

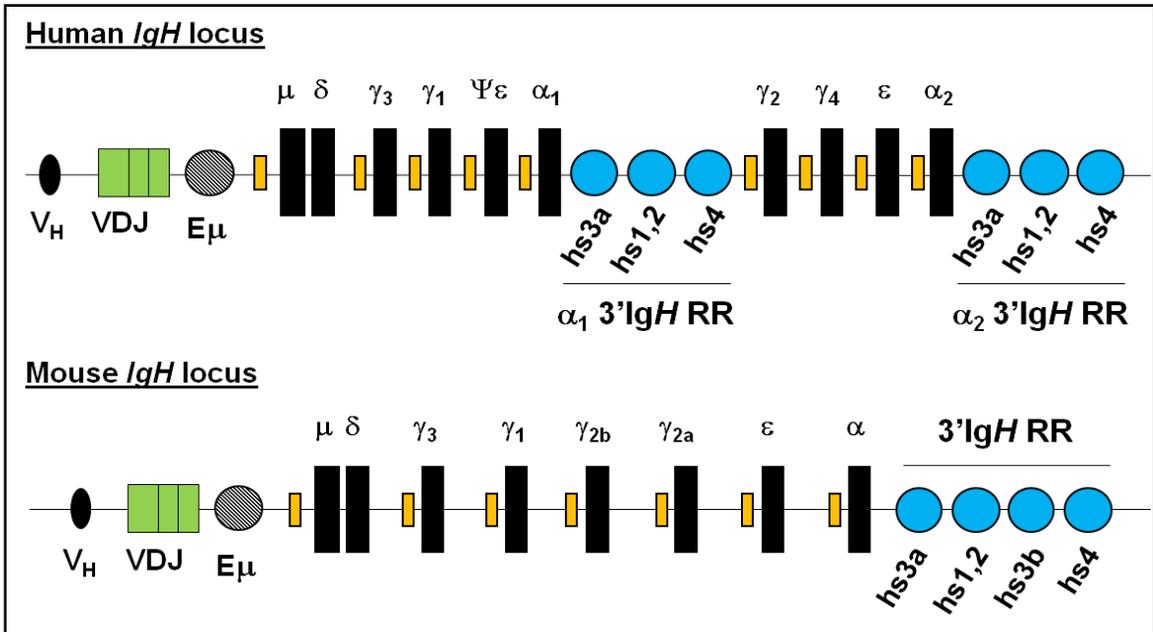


Figure 4. Immunoglobulin heavy chain (*IgH*) gene locus. V_H , variable heavy chain promoter; E_{μ} , intronic or μ enhancer; open rectangles, switch regions upstream of heavy chain constant chain regions.

Past research shows that mouse 3'*IgH* RR transcriptional activity in LPS-stimulated CH12.LX cells is inhibited by TCDD (Sulentic et al., 2004a). This effect parallels TCDD-induced inhibition of μ gene expression and IgM production (Sulentic et al., 2000). The hs4 and hs1,2 enhancers each contain a DRE-like site that demonstrates TCDD-inducible binding of AhR/ARNT by EMSA-Western analysis (Sulentic et al., 2000). Altered binding to these enhancers supports at least a partial role of DRE-dependent regulation; however, the AhR is known to interact directly and indirectly with a number of different transcription factors such as, AP-1, NF- κ B, and SP1 (Suh et al., 2002; Tian et al., 1999; Kobayashi et al., 1996). Even though a link between the AhR and Oct remains unclear, a high frequency of Oct sites were found in AhR-responsive genes using a genetic algorithm thus suggesting a potential role for the transcription factor in mediating a response to TCDD (Kel et al., 2004). Additionally, Oct coordinates with NF- κ B as a repressor of mouse hs1,2 activity in plasma cells and like NF- κ B could therefore be modulated by TCDD or the AhR (Michaelson et al, 1996).

To determine which enhancer(s) may be responsible for mediating TCDD-induced inhibition of 3'*IgH* RR activity, each enhancer was evaluated in isolation. Transient transfections of LPS-stimulated CH12.LX cells with a reporter plasmid containing the hs4 enhancer resulted in increased activity by TCDD (Sulentic et al., 2004b). EMSA analysis indicated AhR/ARNT binding to a region of the hs4 enhancer containing a DRE and overlapping NF- κ B motif, which could explain how TCDD is mediating the enhancer's activity (Sulentic et al., 2004b). This outcome is opposite in comparison to TCDD's effects on the overall mouse 3'*IgH* RR so the hs4 enhancer may have a distinct or unrelated function. Since the mouse hs4 enhancer has the most activity

of the four enhancers in pre-B cells, it may have greater influence on early B-cell development and VDJ recombination (Chauveau et al, 1998). One study indicates that deletion of the hs4 enhancer does not affect *in vitro* class switch recombination or Ig secretion in response to cytokine or LPS stimulation (Vincent-Fabert et al., 2009). When the mouse hs1,2 enhancer was evaluated in CH12.LX cells similarly to the hs4 enhancer, TCDD inhibited transcriptional activity of the enhancer (Fernando et al., 2012). The effects on the hs1,2 enhancer mirrors the suppression of LPS-induced 3'*IgH* RR activity by TCDD making it possible that the hs1,2 enhancer plays an important role in mediating transcriptional inhibition of the 3'*IgH* RR.

The hs1,2 Enhancer

While much effort has focused on studying TCDD-induced responses of the mouse 3'*IgH* RR and its enhancers, recent efforts have turned towards human. Unlike the mouse hs1,2 enhancer, the human hs1,2 enhancer is activated by TCDD in LPS-stimulated CH12.LX cells (Fernando et al., 2012). Activation of the human hs1,2 enhancer is similarly demonstrated in a human B-cell line (IM-9) (Fernando et al., 2012). The explanation to this interesting dichotomy may be found in the DNA sequences. While the core region of the mouse and human hs1,2 enhancers is about 90% similar, there are some notable differences (Mills et al., 1997). The mouse hs1,2 enhancer contains two binding sites for Pax5: one low-affinity and one high-affinity (Singh and Birshstein, 1993) (Fig. 5). Pax5, also known as B cell-specific activator protein (BSAP), is an important transcriptional regulator involved in determining B cell lineage identity and function. The Pax 5 transcription factor is critical for progenitors to commit to the B-

cell pathway in early stages while its eventual downregulation permits terminal plasma cell differentiation (Cobaleda et al., 2007). In LPS-stimulated CH12.LX cells Pax5 levels decrease, but this down-regulation is inhibited by the presence of TCDD co-treatment (Yoo et al., 2004; Schneider et al., 2008). Deregulation of Pax5 and three of its downstream targets (IgH, Ig κ , IgJ) imply a role for the transcription factor in TCDD-mediated impairment of B cell differentiation and Ig expression (Schneider et al., 2009).

The human $\alpha 1$ hs1,2 enhancer has a polymorphic region that is absent in the mouse hs1,2 enhancer. The polymorphism consists of an invariant sequence (IS) of approximately 55 bp, which can exist one (α_{1A}), two (α_{1B}), three (α_{1C}) or four (α_{1D}) times and may alter transcriptional activity (Denizot et al., 2001) (Fig. 5). Within the IS are several binding sites for transcription factors (AP-1, NF1, NF- κ B) and also a DRE core-motif analogous to the functional DRE found in the mouse hs1,2 enhancer (Chen and Birshstein, 1997; Denizot et al., 2001; Giambra et al., 2005; Fernando et al., 2012) (Fig. 5). This polymorphic region has drawn particular interest, because of its association with a number of diseases involving Ig secretion: celiac disease, IgA nephropathy, and cutaneous immune-related disorders (Aupetit et al., 2000; Frezza et al., 2004; Cianci et al., 2008). Of the four alleles α_{1B} correlates with increased prevalence or severity of these diseases (Aupetit et al., 2000; Frezza et al., 2004; Cianci et al., 2008). However, there were few observed genomes containing the α_{1C} and α_{1D} so their importance should not be dismissed (Aupetit et al., 2000; Frezza et al., 2004; Cianci et al., 2008). It is possible that increased transcriptional strength from redundant binding sites affects outcomes to correlated diseases. Furthermore, frequency of the α_{1A} , α_{1B} or α_{1C} alleles is distributed differently among various population groups indicating the polymorphism

may be considered a reliable anthropogenetic marker (Giambra et al., 2006). African populations have the highest frequency of the α_{1C} allele while Asian and European populations have a higher frequency of the α_{1B} allele (Giambra et al., 2006).

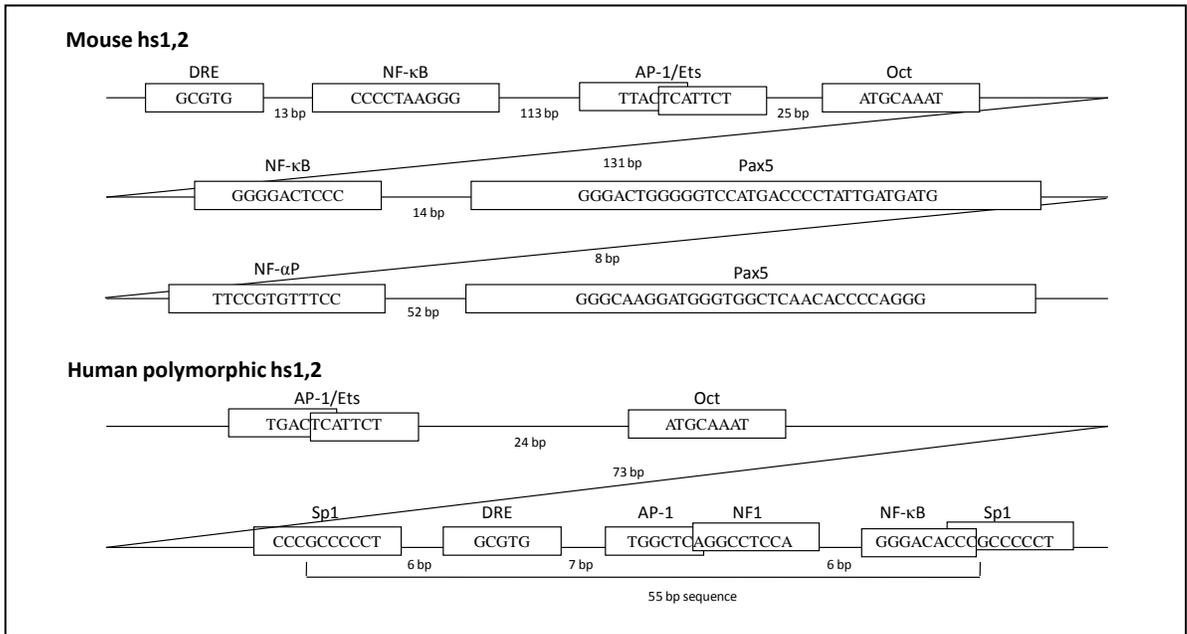


Figure 5. DNA sequences for the mouse and human hs1,2 enhancer (α_{1A}).

Significance and Objective

Previous studies showed inhibition of the mouse hs1,2 enhancer by TCDD whereas the human hs1,2 enhancer is activated (Fernando et al., 2012). It is reasonable that these diverging results may be due to differences in the DNA sequence. As such, it is hypothesized that specific transcription factor binding sites of the mouse hs1,2 enhancer and the human polymorphic hs1,2 enhancer differentially modulate TCDD-induced activity. **Objective one** was to evaluate the transcriptional activity of the Pax5 binding site that is present in the mouse hs1,2 enhancer yet absent in the human. Having two Pax5 binding sites in the mouse hs1,2 enhancer and none in the human hs1,2 enhancer may explain why TCDD inhibits the enhancer's activity in the mouse yet activates it in the human. **Objective two** was to determine what role the AhR plays in TCDD-inducibility of human hs1,2 enhancer activity. Since the human hs1,2 enhancer contains binding sites that are directly or indirectly effected by TCDD or the AhR, it is important to establish what involvement the AhR has in enhancer modulation. **Objective three** was to evaluate the role of specific transcription factors in the human polymorphic hs1,2 enhancer. The IS is especially interesting, because it is associated with several immune-related diseases (e.g. celiac disease, IgA nephropathy, and cutaneous immune-related disorders) and contains transcription factor binding motifs that have been shown to interact with the AhR or be modulated by TCDD.

