JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, an Exogenous Modulator of the 3'α Immunoglobulin Heavy Chain Enhancer in the CH12.LX Mouse Cell Line*

Courtney E. W. Sulentic, Wei Zhang, Yong Joo Na and Norbert E. Kaminski*

Department of Pharmacology & Toxicology and National Food Safety & Toxicology Center,

Michigan State University, East Lansing, MI, 48824 USA CEWS, WZ, NEK

Korea Advanced Institute of Science and Technology, Daejeon, KOREA YJN

Running Title: TCDD inhibits 3'α enhancer activity

[*] *Corresponding author:* Dr. Norbert E. Kaminski, Dept. of Pharmacology & Toxicology, 315 National Food Safety & Toxicology Center, Michigan State University, East Lansing, Michigan 48824, phone: 517-353-3786, fax: 517-432-3218, email: kamins11@msu.edu

Number of text pages: 14

Number of tables: 0

Number of figures: 5

Number of references: 40

Number of words in abstract: 211

Number of words in introduction: 894

Number of words in discussion: 1,640

[3] *Abbreviations:* IgH, immunoglobulin heavy chain; Eμ, intronic enhancer; BSAP, B-cell specific activator protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin-responsive element; DMSO, dimethyl sulfoxide; ChIP, chromatin immunoprecipitation.

Abstract

Transcriptional regulation of the Ig heavy chain gene involves several regulatory elements including the 3'α enhancer, which is composed of four distinct regulatory domains. DNA binding sites for several transcription factors, including B cell-specific activator protein, NF-κB and Oct have been identified within the 3'α enhancer domains and are believed to be important in regulating 3'α enhancer activity. We have identified an additional DNA binding motif, the dioxin responsive element (DRE) which can contribute to 3'α enhancer regulation. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a known disruptor of B cell differentiation (i.e., decreased plasma cell formation, inhibition of μ heavy chain expression and suppression of IgM secretion), induces binding of the aryl hydrocarbon receptor (AhR) nuclear complex to DREs. TCDD also induces AhR binding to the hs4 domain of the 3'α enhancer. Interestingly, TCDD enhances LPS-induced activation of the hs4 domain but profoundly inhibits LPS-induced activation of the complete 3'α enhancer. Furthermore, site-directed mutational analysis demonstrated that a DRE and κB element in the hs4 domain is modulated by TCDD in LPS-activated B cells. We propose that the AhR is a novel transcriptional regulator of the 3'α enhancer, which can mediate, at least in part, the effects of TCDD on the 3'α enhancer and its domains, putatively contributing to a marked suppression of IgM production.

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

Regulation of the murine immunoglobulin heavy chain (IgH)[3] locus is governed through a complex interaction of several regulatory elements whose activity is B cell specific and dependent on the state of B cell maturation. The most 5' regulatory element is the V_H promoter, which lies immediately upstream of each variable region and contributes to B cell specific activity of the Ig heavy chain locus. Located between the rearranged VDJ segments and the Cμ constant region is the intronic enhancer (Eμ) which contributes to B cell specific activity and is involved early in B cell development where it regulates V to D-J joining and μ heavy chain gene expression (Calame and Eaton, 1988; Chen et al., 1993; Serwe and Sablitzky, 1993). However, studies with mice and B cell lines that lack Eμ demonstrate normal Ig heavy chain expression implicating the involvement of yet another regulatory element(s) important to processes late in B cell differentiation such as up-regulation of IgH expression and secretion as well as class switching (Klein et al., 1984; Wabl and Burrows, 1984). A 40 kb region, downstream of the α heavy chain gene, demonstrates enhancer activity that is largely restricted to plasma cells and appears to regulate the above processes (Singh and Birshtein, 1993; Madisen and Groudine, 1994; Saleque et al., 1997). Within this region, four separate enhancer domains, hs3A, hs1,2, hs3B and hs4, corresponding to DNase1 hypersensitive (hs) sites, were identified and are collectively termed the 3'α enhancer. Activity of these enhancer domains is dependent on the developmental stage of the B cell with hs3A, hs1,2 and hs3B primarily active in activated B cells or plasma cells (Chauveau et al., 1998; Ong et al., 1998; Stevens et al., 2000). In contrast, hs4 is active from a pre-B cell to the plasma cell stage (Chauveau et al., 1998). Several laboratories have reported a synergistic effect when the various 3'α enhancer domains are linked in tandem as demonstrated by transient transfection assays with reporter constructs; however, the greatest activity occurs when all four enhancer domains are linked (Chauveau et al., 1998; Ong

et al., 1998; Stevens et al., 2000). In addition, DNA binding sites for several transcription factors, including B cell-specific activator protein (BSAP), NF-κB and Oct, have been identified within the 3'α enhancer and appear to be important regulators of enhancer activity (Michaelson et al., 1996; Saleque et al., 1999; Kanda et al., 2000; Stevens et al., 2000). We have recently identified an additional DNA binding motif, the dioxin responsive element (DRE) which may also contribute to the regulation of the 3'α enhancer (Sulentic et al., 2000).