The current study has significant implications for several reasons. First of all, it exemplifies the complexity of translating mouse studies to human risk assessment, because there are sequence differences in the DNA. Some transcription factor binding sites are contained within both the mouse and human hs1,2 enhancer (AP-1/Ets, Oct,

DRE, NF- κ B) while other binding motifs are only in the mouse (NF- α P, Pax5) or the human (Sp1, NF1, AP-1) (Fig. 5). Also, this study will further elucidate the mechanism involved in TCDD-induced modulation of the hs1,2 enhancer. Evaluating the transcriptional activity of specific binding motifs will help clarify the pathway. Finally, researching the 3'*IgH* RR and hs1,2 enhancer is important, because they are sensitive to chemical-induced modulation and associated with a number of lymphomas and Ig-secreting diseases. Understanding how this region is governed could provide insight to the etiology of certain disease states and how they could be altered along with how these diseases could be environmentally triggered.

II. MATERIALS AND METHODS

Chemicals and Reagents

TCDD purchased from AccuStandard, Inc. (New Haven, CT) comes dissolved in 100% DMSO and the certificate of analysis reports 99.1% purity. The AhR antagonist 2-methyl-2H-pyrazole-3-carboxylic acid-(2-methyl-4-o-tolyl-azo-phenyl)-amide (CH-223191), previously characterized by Kim et al., (2006) was purchased from Calbiochem (Carlsbad, CA) and dissolved in 100% DMSO. LPS from *Escherichia coli* and DMSO were purchased from Sigma Alrich (St. Louis, MO). The LPS was dissolved in 1x sterile-filtered PBS.

Cell Line Model

The CH12.LX mouse B cell line, compliments of Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC), is derived from the murine CH12 B-cell lymphoma arising in a B10.H-2^aH-4^bp/Wts (2^a4^b) mouse. The CH12.LX cell was characterized by Bishop and Haughton (1986) and has been used extensively in immunological and toxicological research. There is high AhR expression and a functional AhR signaling pathway as well as inhibition of LPS-induced Ig expression by TCDD (Sulentic et al., 1998, 2000). For these reasons using the CH12.LX cell line for dioxin studies is useful and will help initiate further studies in human cell lines.

Cell Culture Conditions

Cells were kept at 37°C in a 5% CO₂ atmosphere and grown in complete media. The complete media consisted of RPMI-1640 (Mediatech, Herndon, VA) enhanced with 2 mM L-glutamine, 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol and 23.8 mM sodium bicarbonate.

Reporter Plasmid Constructs

The human polymorphic plasmids utilize the pGL3 basic luciferase reporter construct (Promega, Madison, WI) containing ampicillin resistance and the luciferase gene. The enhancerless variable heavy chain promoter (V_H) plasmid and the α_{1A} , α_{1B} and α_{1C} plasmids were a generous gift from Dr. Michel Cogné (Université de Limoges, France) (Fig. 6). Of the plasmids that contain the human hs1,2 enhancer there is an invariant sequence (IS) that may be present one (α_{1A}), two (α_{1B}) or three (α_{1C}) times (Fig. 6). The IS can also be present four (α_{1D}) times, but was not studied because of low prevalence. Each IS is approximately 55 bp in length and contains several transcription factor binding sites (AP-1, NF1, NF-κB, DRE), as previously mentioned (Chen and Birshstein, 1997; Denizot et al., 2001; Giambra et al., 2005; Fernando et al., 2012). The DRE and AP-1 binding sites found in the third IS of the α_{1C} are not conserved. However, isolation of genomic DNA from human buccal cells indicated conservation of both these sites.

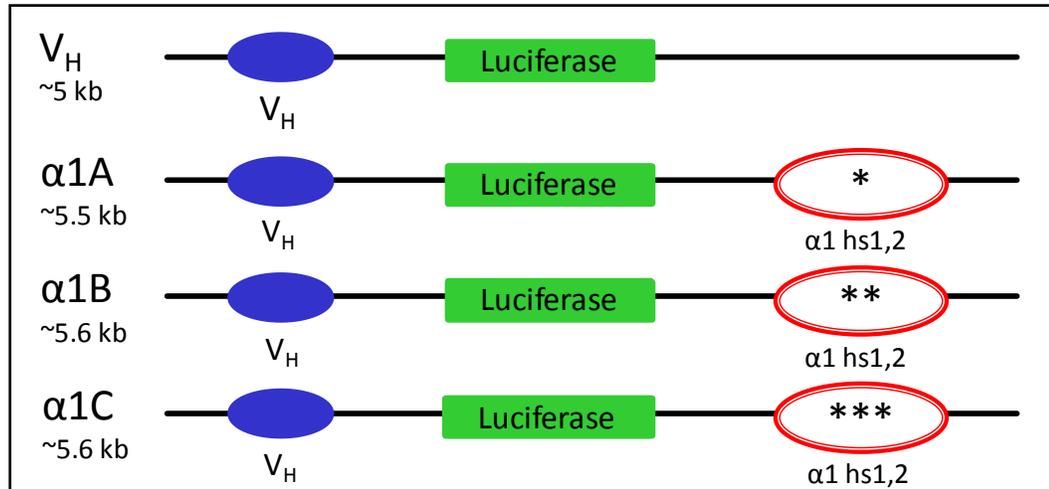


Figure 6. Human polymorphic hs1,2 reporter plasmid constructs. The asterick (*) represents the IS that may be present one (α_{1A}), two (α_{1B}) or three (α_{1C}) times.

Site-directed Mutagenesis

The QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) consists of a four-step process involving plasmid preparation, temperature cycling, digestion, and finally transformation. Each mutation reaction used 5 μ l of 10X reaction buffer, 100 ng of parental template, 125 ng each of forward and reverse mutation primers, 1 μ l of dNTP mix, 1.5 μ l of QuikSolution reagent and ddH₂O for a final volume of 50 μ l. 1 μ l of QuikChange Lightning Enzyme was added before thermal cycling. PCR conditions used are as follows: 95°C for 2 min followed by 18 cycles at 95°C for 20 s, 60°C for 10 s, 68°C for 2.75 min (30 sec/kb of plasmid length), then 68°C for 5 min. After the PCR reaction, the parental plasmid was digested by incubating the PCR product with *Dpn* I (2 μ L/50 μ L reaction) for 5 min at 37°C. 2 μ l of *Dpn* I-treated DNA was used to transform 45 μ l of XL10-Gold® ultracompetent cells. Plasmid DNA was isolated from transformed colonies and sequenced to ensure quality and accuracy of the mutations (Retrogen, Inc., San Diego, CA). For the α_{1A} plasmid one high-affinity Pax5 site was added and the AP-1, NF κ B, DRE, Oct, AP1.ETS, 5' SP1 and 3' SP1 sites were mutated: termed $\alpha_{1A}+\text{Pax5}$, $\alpha_{1A}\text{AP1mut}$, $\alpha_{1A}\text{NF}\kappa\text{Bmut}$, $\alpha_{1A}\text{DREmut}$, $\alpha_{1A}\text{Octmut}$, $\alpha_{1A}\text{AP1.ETSmut}$, $\alpha_{1A}\text{SP1.1mut}$ and $\alpha_{1A}\text{SP1.2mut}$, respectively (Table 1). Additionally, the invariant sequence (IS) was deleted from the α_{1A} ($\alpha_{1A}\text{ISdel}$) and mutants containing the IS deleted plus the Oct site mutated ($\alpha_{1A}\text{ISdelOctmut}$) and the DRE site plus NF- κ B mutated ($\alpha_{1A}\text{DRE.NF}\kappa\text{Bmut}$) were also generated (Table 1). In the α_{1B} plasmid the first IS was deleted ($\alpha_{1B}\text{IS1del}$) or the AP-1 ($\alpha_{1B}\text{AP1.1mut}$), NF- κ B ($\alpha_{1B}\text{NF}\kappa\text{B.1mut}$), DRE ($\alpha_{1B}\text{DRE.1mut}$) sites were individually mutated from the first IS (Table 2). Lastly, for the α_{1C} plasmid the third IS was deleted ($\alpha_{1C}\text{IS3del}$) and the NF- κ B site in the first IS was

mutated ($\alpha_{1C}NF\kappa B.1mut$) (Table 2). A sequenced human sample containing the α_{1C} hs1,2 enhancer showed consensus-matching DRE and AP-1 sites in the third IS so both sites were mutated and restored back to their respective consensus sequences ($\alpha_{1C}Clinical$) (Table 2). Mutations of transcription factor binding sites were determined based off of enzyme mobility shift assay data and TFSEARCH, an online transcription factor profile database (Grant et al., 1995; Heinemeyer et al., 1998; Yao and Denison, 1992; Lenardo et al., 1987)

$\alpha_{1A} + \text{Pax5}$	F: 5'- GTGGTCCCAGTGTGAGCCCTGGGGTGTGAGCCACCCATCCTTGC CCTAACCCAAGTGGGCCT -3' R: 5'- AGGCCCACTTGGGTTAGGGCAAGGATGGGTGGCTGCACACCCCA GGGCTGACACTGGGACCAC -3'
$\alpha_{1A} \text{API mut}$	F: 5'-TGTCCCCGAATCTGGAGGCCCTAGATGCCTGGCCACGCTGGGGGAG-3 CCGG <u>ACTCGG</u> TCCG GGCCTGAGCCAGCC R: 3'-ACAGGGGCTTAGACCTCCGGGAATCTACGGACCGGTGCGACCCCTC-5'
$\alpha_{1A} \text{NFkB mut}$	F: 5'-CCTGTCCTGGGGAGGGGGCAGTTCCTTCTACAATCTGGAGGCCTGAGCCAG-3' CGCCACAGGGGCTTA GCGGGTGTCCCGAAT R: 3'-GGACAGGACCCCTCCCGTCAAGAAGATGTTAGACCTCCGGACTCGGTC-5'
$\alpha_{1A} \text{DRE mut}$	F: 5'-CCTGAGCCAGCCTGGCAGACTTGGGGAGGGGGCGGG-3' ACCGGTGGGACCC TGGCCACGCTGGG R: 3'-GGACTCGGTGCGACCGTCTGAACCCCTCCCGCC-5'
$\alpha_{1A} \text{Oct mut}$	F: 5'-CATGTTTTTCGGTGTGGAACAAACAACCGACTTAGGGTGCCTGAGGGCCAAGTCTGCCAGA-3' TGGTAAACGTACACG ACCATTGCATGTGC R: 3'-GTACAAAAGCCACACCTTGTTGTTGGCTGAATCCACGGGACTCCCGTTTCAGACGGGTCT-5'
$\alpha_{1A} \text{ISdel}$	F: 5'-GGGGAGGGGGC.....GGGAGAATCGTG -3' CCCCGCC.....CGACCCT GGCGGG.....GCTGGGA R: 3'-CCCCCTCCCCG.....CCCTCTAGCAC-5'
$\alpha_{1A} \text{API.ETS mut}$	F: 5'-GTGCCCTGAGGGCCAAGTCTGCCCTAGTTGGCCCTCAAGGAGAAACAGATCAGAGCC-3' GGTCTTACTCAGTGA CCAGAATGAGTCACT R: 3'-CACGGGACTCCCGTTTCAGACGGGGATCAACCGGGGAGTTCCTCTTGTCTAGTCTCGG-5'
$\alpha_{1A} \text{SP1.1 mut}$	F: 5'-GGGCCTGTCCTGGGGAGGATCGACTTGTCCCGAATCTGGAGGC-3' CTCCCCGCCAC GAGGGGGCGGTG R: 3'-CCCGACAGGACCCCTCCTAGCTGAACAGGGGCTTAGACCTCCG-5'
$\alpha_{1A} \text{SP1.2 mut}$	F: 5'-CAGCCTGGCCACGCTGGGGAGGATCGACTAGAATCGTCAAGCTATTCTGG-3' CTCCCCGCCCT GAGGGGGCGGA R: 3'-GTCCGACCGGTGCGACCCCTCCTAGCTGATCTTAGCACGTTTCGATAAAGACC-5'
$\alpha_{1A} \text{DRE.NFkB mut}$	Serial mutagenesis performed using primer sets from $\alpha_{1A} \text{DRE mut}$ and $\alpha_{1A} \text{NFkB mut}$
$\alpha_{1A} \text{ISdelOct mut}$	Serial mutagenesis performed using primer sets from $\alpha_{1A} \text{ISdel}$ and $\alpha_{1A} \text{Oct mut}$

Table 1. α_{1A} site-directed mutagenesis primers. Bolded sequence denotes addition or mutation. Underlined sequence denotes original sequence before mutation or deletion.

α_{1B} API.1mut	F: 5'-CTCCCCAGCGTGCCAGGCATCTAAGGGCCTCCAGATTCGGGGACA-3' CGACCGAGTC GCTGGCTCAG R: 3'-GAGGGGGTCGCACCGTCCGTAGATTCCCGGAGGTCTAAGCCCCTGT-5'
α_{1B} NFkB.1mut	F: 5'-GGCTCAGGCCTCCAGATTCGGATCTTAGCCCCACCACAGCGTGGCCAG-3' GCCCTGTGGGG CGGGACACCCC R: 3'-CCGAGTCCGGAGGTCTAAGCCTAGAATCGGGGGTGGTGTGCACCGGTC-5'
α_{1B} DRE.1mut	F: 5'-TCTCCCCCCCCTCCCCAAGACTGCCAGGCTGGTC-3' GTCCGACCG CAGCGTGGC R: 3'-AGAGGGCGGGGAGGGGGTCTGACGGTCCGACCGAG-5'
α_{1B} IS1del	F: 5'- GCACGATTCTCCC.....CCCCACCACAGCG--3' GGGCGGGGGGGGGGT CCCGCC.....CCCCCA R: 3'-CGTGCTAAGAGGG.....GGGGTGGTGTCCG-5'
α_{1C} NFkB.1mut	F: 5'-GTCCTGGGGAGGGGGCAGTCTTCTCGAATCTGGAGGCTGA-3' GCCACAGGGGC CGGGTGTCCCG R: 3'-CAGGACCCCTCCCCGTCAAGAAGAGCTTAGACCTCCGGACT-5'
α_{1C} Clinical	F: 5'-CTGGAGGC TGAGCC AGCCTGGCC CAGC CTGGTGGGTGGCGGTGCAGCT-3' GGTGGCCAC.....GCACACA CCACCGGTG.....CGTGTGT R: 3'-GACCTCCGGACTCGGTCCGACCGGTGCGACCCACCCACCGCCGACGTCA-5'
α_{1C} IS3del	F: 5'-CGCTGTGGTGGGG.....GCTGCAGCTGCAG-3' CCCGGG .CGCGCA GGGCC .CGCGCT R: 3'-GCGACACCACCC.....CGACGTCGACGTC-5'

Table 2. α_{1B} and α_{1C} site-directed mutagenesis primers. Bolded sequence denotes addition or mutation. Underlined sequence denotes original sequence before mutation or deletion.

Transient Transfection

1.0 x 10⁷ cells were pelleted by centrifugation at 300 x g for 5 minutes at 4°C. Media was removed and cells were resuspended with 10 µg of plasmid and enough media to bring the final volume up to 200 µl. 200 µL (1.0 x 10⁷ cells) was transferred to a 2mm electroporation cuvette and electroporated at 250 V, 150 µF, and 75 ohms. Each plasmid was transfected multiple times and cells were pooled and diluted to obtain 2 x 10⁵ cells/mL. Transfected cells were cultured in the absence of any additional treatment (naïve, NA) or treated with 0.01% DMSO (vehicle control-0 nM TCDD) or TCDD (0.01 nM, 0.1 nM, 1.0 nM, 10 nM) in the absence or presence of LPS (1.0 or 0.1 µg/ml) stimulation. Different LPS concentrations were used, because different lots of LPS varied in potency. Cells were aliquoted into 12-well plates (n=3) and incubated at 37°C in 5% CO₂ for 24 hours. In AhR antagonist studies, cells were pre-treated with the antagonist (15µM) for 1 hour prior to additional treatments.

Luciferase Assay System

After the 24 hour incubation period, cell culture plates were centrifuged at 1800 x g for 5 minutes at 4°C. Supernatant was removed and cells were lysed with 1x lysis buffer (Promega, Madison, WI) and immediately frozen at -80°C for no less than 1 hour. To quantify gene expression the Luciferase Assay System (Promega, Madison, WI) was used to measure luciferase enzyme activity. Samples were thawed to room temperature and centrifuged at 20,000 x g for 5 minutes at 4°C. 20 µl of sample lysate was mixed with 100 µl of luciferase substrate reagent and a single-tube luminometer (Berthold

Detection Systems, Oak Ridge, TN) reported light measurements as relative light units (RLUs) following each reaction.

Transfection Efficiency

An additional group of naïve cells were seeded for each transfected plasmid to determine transfection efficiency. TCDD or LPS treatments do not affect transfection efficiency (data not shown). DNA was isolated through a genomic DNA miniprep kit (Sigma Aldrich, St. Louis, MO) at 4 hrs post-transfection and diluted 10-fold. Real-time polymerase chain reaction (RT-PCR) was performed by using absolute quantification. In a 96-well plate each sample well contained 23 μl of the reaction master mix [12.5 μl 2x SYBR Green (Applied Biosystems, Warrington, UK), 1 μl 10 pmol/ μl forward primer-pGL3 luciferase, 1 μl 10 pmol/ μl reverse primer-pGL3 luciferase, and 8.5 μl purified water] and 2 μl of sample DNA. The forward and reverse primers are 5'-ACTGGGACGAAGACGAACACTT-3' and 5'-TCAGAGACTTCAGGCGGTCAA-3', respectively. Sample PCR data was compared to a standard luciferase reporter plasmid with concentrations ranging from 0.1 ng/ μl to 1×10^{-6} ng/ μl . Amount of transfected plasmid (ng) was calculated by taking the concentration of DNA from the PCR results (ng/ μl) x the volume of DNA added (2 μl) x the fold dilution (10). Number of plasmids per cell was calculated from the equation: [ng of plasmid DNA x number of plasmids/ng] \div total number of cells isolated (previously described in Sulentic et al., 2004). Luciferase activity was normalized to the wildtype plasmid based on plasmids per cell.

Statistical Analyses of Data

Comparisons between treatment groups (n=3) of the same reporter plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Significant differences in luciferase activity (mean \pm SEM) were compared to the corresponding vehicle control and represented by “*”, “**”, “***” at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Differences in TCDD-induced fold change was determined in the same manner and denoted by “‡”, “‡‡”, “‡‡‡” at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Differences in luciferase activity or fold change between reporter plasmids were analyzed using a 2-way ANOVA with Bonferroni post test. Daggers, “†”, “††”, “†††”, denote significant differences between different reporter plasmids at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Multiple, separate experiments (n=3 for each treatment group) were used to generate TCDD-induced fold change results (mean \pm SEM). Synergism was calculated by normalizing relative light units (RLUs) of plasmids to the average NA RLU of the wildtype plasmid (V_H or α_{1A}). RLU data has already undergone normalization to transfection efficiency. The average DMSO vehicle RLU was subtracted from treatment RLUs of its respective plasmid enable to compare an additive effect (i.e. “L” + “TCDD”) versus a co-treatment effect (i.e. “LPS + TCDD”).

III. RESULTS

Insertion of a Pax5 binding site lowers transcriptional activity of the human hs1,2 enhancer

Previous transient transfections of CH12.LX cells show that TCDD inhibits transcriptional activity of the mouse hs1,2 enhancer yet activates the human hs1,2 enhancer (Fernando et al., 2012). It is possible that these diverging outcomes may be due to the presence of two Pax5 binding sites (one high-affinity and one low-affinity) in the mouse hs1,2 enhancer and none in the human hs1,2 enhancer. The B-cell lineage specific activator protein (BSAP) that binds Pax5 sites is expressed early in B-cell differentiation, but is consequently down-regulated following B-cell activation (e.g. LPS). TCDD treatment inhibits this natural downregulation of BSAP (Yoo et al., 2004; Schneider et al., 2008).

Using site-directed mutagenesis, one high-affinity Pax5 binding site was added to the human α_{1A} plasmid (α_{1A} + Pax5). Overall transcriptional activity was lowered by the Pax5 site and significant differences in luciferase activity were observed between plasmids for nearly every unstimulated and LPS-stimulated treatment (Fig. 7A). Basal activity was minimally lowered by insertion of the Pax5 site while LPS-induced activity was markedly suppressed (Fig. 7A). Furthermore, α_{1A} demonstrated synergism from LPS/TCDD cotreatment while α_{1A} + Pax5 did not (Fig. 7A). However, contrary to expectation, the Pax5 binding site did not seem to alter TCDD-induced fold-change

activation of the human hs1,2 enhancer (Fig. 7B). Even though TCDD still increased α_{1A} transcriptional activity in a concentration-dependent manner that appeared unrelated to the Pax5 site overall transcription was still lower for $\alpha_{1A} + \text{Pax5}$ (Fig. 7A and 7B). Although these outcomes may dismiss the role Pax5 may have in the inhibition of mouse hs1,2 activity by TCDD further consideration should be warranted, because of the complexity involved in Pax5 regulation.

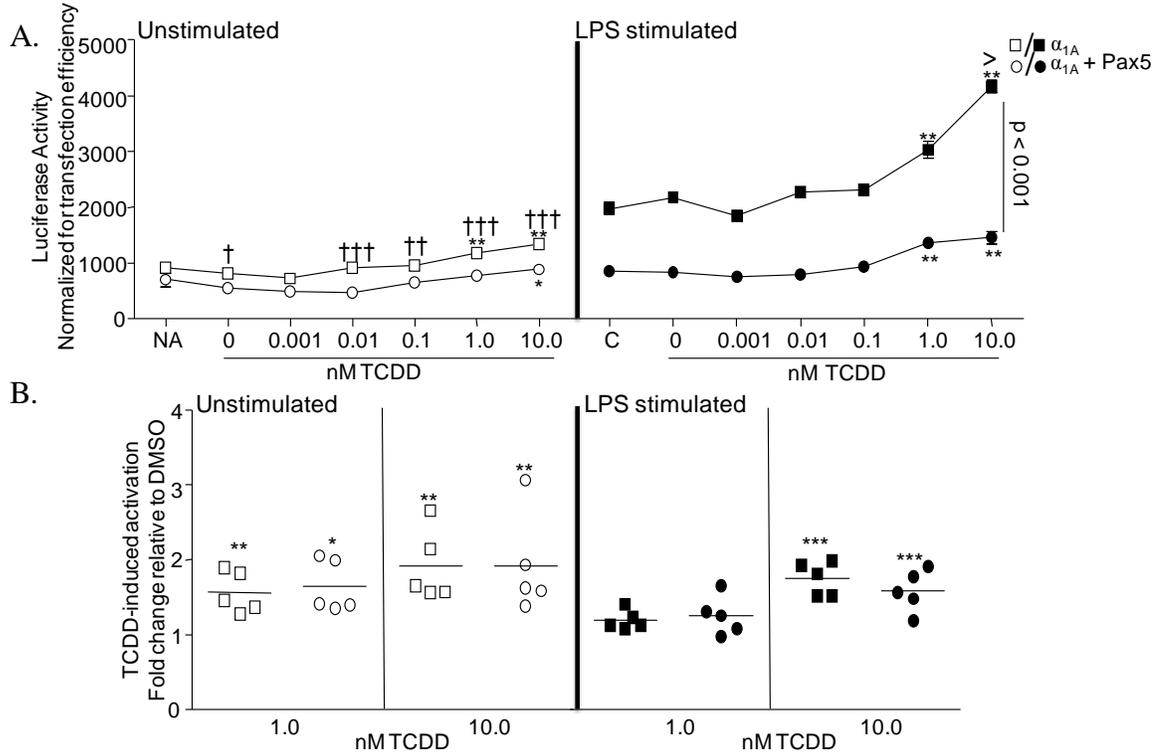


Figure 7. A Pax5 site decreases overall transcriptional activity of the α_{1A} . CH12.LX cells were transiently transfected with α_{1A} or α_{1A} + Pax5 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (1 μ g/ml) stimulation. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency. C represents the LPS alone control. B. Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of independent experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, “*”, “**”, or “***” denote significance compared to the corresponding vehicle set to 1 at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, “†”, “††”, “†††”, denote significant differences between reporter plasmids for each treatment at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Vertical line represents significance difference between all treatment groups between reporter plasmids at $p < 0.001$. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of 5 separate experiments.

TCDD activates the human polymorphic hs1,2 enhancer in an AhR-dependent manner

The antagonist, CH-223191, was used to test if the AhR is essential for TCDD-induced activation of the human polymorphic hs1,2 enhancer. CH12.LX cells were transiently transfected with the V_H , α_{1A} , α_{1B} or α_{1C} luciferase reporter plasmids then treated with the AhR antagonist making the receptor inaccessible and unable to translocate to the nucleus. Transcriptional activity of basal and LPS levels of the V_H remained low while α_{1A} and α_{1B} had similarly higher activity and α_{1C} demonstrated the most activity (Fig. 8A). TCDD expectedly activated the human hs1,2 enhancer when containing one, two or three invariant sequences in both unstimulated and LPS-stimulated cells (Fig. 8A and 8B). Cells treated with both the antagonist and TCDD displayed a reversal of enhancer activation thus exemplifying the necessity of the receptor (Fig. 8A and 8B). Also, α_{1A} , α_{1B} and α_{1C} demonstrated synergism from LPS/TCDD cotreatment compared to either treatment by itself (Fig. 8A). The fold change between the human polymorphic reporter plasmids were quite similar, which means that the number of invariant sequences present may be less important to what degree the human hs1,2 enhancer is activated by TCDD (Fig. 8B). In essence, these results indicate that the AhR is necessary for TCDD to activate the enhancer, but whether it is directly or indirectly involved in the mechanism is yet to be determined. Either the TCDD/AhR/ARNT complex is binding the hs1,2 enhancer or TCDD and/or the AhR is altering binding of another transcription factor.

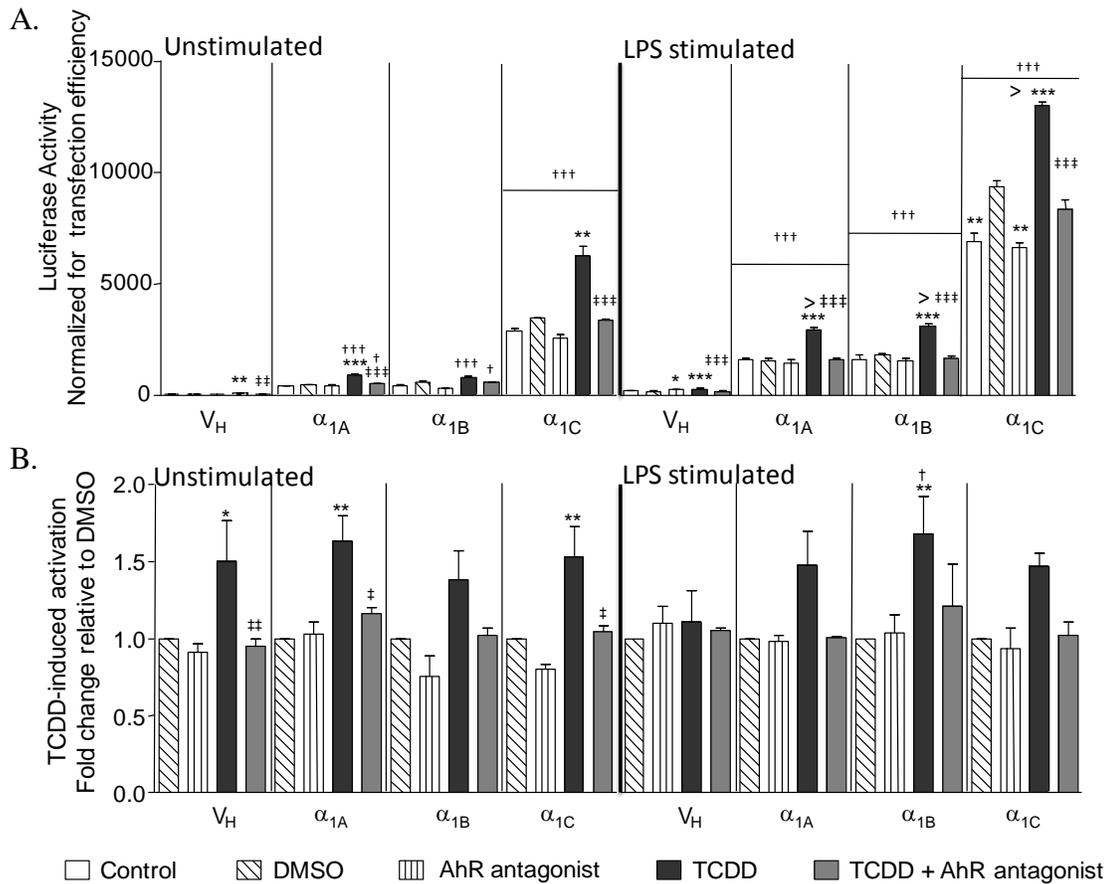


Figure 8. TCDD-induced activation of the human polymorphic enhancer is AhR dependent. CH12.LX cells were transiently transfected with V_H, α_{1A}, α_{1B}, or α_{1C} reporter constructs. Transfected cells were either pre-treated for 1 hr with 15 μM AhR antagonist (CH-223191), 0.15% DMSO, or media alone then cultured for 24 h in the absence or presence of LPS (1.0 μg/mL) stimulation and either media alone, 0.01% DMSO, or 1 nM TCDD. “Control” denotes either unstimulated naive or LPS alone. A. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of independent experiments (n=3 for each treatment group within each experiment). Significance was determined by a 1-way ANOVA followed by a Dunnett’s Multiple Comparison post test: “*”, “**”, “***”, significance compared to the corresponding DMSO vehicle control at *p*<0.05, *p*<0.01 and *p*<0.001, respectively; “‡”, “‡‡”, “‡‡‡”, significant difference between TCDD alone and TCDD + AhR antagonist. Significance compared to the V_H reporter was determined by a 2-way ANOVA with a Bonferroni’s post-hoc test: “†” and “†††”, significance at *p*<0.05 and *p*<0.001, respectively. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of three separate experiments.

Deletion of the invariant sequence lowers overall transcriptional activity of the human hs1,2 enhancer

As previously mentioned the invariant sequence (IS) contains a number of transcription factor binding sites (DRE, AP-1, NF1, NF- κ B) and is flanked by SP1 sites on both the 5' and 3' end (Chen and Birshstein, 1997; Denizot et al., 2001; Giambra et al., 2005; Fernando et al., 2012). TCDD and/or the AhR have been shown to affect these transcription factors so evaluation of this region may explain how TCDD induces the hs1,2 enhancer. The α_{1A} and α_{1B} plasmids contain one and two IS, respectively. Sequencing results of the α_{1C} plasmid indicate the DRE and AP-1 sites of the third IS are not entirely conserved, but this is contrary to human sequencing (Denizot et al., 2001; Fernando et al., 2012). To determine how the polymorphism affects transcriptional activity and TCDD-induced fold change of the hs1,2 enhancer the IS was deleted from the α_{1A} and α_{1B} . Transient transfection into CH12.LX cells of the deleted IS from the α_{1A} (α_{1A} ISdel) showed a significant decrease in overall transcriptional activity of the human hs1,2 enhancer (Fig. 9A). Luciferase activity is decreased for both unstimulated and LPS stimulated treatments when the IS is deleted, but TCDD-induced fold-change activation is still parallel with α_{1A} (Fig. 9A and 9B). Deleting the first IS from the α_{1B} (α_{1B} IS1del) revealed a similar outcome to the α_{1A} ISdel by lowering the transcriptional activity for all treatment groups (Fig 9A). Interestingly, the α_{1B} IS1del still displayed more overall activity than the α_{1A} (Fig 10A). Either the additional IS cannot account for all of the activity of the enhancer and the other transcription factors found outside the polymorphism are involved or the deletion generates a compensating mechanism. Furthermore, the α_{1A} , α_{1A} ISdel, α_{1B} IS1del and α_{1B} all showed similar profiles in

synergistic activation from LPS and TCDD cotreatment (Fig. 9A and 10A). Again, no significant differences in the magnitude of TCDD-induced fold-change activation were observed between the α_{1B} IS1del, α_{1A} or α_{1B} , but there is a noticeable decrease in LPS stimulated fold-changes of the α_{1B} IS1del when compared to the α_{1B} (Fig. 10B). A slight decrease in α_{1A} ISdel stimulated fold-changes is also seen when compared to the α_{1A} , which may suggest that multiple transcription factors both within and outside the IS work together to induce activation of the human hs1,2 enhancer by TCDD (Fig. 10B).

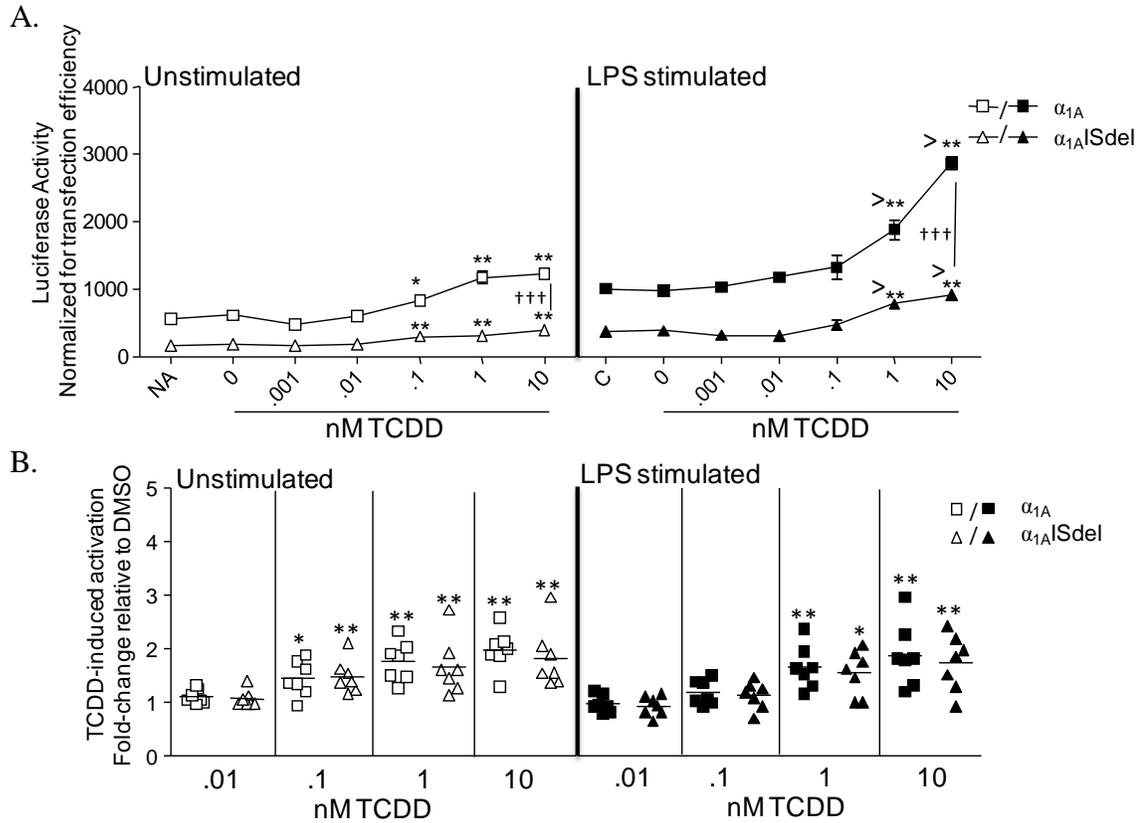


Figure 9. Deletion of the IS from the α_{1A} reduces overall transcriptional activity. CH12.LX cells were transiently transfected with α_{1A} or $\alpha_{1A}ISdel$ reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of independent experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*" or "**", denote significance compared to the corresponding vehicle control at $p < 0.05$ or $p < 0.01$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.001$. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of seven experiments.

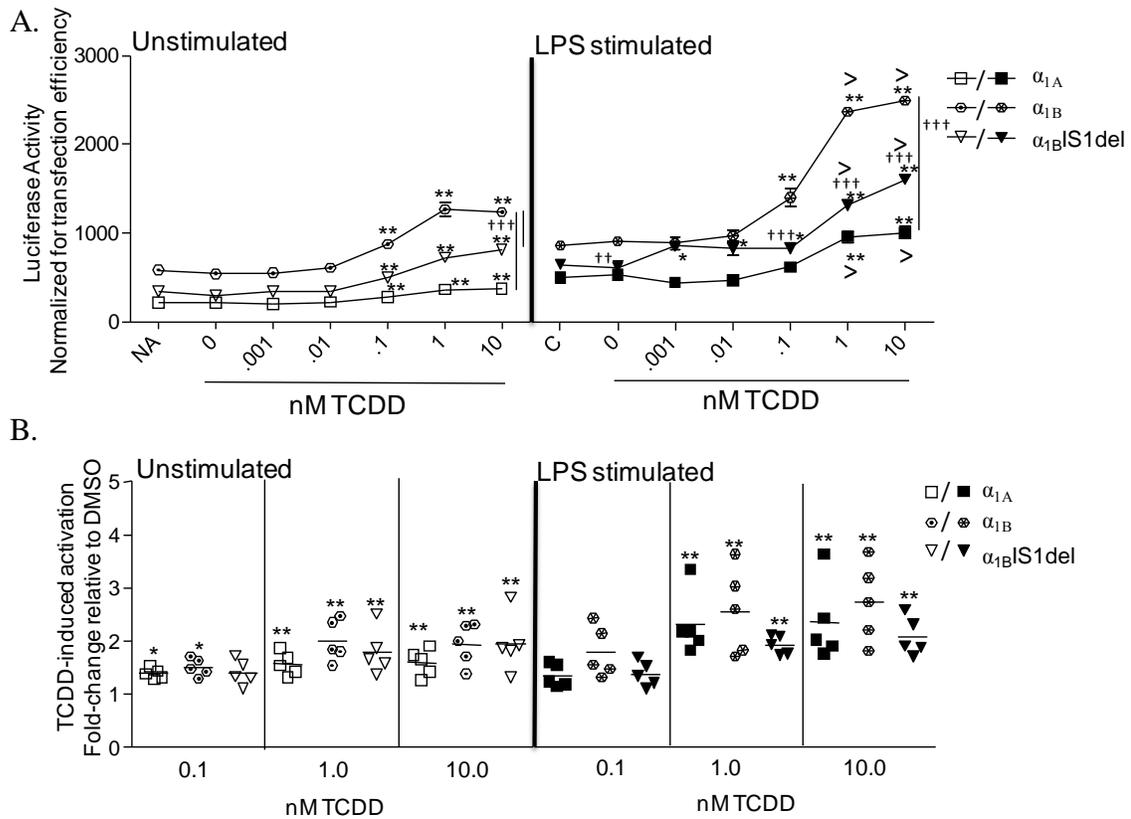


Figure 10. Deletion of the first IS from the α_{1B} lowers overall transcriptional activity of the human hs1,2 enhancer. CH12.LX cells were transiently transfected with α_{1A} , $\alpha_{1BIS1del}$ or α_{1B} reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of independent experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*" or "**", denote significance compared to the corresponding vehicle control at $p < 0.05$ or $p < 0.01$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "††" or "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.01$ or $p < 0.001$. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of five experiments.

Mutation of the invariant sequence AP-1 or NF- κ B site increases overall transcription of the human hs1,2 enhancer

The activator protein-1 (AP-1) binding site (TGGCTCA) and nuclear factor kappa B (NF- κ B) site (GGGACACCC) are located within the invariant sequence (IS) of the human polymorphic hs1,2 enhancer (Denizot, 2001). It has been shown that AP-1 or NF- κ B are affected by the AhR or TCDD through cross-talk interactions or altered binding and expression (Suh et al., 2002; Tian, 2002). Evaluation of these sites is therefore necessary, because each may be involved in the TCDD-induced activation of the human hs1,2 enhancer.

A number of studies have reported TCDD-induced influence on AP-1, but outcomes vary on whether the transcription factor's activity increases or decreases. In LPS-activated CH12.LX cells, one study showed that TCDD upregulated AP-1 binding within the promoter of B lymphocyte-induced maturation protein-1 (Blimp-1), a critical regulator of B-cell differentiation and a negative transcriptional repressor of Pax5 (Schneider et al., 2009). In this same study a link was made between TCDD-mediated suppression of Blimp-1 through AP-1 binding and Pax5 dysregulation (Schneider et al., 2009). Also, other studies conducted in multiple liver cell types showed an increase in AP-1 DNA binding activity resulting from genes induced by AhR agonists (Puga et al., 1992; Ashida et al., 2000). In opposition Suh and coworkers demonstrated that TCDD inhibited DNA binding and transcriptional activity of AP-1 in LPS-activated CH12.LX cells (2002). In the same study TCDD was unable to inhibit AP-1 activity in an AhR-

deficient murine B cell line, BCL-1 (Suh et al., 2002). Even though TCDD-induced effects on AP-1 differ, it is clear that the transcription factor is targeted.

Site-directed mutagenesis was used to mutate the AP-1 site in the α_{1A} reporter plasmid (α_{1A} AP1mut), which was then transiently transfected into CH12.LX cells. The α_{1A} AP1mut resulted in significantly higher transcription of the human hs1,2 enhancer in both unstimulated and LPS-stimulated CH12.LX cells (Fig. 11A). Also, synergism was similarly exhibited by α_{1A} and α_{1A} AP1mut. Although differences were not significant, averaged TCDD-induced fold-change data from several experiments show a trend for greater activation of α_{1A} as compares to α_{1A} AP1mut at higher concentrations of TCDD in LPS-stimulated CH12.LX cells (Fig. 11B). The AP-1 site seems to play an important role in the overall transcription of the human hs1,2 enhancer, but may not play a dominant role in TCDD-induced modulation.

Like the IS AP-1 site, TCDD-induced modulation of the human polymorphic hs1,2 enhancer may involve the IS NF- κ B site. NF- κ B activity contributes to the activation of hs1,2 enhancer at the plasma cell stage (Michaelson et al., 1996). Also, the transcription factor has been found to modulate AhR signaling, which may explain how TCDD-induced immunotoxicity is mediated (Tian, 2009). Transcriptional activity of the AhR and NF- κ B has been shown to be regulated by the same corepressors and coactivators, such as Steroid receptor coactivator-1 (SRC-1) and p300/CBP (Tian, 2009). Because coregulators are required for both pathways, it is possible that competition for binding occurs causing one pathway to be active while the other pathway is suppressed (Tian, 2009).

In its evaluation the NF- κ B site was mutated from the α_{1A} reporter plasmid (α_{1A} NF κ Bmut). Transient transfections of CH12.LX cells resulted in significantly higher transcriptional activity from mutation of the NF- κ B site in both unstimulated and LPS-stimulated cells (Fig. 12A). Although the overall transcriptional activity differed between mutated and nonmutated plasmids, TCDD-induced activation and synergism still occurred in both (Fig. 12A and 12B). In unstimulated cells mutating the NF- κ B site did not affect fold-change activation when compared to α_{1A} (Fig. 12B). However, in LPS-stimulated cells the mutation appears to show a lesser degree of fold change activation for all concentrations of TCDD (Fig. 12B).

Overall transcriptional activity is clearly affected by the AP-1 and NF- κ B mutations. TCDD positively regulates hs1,2 enhancer activity and mutation of these IS sites further increases TCDD-induced activity. However, mutations of the AP-1 site and NF- κ B site each showed slightly lower TCDD-induced fold changes in LPS-stimulated CH12.LX cells (Fig. 11B and 12B). Each site may naturally repress overall hs1,2 activity, but still assist in TCDD-induced enhancer activation. It seems likely that several transcription factors are coordinating TCDD's ability to activate the human hs1,2 enhancer.

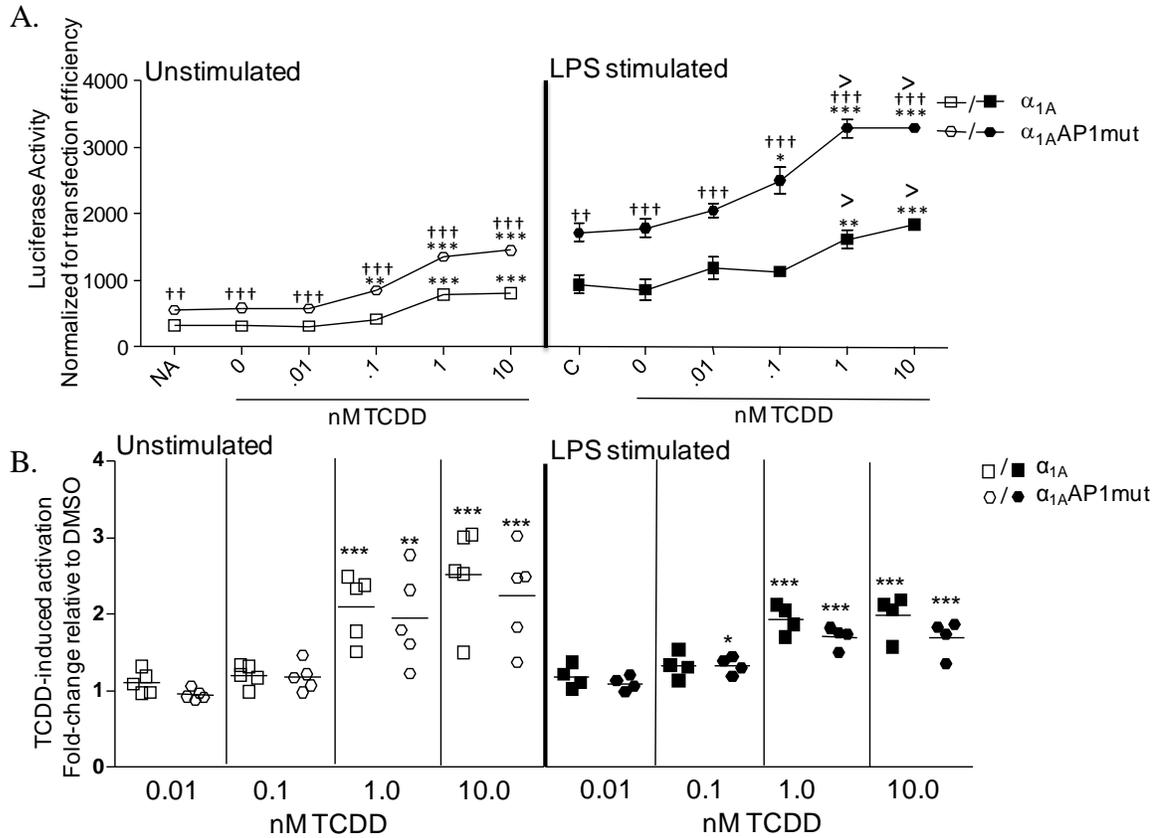


Figure 11. Mutation of the IS AP-1 site increases overall transcriptional activity and slightly decreases TCDD-induced activation of the human polymorphic hs1,2 enhancer. CH12.LX cells were transiently transfected with α_{1A} or $\alpha_{1A}AP1mut$ reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.01-10.0 nM) in the absence or presence of LPS (0.1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. TCDD-induced activation is represented on the y-axis as fold change relative to the DMSO vehicle. Results (mean \pm SEM) were generated from separate experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**" or "***", denote significance compared to the corresponding vehicle control at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "††", "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.01$ and $p < 0.001$, respectively. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of at least 4 separate experiments.

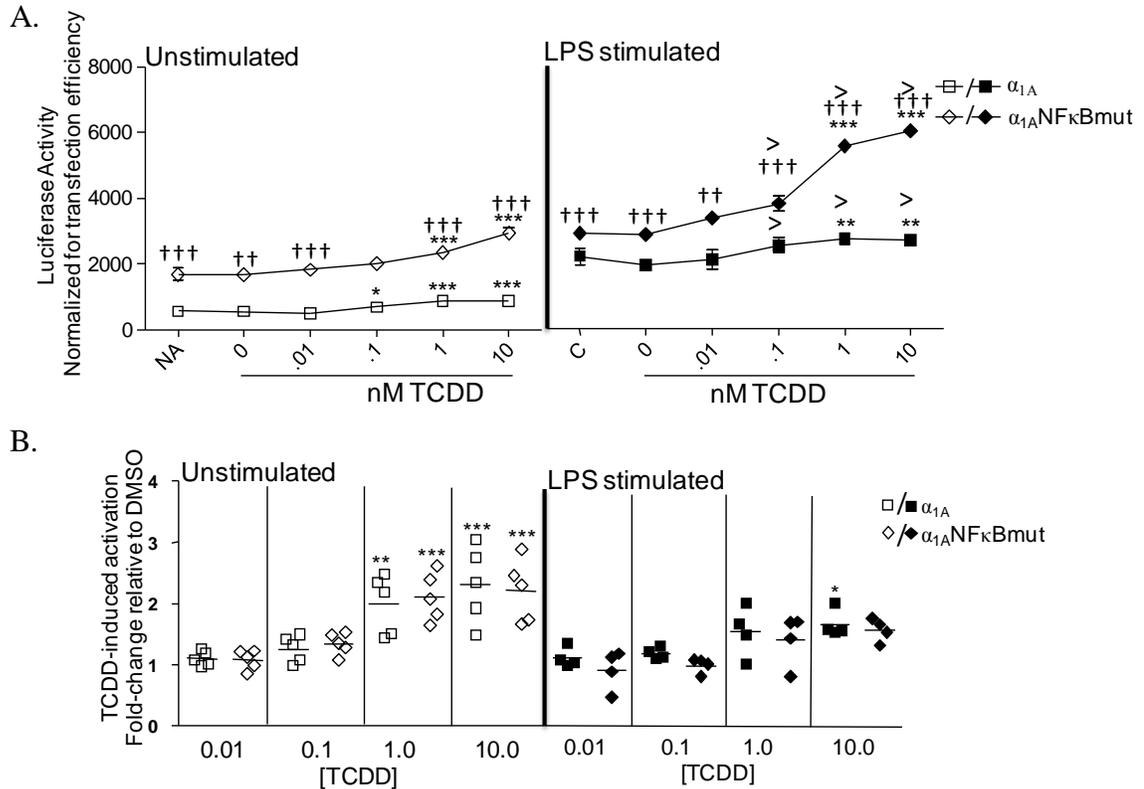


Figure 12. Mutation of the IS NF κ B site of the α_{1A} increases overall transcription and slightly lowers stimulated TCDD-induced fold change. CH12.LX cells were transiently transfected with α_{1A} or NF κ Bmut α_{1A} reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.01-10.0 nM) in the absence or presence of LPS (0.1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. TCDD-induced activation is represented on the y-axis as fold change relative to the DMSO vehicle. Results (mean \pm SEM) were generated from separate experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**" or "***", denote significance compared to the corresponding vehicle control at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "††", "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.01$ and $p < 0.001$, respectively. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of at least 4 separate experiments.

Mutating the AP1.ETS site decreases overall transcriptional activity of the human hs1,2 enhancer

The AP1.ETS (TGACTCATTCT) site is located 5' to the invariant sequence (IS). There is a single nucleotide difference between the mouse and human AP1.ETS site, which causes the human AP-1 site to match the consensus (Mills et al., 1997). In the murine hs1,2 enhancer the AP1.ETS site confers responsiveness to B-cell receptor cross-linking and has a functional role in 3'IgH RR activity (Grant et al., 1995). Following IgM receptor activation of primary B lymphocytes or BAL-17 cells enhancer activation was concurrent with recruitment and binding of nuclear factor of activated B cells (NFAB) to the AP1.ETS site (Grant et al., 1995). Because of its demonstrated role in murine enhancer activity, it is necessary to explore what role the AP1.ETS site plays in the human hs1,2 enhancer and how TCDD-induced modulation is altered. Mutation of the AP1.ETS site (α_{1A} AP1.ETSmut) within the α_{1A} reporter plasmid followed by transient transfection of CH12.LX cells resulted in a markedly lower overall transcriptional activity for unstimulated and LPS-stimulated treatments with or without TCDD (Fig. 13A). Additionally, α_{1A} demonstrated more synergism than α_{1A} AP1.ETSmut (Fig. 13A). Of all the individual transcription factor binding sites evaluated in this study, mutation of the AP1.ETS was the only one that decreased luciferase activity. Again, no significant differences in TCDD-induced fold-change were observed between the α_{1A} AP1.ETSmut and α_{1A} , but the motif seems to account for much activity of the human hs1,2 enhancer (Fig. 13A & 13B).

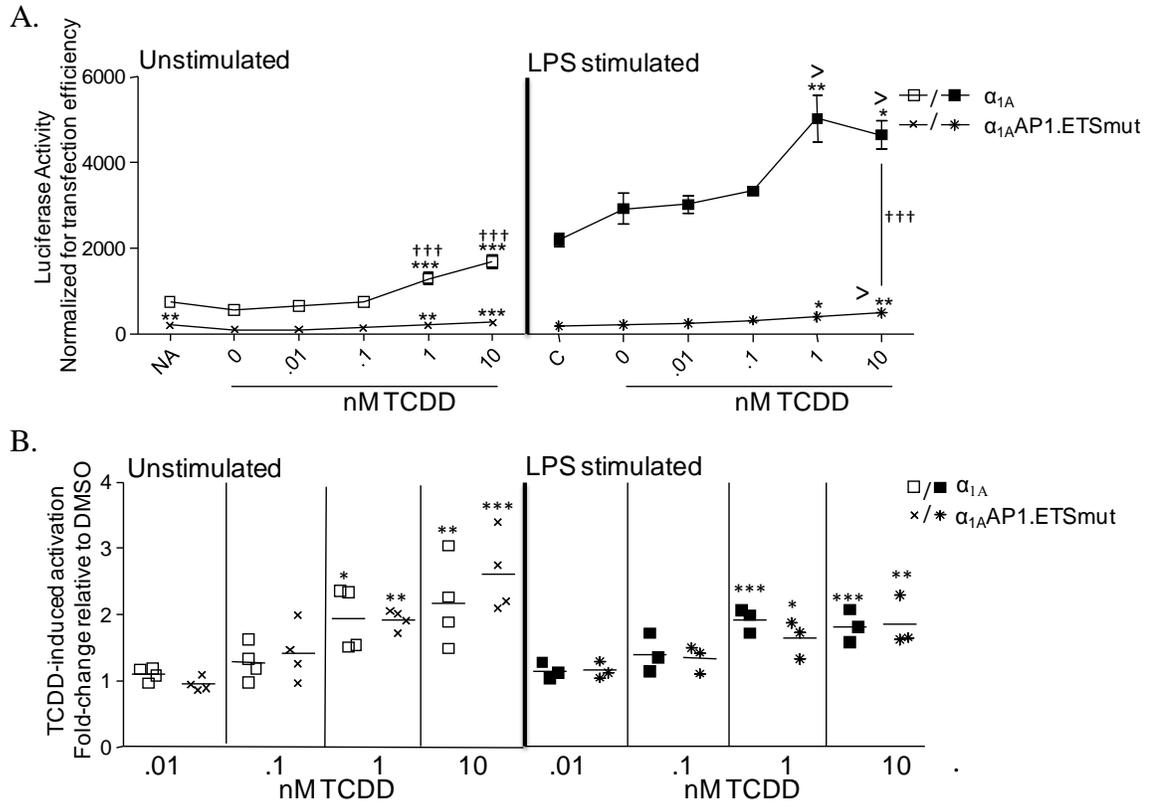


Figure 13. Mutation of the AP1.ETS site decreases overall transcriptional activity of the human hs1,2 enhancer. CH12.LX cells were transiently transfected with α_{1A} or $\alpha_{1A}AP1.ETSmut$ reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.01-10.0 nM) in the absence or presence of LPS (0.1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. TCDD-induced activation is represented on the y-axis as fold change relative to the DMSO vehicle. Results (mean \pm SEM) were generated from separate experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**" or "***", denote significance compared to the corresponding vehicle control at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.001$. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of at least three experiments.

Involvement of Oct and AP1.ETS motifs and the invariant sequence in human hs1,2 enhancer activity

Like the AP1.ETS site the transcription factor, octamer (Oct-ATGCAAAT), is located 5' of the invariant sequence (IS). Oct contributes to mouse hs1,2 enhancer activity and is conserved between mouse and human (Mills et al., 1997). In B cells Oct in collaboration with G-rich, κ B-like motifs and BSAP repress transcription of the murine hs1,2 enhancer (Singh and Birshstein, 1996). Even though little is known of TCDD-induced effects on Oct, its active role in enhancer activity makes it a site of interest. Furthermore, the binding motif is frequently present in AhR-sensitive genes (Kel et al., 2004), which means that it could have a vital role in AhR signaling and TCDD-induced modulation. When Oct was mutated (α_{1A} Octmut) from the α_{1A} plasmid and transiently transfected into CH12.LX cells, transcriptional activity increased for unstimulated and LPS-stimulated treatments both with and without TCDD (Fig. 14A). Interestingly, mutation of Oct generated a significant increase in unstimulated TCDD-induced fold-change activation compared to α_{1A} (Fig. 14B). When the Oct was mutated from the α_{1A} ISdel (α_{1A} OctmutISdel) there was still more luciferase activity than the α_{1A} wildtype and even the α_{1A} Octmut (Fig. 14A). Also, stimulated TCDD-induced fold changes of the α_{1A} OctmutISdel were not as high as the α_{1A} (Fig. 14B).

To assess relative differences in transcriptional activity V_H , α_{1A} , α_{1A} AP1.ETSmut, α_{1A} OctmutISdel, and α_{1A} ISdel were transiently transfected into CH12.LX cells. Transfecting the α_{1A} AP1.ETSmut and α_{1A} ISdel together will indicate the degree of transcriptional suppression between one another in comparison to V_H and α_{1A} activity. The α_{1A} AP1.ETSmut showed luciferase activity only slightly higher than V_H levels, but

noticeably lower than $\alpha_{1A}ISdel$, which suggests the AP1.ETS site is responsible for more enhancer activity than the IS (Fig. 15). Also, evaluating the $\alpha_{1A}OctmutISdel$ and $\alpha_{1A}ISdel$ together in this experimental design will address what impact mutating the Oct site has in combination with the IS deletion. Deletion of the IS by itself lowered transcriptional activity of the hs1,2 enhancer, as previously seen, but with the Oct additionally mutated there seems to be a dramatic increase in activity (Fig. 15). It is possible that deletion of the IS and mutation of the Oct site together acquit the enhancer of any inhibition and allow for full activity of the AP1.ETS site.

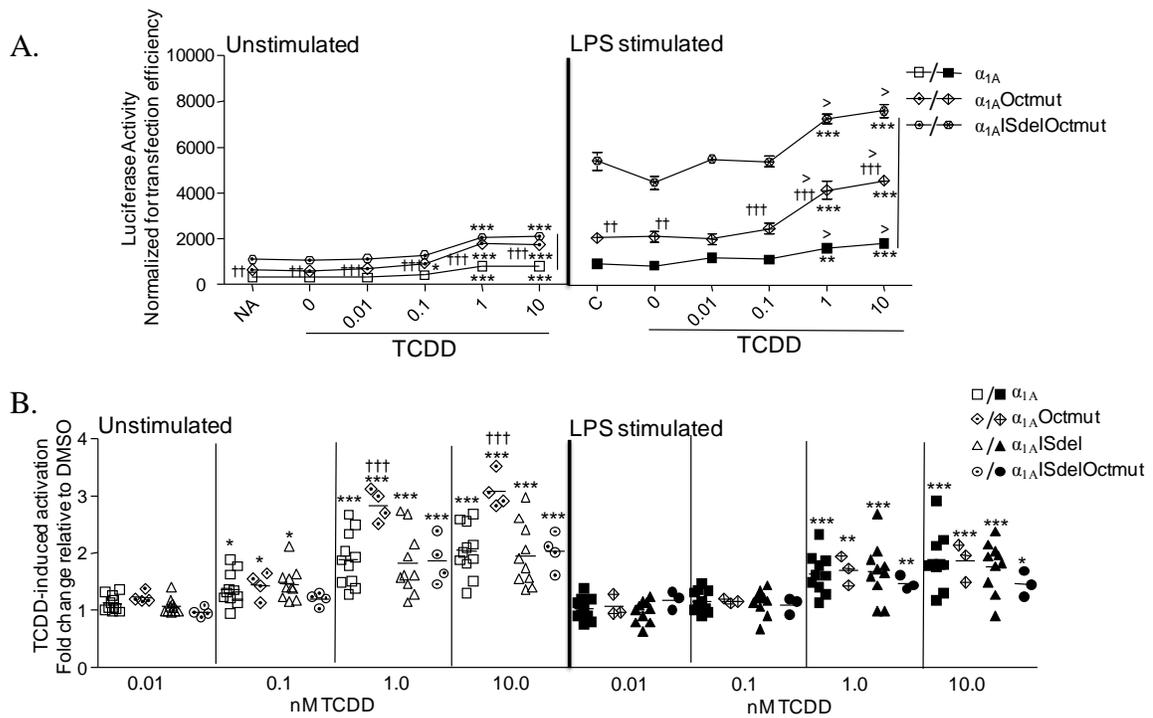


Figure 14. Mutations involving Oct increase transcriptional activity of the hs1,2 enhancer. CH12.LX cells were transiently transfected with α_{1A} , $\alpha_{1A}Octmut$, $\alpha_{1A}ISdelOctmut$ or $\alpha_{1A}ISdel$ reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.01-10.0 nM) in the absence or presence of LPS (0.1 μ g/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean \pm SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. TCDD-induced activation is represented on the y-axis as fold change relative to the DMSO vehicle. Results (mean \pm SEM) were generated from separate experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**" or "***", denote significance compared to the corresponding vehicle control at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA Bonferroni post test. Daggers, "††" or "†††", denote significant differences between reporter plasmids for each treatment at $p < 0.01$ and $p < 0.001$, respectively. Vertical line also represents significance at $p < 0.001$. > denotes synergistic activation by LPS and TCDD cotreatment compared to the activation of either treatment alone. Results are representative of at least three experiments.

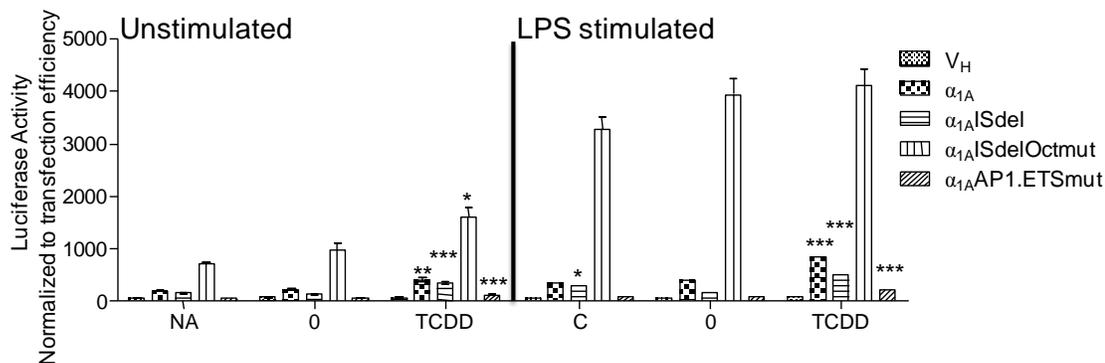


Figure 15. Mutating the AP1.ETS site decreases hs1,2 transcriptional activity more than deletion of the IS. CH12.LX cells were transiently transfected with V_H, α_{1A}, α_{1A}ISdel, α_{1A}ISdelOctmut or α_{1A}AP1.ETSmut. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (10.0 nM) in the absence or presence of LPS (0.1 μg/ml) stimulation. C represents the LPS alone control. A. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units (RLUs) normalized to transfection efficiency. B. TCDD-induced activation is represented on the y-axis as fold change relative to the DMSO vehicle. Results (mean ± SEM) were generated from separate experiments (n=3 for each treatment group within each experiment). Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**" or "***", denote significance compared to the corresponding vehicle control at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively. Results are representative of three experiments.

IV. DISCUSSION

The role of Pax5 in mediating TCDD-induced activity of the human hs1,2 enhancer

Pax5 (BSAP) is a transcription factor that plays a vital role in B-cell development and differentiation. It is present in pro-B, pre-B and mature B cells yet absent in plasma cells. Pax5 represses IgH expression by suppressing the 3'IgH RR and its natural loss in the plasma cell stage resultantly contributes to the activation of the murine 3'IgH RR and Ig expression (Singh and Birshtein, 1993; Neurath, 1994). Opposite of the murine hs1,2 enhancer, hs4 enhancer activity is upregulated by BSAP binding, which occurs in early B cell lineage (Mills et al., 1997). The one BSAP site found in the mouse hs4 enhancer is not found in the human hs4 enhancer indicating its presence is unnecessary for human hs4 activation (Mills et al., 1997). A previous study indicated inhibition of mouse hs1,2 enhancer activity through collaborative binding involving BSAP sites, an octamer sequence, a G-rich sequence and possibly an NF- κ B binding site (Michaelson et al., 1996; Singh and Birshtein, 1996). Altered binding of these same sites allows for activation of the mouse hs1,2 enhancer following the loss of Pax5 that occurs following B-cell activation (Singh and Birshtein, 1996). It is possible that absence of a Pax5 binding site in the human hs1,2 enhancer may partially explain TCDD-induced inhibition of the mouse hs1,2 enhancer versus TCDD-induced activation of the human hs1,2 enhancer.

LPS stimulation of mouse B cells decreases Pax5 mRNA and protein levels and binding beginning at 48 hrs post-stimulation (Yoo et al., 2004). When B cells are activated by LPS in the presence of TCDD, inhibition of the expected decrease in Pax5 levels is observed beginning at 48 hrs post-stimulation (Yoo et al., 2004). Disturbance in the downregulation of Pax5 by TCDD is also consistent with repression of IgH, Ig κ and J chain mRNA, which provides evidence that Pax5 is a key player in TCDD-induced suppression of the IgM response (Yoo et al., 2004, Schneider et al., 2008). It is possible that a DRE site in the Pax5 promoter is mediating TCDD-induced effects. Like mouse, Pax5 is similarly present in human (e.g. IM-9 B lymphoblasts). A previous study indicated the TCDD-induced AhR recognized a Pax5 site in the promoter region of the CD19 gene, but this was because of a DRE site contained within the Pax5 site (Masten and Shiverick, 1995). While Pax5 is seemingly involved in TCDD/AhR signaling in mouse, its role in human Ig production remains largely in question.

As expected, addition of the high-affinity Pax5 binding site to the human hs1,2 enhancer demonstrated a repressor role by decreasing overall transcriptional activity of the enhancer (Fig. 7A). Also, synergism from cotreatment of LPS and TCDD was not evident with α_{1A} + Pax5 (Fig. 7A). TCDD-induced fold-change activation of the human α_{1A} , however, remained unaffected by the presence of the Pax5 binding site (Fig. 7B). Even though inhibition of hs1,2 enhancer activity by TCDD as seen in the mouse was not similarly demonstrated by the α_{1A} + Pax5, Pax5 signaling is highly complex. Not only does Pax5 interact with other transcription factors, as previously mentioned, but it can display dual functions in B lymphocytes or undergo alternative splicing (Pinaud et al., 2011). Pax proteins are also able to regulate responses by inducing conformational

changes to DNA so it is possible that the murine high-affinity Pax5 site by itself (i.e. without low-affinity Pax5) was not sufficient in generating TCDD-mediated inhibition of the human hs1,2 enhancer (Chalepakis, 1994; Epstein et.al., 1994). Furthering the current study by adding the low affinity Pax5 site of the murine hs1,2 enhancer to the α_{1A} + Pax5 would help rule out this possibility. Also, transient transfections were conducted at 24 hrs and LPS decreases Pax5 activity beginning at 48 hrs (Yoo et al., 2004). Even though TCDD-induced activation remained unaffected between α_{1A} and α_{1A} + Pax5 in this study, the role of Pax5 in how TCDD differentially modulates the mouse and human hs1,2 enhancer cannot be disregarded.

Mutation of the high-affinity Pax5 site in the mouse hs1,2 reporter plasmid was also attempted throughout the duration of this project, but efforts remained unsuccessful. Several different approaches were taken to improve problems with low transformation efficiency and sequencing failure, such as increasing amounts of parental template, dNTP, mutant primers and/or adjusting PCR conditions. The size of the reporter plasmid (~9 kb) in conjunction with repetitive DNA regions may be generating problematic secondary structures that hinder mutant plasmid extension in the thermal cycling step. Sequencing of the mouse hs1,2 reporter plasmid has ensured its quality so future attempts would be worthwhile.

Activation of the human polymorphic hs1,2 enhancer by TCDD is AhR-dependent

The mechanism of TCDD-induced toxicity has been most well-defined through the aryl hydrocarbon receptor signaling pathway. Historically, this pathway has been mapped out with the TCDD/AhR complex binding DRE sites in the promoter or enhancer

regions of genes for drug-metabolizing enzymes. By using the AhR antagonist, CH-223191, it was confirmed that TCDD-induced activation of the human polymorphic hs1,2 enhancer requires the AhR. It still remains unclear whether the AhR is directly or indirectly involved in the pathway, since the receptor complex can directly bind DRE sites or alter DNA binding or expression of other transcription factors such as AP-1, NF- κ B and SP1 (Kobayashi, 1996; Tian et al., 1999; Suh, 2002).

Experimental outcomes show the magnitude of TCDD-induced fold change activation did not change between α_{1A} , α_{1B} and α_{1C} . However, the amount of overall transcriptional activity is dependent upon the number of IS present. The reporter plasmids all experienced synergistic activation from cotreatment of LPS and 10 nM TCDD as well. Either one invariant sequence is enough to drive activation of the enhancer by TCDD and/or one or more binding sites outside of the polymorphic is involved.

As previously noted, α_{1C} does not have a conserved DRE or AP-1 site in the third IS so a mutant plasmid (α_{1C} Clinical) has been generated restoring both sites back to their respective, consensus sequences. Transient transfection and analysis of this plasmid will further establish whether or not there is any correlation between number of IS and degree of TCDD-induced fold change. Additionally, the third IS has been deleted from the α_{1C} (IS3del α_{1C}). Evaluation of this mutant plasmid may show a decrease in basal and LPS levels as seen by the α_{1A} ISdel and α_{1B} IS1del and further credit the IS for possessing a significant amount of transcriptional activity. As such, individual mutation of transcription factor binding motifs of the human hs1,2 enhancer will better pinpoint

which sites are involved in TCDD-induced modulation of the enhancer and its overall activity.

Involvement of the transcription factors of the invariant sequence in human hs1,2 activity

The invariant sequence contains a DRE, AP-1, NF1 and NF- κ B site flanked by SP1 sites on both ends of the polymorphism (Chen and Birshtein, 1997; Denizot et al., 2001; Giambra et al., 2005; Fernando et al., 2012). Activator protein 1 (AP-1) is a heterodimer protein composed of c-Fos and c-Jun while nuclear factor kappa B (NF- κ B) is also a heterodimer protein composed of different members of Rel family transcription factors. Both have been associated with altered binding or regulation by the AhR or TCDD (Tian et al., 1999; Suh et al., 2002). In LPS-activated CH12.LX cells TCDD suppressed AP-1 binding to AP-1 recognition sites within the B lymphocyte-induced maturation protein-1 (Blimp-1) promoter, which was in concordance with suppression of *Blimp-1* by TCDD (Schneider et al., 2009). Blimp-1 is a critical regulator of B cell differentiation and also acts as a transcriptional repressor to Pax5. Following this suppression by AP-1, *Blimp-1* mRNA and DNA-binding activity within the Pax5 promoter dysregulated Pax5 (Schneider et al., 2009). In a different study, TCDD-treated guinea pigs showed changes in protein phosphorylation that were accompanied by increased AP-1 DNA-binding activity in liver tissue (Ashida et al., 2000). Outcomes from these separate studies indicate that TCDD may downregulate or upregulate AP-1 activities.

NF- κ B has been identified as a key regulator of the murine hs1,2 enhancer and physical interactions between the AhR and NF- κ B have been demonstrated (Tian et al., 2002; Michaelson et al., 1996). Mutation of the NF- κ B site from a murine hs1,2 enhancer reporter construct increased or decreased enhancer activity in a cell-stage dependent manner thus suggesting an important role for κ B binding proteins in modulating the hs1,2 enhancer (Michaelson et al., 1996). Also, EMSA-Western Analysis showed TCDD-induced binding of NF- κ B/Rel proteins to a κ B site with an overlapping DRE in the hs4 enhancer (Sulentic et al., 2000). The potential for a check-and-balance relationship between the NF- κ B and AhR pathway has further been demonstrated, which may occur through negative regulation when one receptor binds within the other receptor's promoter (Tian et al., 2002)

In the current study mutation of the AP-1 site and NF- κ B site increased basal and LPS levels of the α_{1A} human hs1,2 enhancer (Fig. 11A and 12A). Like the α_{1A} , a TCDD-concentration response was also observed with both the α_{1A} AP1mut and α_{1A} NF κ Bmut (Fig. 11A and 12A). An increase in overall transcriptional activity caused by mutation of the AP-1 or NF- κ B could mean that these sites naturally have repressor roles. Alternatively, other sites present in the enhancer (i.e. AP1.ETS, Oct, SP1, DRE, NF1) may compensate for any loss in transcription from the AP-1 or NF- κ B site and overly adjust activity as a result. Since the previously mentioned studies imply that AP-1 and NF- κ B can be affected by the AhR and/or TCDD, it is reasonable to think that either one of these binding motifs are responsible for TCDD's ability to activate the human hs1,2 enhancer. Outcomes from this study, however, suggest otherwise. TCDD-induced fold-changes of α_{1A} AP1mut and α_{1A} NF κ Bmut had slightly lower stimulated fold-changes

compared to the α_{1A} . This may suggest that the AP-1 and NF- κ B binding-motifs each are one of several binding-motifs that coordinate TCDD-induced hs1,2 enhancer activation in stimulated CH12.LX cells in addition to repressing basal and stimulated enhancer activity.

Because slightly lower TCDD-induced fold changes resulted from mutation of the AP-1 and NF- κ B sites, creating a plasmid from the α_{1A} that has both of these sites mutated would be beneficial. A double mutant of the α_{1A} containing a mutated DRE and NF- κ B has already been created, but still needs to be tested. One study indicated the importance of multiple binding sites when it demonstrated that in addition to a DRE, an NF- κ B site was involved in TCDD-induced regulation of Fas and FasL promoters (Singh, 2007). As such, plasmids containing mutations of two or more transcription factor binding sites would help tease apart the complexity of how TCDD induces the human hs1,2 enhancer.

At this time concluding remarks cannot be made about the role the DRE may play in modulating TCDD-induced activation of the human hs1,2 enhancer. Nevertheless, a plasmid containing a mutated DRE site from the α_{1A} (α_{1A} DREmut) has been created and initial efforts are suggesting an increase in overall transcriptional activity with no difference in fold change. Additional DREs do not seem to increase the magnitude of TCDD-induced fold change, but the AhR certainly mediates TCDD-induced activity as seen from antagonist experiments (Fig. 8B). It may be that one DRE site is enough to drive TCDD's activation of the hs1,2 enhancer or it simply has minimal involvement. Also, the IS DRE site may affect basal and LPS-induced transcriptional activity since overall hs1,2 enhancer activity increases with the number of IS (Fig. 8A). Results from

transient transfections of the α_{1A} DREmut will determine if the drop in transcription that resulted from deletion of the IS is related to the DRE site.

The NF1, also located within the α_{1A} IS, has not yet been mutated. Analysis of this binding motif is critical, because a complete and comprehensive study will have been performed on every IS binding site. Deletion of an IS from the α_{1A} and α_{1B} showed a decrease in overall transcriptional activity whereas mutation of the AP-1 and NF- κ B sites showed an increase. Analysis of the DRE and NF1 site may better explain this contradictory outcome. Upon individual evaluation of the NF- κ B, DRE, AP-1 and NF1 it will also be known which sites impact overall transcriptional activity the most and how. Additional mutation studies will determine if the DRE and NF1 mediate TCDD-induced fold change of the human hs1,2 enhancer is substantially altered. Following a thorough assessment of each IS binding-motif, greater care can be taken as to which combination of multiple binding sites should be mutated from the parental α_{1A} . At this time it seems that TCDD-induced modulation of the hs1,2 enhancer is not driven solely through one binding site, but rather two or more.

Transcriptional role of binding-motifs located outside the invariant sequence

Located 5' and furthest from the IS is an AP1.ETS binding site. An earlier study identified binding of a novel DNA binding complex, nuclear factor of activated B cells (NFAB), to an AP1.ETS site of the mouse 3'*IgH* RR that supported enhancer activation following cross-linking of surface IgM (Grant et al., 1995). Also, in a study involving a melanoma cell line, matrix metalloproteinase promoter activity was increased when an AP1.ETS site was formed from an AP-1 site adjacent to a polymorphic ETS element

(Villano et al., 2006). Despite increased promoter activity, however, the ETS polymorphism did not alter TCDD-induced activity compared to the wildtype (Villano et al., 2006). Outcomes from these studies certainly highlight potential for the human hs1,2 AP1.ETS site to possess an active role in the enhancer's activity. Out of all of the individual binding sites mutated and analyzed thus far, mutation of the AP1.ETS site (α_{1A} AP1.ETSmut) was the only one that showed a decrease in transcriptional activity of the hs1,2 enhancer when transiently transfected into CH12.LX cells (Fig. 13A). A drop in luciferase activity in both unstimulated and LPS-stimulated cells underscores the importance this site has in mediating human hs1,2 enhancer activity. Evaluation of the α_{1A} AP1.ETSmut still showed that TCDD-induced fold change did not differ from the α_{1A} , but α_{1A} did experience more synergism (Fig. 13A and 13B).

Also located 5' and outside the IS is an octamer (Oct) site, which has been less studied in regards to AhR or TCDD-induced modulation. Nevertheless, NF- κ B in combination with Oct transcription factors seem to contribute to the activity of the mouse hs1,2 enhancer in plasma cells while Pax5 is concurrently downregulated (Michaelson, 1996). Also, Oct binding sites are frequently found in AhR-sensitive genes suggesting a possible role in the AhR signaling network (Kel et al., 2004). Mutation of the Oct site (α_{1A} Octmut) resulted in an increase in overall transcriptional activity of the human hs1,2 enhancer when transiently transfected into CH12.LX cells suggesting an innate repressor role of the enhancer's activity (Fig. 14A). Interestingly, the α_{1A} Octmut showed a significantly higher degree of TCDD-induced activation when compared to the α_{1A} , but this was only seen in unstimulated cells. Under this circumstance, Oct may naturally hinder hs1,2 activation by TCDD when LPS is not involved. When transiently

transfected cells are LPS-stimulated, however, there appears to be no differences in TCDD-induced fold changes between the α_{1A} Octmut and α_{1A} (Fig. 14B). LPS signaling must somehow disturb the mechanism that was involved in the increased fold-change of α_{1A} Octmut activation by TCDD experienced in unstimulated cells.

Interestingly, mutating the Oct site in conjunction with deletion of the IS from the α_{1A} (α_{1A} OctmutISdel) experienced more overall transcriptional activity than α_{1A} Octmut (Fig. 14A). The α_{1A} OctmutISdel did not always experience consistent TCDD-induced activation and stimulated fold changes were on average lower than α_{1A} (Fig. 14A, 14B and 15). One of the binding sites deleted or mutated must certainly be responsible for TCDD-induced activation of the hs1,2 enhancer, since the only remaining binding site is the AP1.ETS. Otherwise some sort of inconsistent compensating mechanism may be taking place. The IS deletion could have allowed for the flanking SP1 sites of the IS to come together to create one functional SP1 site that together with the remaining AP1.ETS site may have generated a highly transcriptionally active reporter plasmid.

In regards to SP1, one study indicated a synergistic effect between AhR/ARNT and SP1, which increased the drug-inducible expression of CYP1A1 in a *Drosophila* cell line (Kobayashi, 1996). Another study credited an SP1-based transcription mechanism for mediating TCDD-induced protein and mRNA expression of mucin-5AC in human bronchial epithelial cells and an immortalized cell line that was independent of the AhR (Lee et al., 2010). It is possible that either of the two SP1 transcription factor binding sites play an important role in modulating the TCDD-induced activation of the human hs1,2 enhancer. As such, both the 5' (α_{1A} SP1.1mut) and 3' (α_{1A} SP1.2mut) SP1 sites have been mutated from the α_{1A} . Upon evaluation, these mutagenic plasmids will provide a

more in-depth analysis on how these sites affect human hs1,2 transcriptional activity or TCDD-induced modulation.

Conclusion

Ultimately, it is difficult to pinpoint the transcription factor binding sites responsible for TCDD-induced modulation of the human hs1,2 enhancer. Differences in the DNA sequence between the mouse and human hs1,2 enhancer and how they are oppositely modulated by TCDD certainly emphasizes the potential difficulty in translating mouse data to human-assessment. While addition of the mouse Pax5 site to the α_{1A} decreased the enhancer's transcriptional activity, TCDD was still able to activate. The presence of two Pax5 binding sites in the mouse hs1,2 enhancer versus none in the human may still explain why TCDD inhibits mouse activity yet activates human. The high-affinity Pax5 was added to the α_{1A} in a way that best mirrored the mouse hs1,2 enhancer, but changes in the double helix or secondary structures of the mutant plasmid may have somehow masked or overridden TCDD-induced inhibition. Otherwise, the involvement Pax5 has in mouse hs1,2 enhancer activity and TCDD responsiveness could simply be unrelated or unnecessary in the human hs1,2 enhancer.

Based on the other binding sites that have been evaluated in this study (AP-1, NF- κ B, Oct, AP1.ETS), none seem to be the sole driving force behind TCDD's ability to activate the human hs1,2 enhancer. Mutation of the AP-1 and NF- κ B site showed slight decreases in TCDD-induced fold-change activation, however, outcomes were not statistically significant. It seems very likely that several transcription factors are simultaneously involved in mediating the effect of TCDD. Even though deletion of the IS shows no change in TCDD-induced activation of the human hs1,2 enhancer, EMSA

analyses indicate that TCDD induces binding within the IS in LPS-stimulated CH12.LX cells (data not shown). Future efforts will focus on isolating and identifying proteins bound to the IS by mass spectrometry.

Individual site mutations of AP-1, NF- κ B and Oct increased human hs1,2 transcriptional activity suggesting repressor roles while mutation of AP1.ETS lowered overall enhancer activity (Fig. 16). The AP1.ETS site seems to control much human hs1,2 enhancer activity, since it is the only site thus far that has shown a drop in transcription when mutated. Deletion of the IS lowered transcriptional activity as well, but again it is difficult to discern which IS binding site(s) is/are of cause (Fig. 16). Furthermore, deleting rather than mutating the IS could be creating an inauthentic outcome due to changes in the secondary structure. It would be worthwhile to generate one plasmid where all the IS binding sites have undergone mutation in order to reinforce results. So far it seems the AP-1 and NF- κ B sites may be involved in TCDD-induced fold-change activation of the enhancer underscoring the likelihood of a complex network of interacting transcription factors. Like AP-1 and NF- κ B, the Oct site also showed a drop in fold change, but only in LPS-stimulated cells thus exemplifying signaling differences between unstimulated and stimulated hs1,2 enhancer modulation.

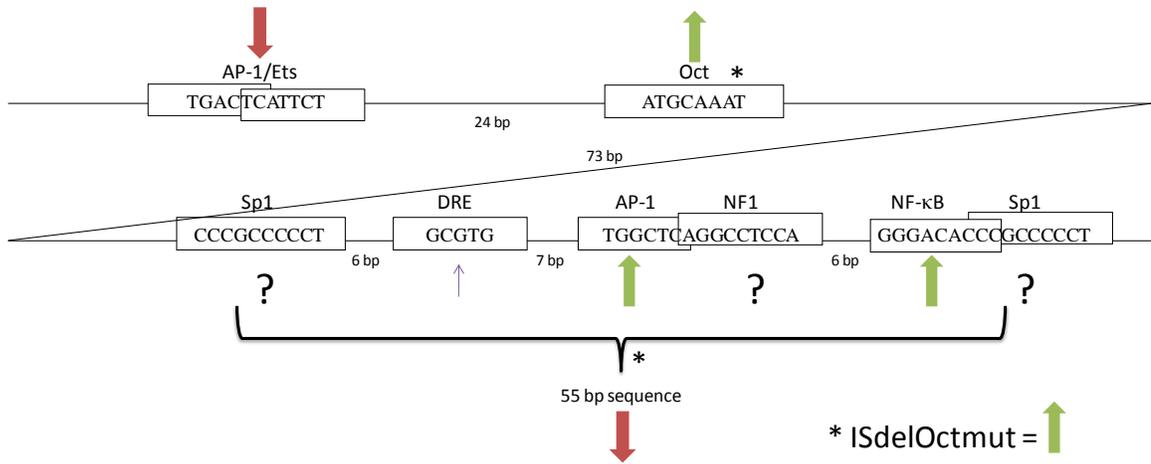


Figure 16. Overall transcriptional activity outcomes of deletion/mutagenic plasmids compared to the human α_{1A} . Up or down arrow means greater or less overall transcriptional activity than α_{1A} . Question mark: results to be determined.

A number of limitations persist in this study in addition to those already reported. First, fold change calculations do not provide a tell-all tale to TCDD-induced regulation. Overall increases or decreases in TCDD-induced transcription translate into real effects, which is a different perspective than simply magnitude of fold change. Second, there may be transcription factors or binding sites present in the hs1,2 enhancer that have not been identified. Enhancer activity and TCDD-induced regulation could possibly be driven through other unrecognized means. Finally, it is uncertain how accurately this research reflects the endogenous human *IgH*. Not only are outcomes not in the context of chromatin, but transcriptional activity is based on reporter plasmids containing a human gene transfected into murine B cells. While the CH12.LX cells have been used extensively and contain a functional AhR pathway, current efforts are being made to characterize and develop a human B cell line that can be ligand-activated. In spite of these limitations, this study lays the groundwork for uncovering hs1,2 regulation.

Overall, the hs1,2 enhancer has an important role in 3'*IgH* RR functioning, so understanding how TCDD or other AhR-ligands modulate it will provide greater insight into how chemically-induced immunotoxic effects are mediated. Also, its involvement in the severity and prevalence of a number of autoimmune diseases makes it an area of interest. Teasing apart the mechanism at hand could ultimately lead to the manipulation of the many disease states associated with the 3'*IgH* RR and human hs1,2 enhancer.

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