Considerable effort has led to the characterization of the transcription factors and the signaling pathway that leads to DRE binding (for review, see Swanson, 2002). DRE signaling is similar to the steroid signaling pathway in that a cytosolic receptor, the aryl hydrocarbon receptor (AhR), translocates to the nucleus following ligand binding where it disassociates from two heat shock proteins 90 (hsp90) and forms a heterodimer with the AhR nuclear translocator (ARNT). The AhR nuclear complex is then capable of binding to DREs in regulatory regions of dioxin sensitive genes. Although the endogenous ligand for the AhR has yet to be identified, halogenated aromatic hydrocarbons, of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the prototype, bind with varying affinity to the AhR. TCDD is a potent and persistent environmental toxicant, which produces a variety of biological effects in animal and cellular models (Birnbaum and Tuomisto, 2000). Of these effects, immune suppression is one of the most sensitive consequences of TCDD exposure (Holsapple et al., 1991). Inhibition of IgM secretion and of the antibody forming cell response has been well documented with several studies supporting the involvement of the AhR; however, the specific mechanism remains unclear (Tucker et al., 1986; Dooley and Holsapple, 1988; Luster et al., 1988; Morris and Holsapple, 1991; Morris et al., 1993; Williams et al., 1996; Sulentic et al., 1998; Sulentic et al., 2000; Vorderstrasse et al., 2001).

Characterization of the effects of TCDD on the B cell line, CH12.LX, has supported an AhR-dependent mechanism for the inhibition of B cell function by TCDD (Sulentic et al., 1998;

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

Sulentic et al., 2000) and is also consistent with measurements of humoral immunity in AhR-null mice (Vorderstrasse et al., 2001). In addition to IgM secretion, TCDD strongly inhibits μ heavy chain expression in the CH12.LX cells which also appears to be AhR-dependent. Interestingly, sequence analysis of the 3'α enhancer identified DRE-like sites within the hs1,2 and hs4 domains which are capable of binding AhR/ARNT dimers as demonstrated by an EMSA-Western analysis (Sulentic et al., 2000). These findings implicate a novel regulator of the 3'α enhancer. The purpose of the present studies was to characterize the effect of TCDD on 3'α enhancer activity in LPS-activated CH12.LX cells by utilizing transient transfections with luciferase reporter constructs containing a V_H promoter 5' of the luciferase gene and either the 3'α enhancer, hs1,2 or hs4 inserted 3' of the luciferase gene. Results from these studies demonstrate a sensitive and profound inhibition by TCDD of LPS-induced 3'α enhancer activity. Interestingly, TCDD produced a concentration-dependent enhancement of LPS-induced hs4 activity. In addition, a chromatin immunoprecipitation (ChIP) assay confirmed TCDD-inducible AhR binding to the hs4 enhancer domain and site-directed mutation of the hs4 DRE and/or κB decreased the effect of TCDD and LPS on hs4 activity. Collectively, these data demonstrate TCDD-mediated modulation of the 3'α enhancer, which may be produced, at least in part, through transcriptional regulation by the AhR.

Materials and Methods

Chemicals and reagents

TCDD, in 100% DMSO, was purchased from AccuStandard Inc. (New Haven, CT). The certificate of product analysis stated the purity of TCDD to be 99.1%, as determined by AccuStandard using gas chromatography/mass spectrometry. DMSO and LPS (Salmonella typhosa) were purchased from Sigma - Aldrich (St. Louis, MO).

Cell line

The CH12.LX B cell line derived from the murine CH12 B-cell lymphoma (Arnold et al., 1983), which arose in B10.H-2^{a}H-4^{b}p/Wts mice (B10.A x B10.129), has been previously characterized by Bishop *et al*. (Bishop and Haughton, 1986) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina). CH12.LX cells were grown in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 50 µM β-mercaptoethanol. Cells were maintained at 37°C in an atmosphere of 5% CO_2.

Transient transfection

Luciferase reporter plasmids were generously provided by Dr. Robert Roeder (Rockefeller University) and included the 5 kb V_H luc (V_H), 9 kb V_H luc1,2 (hs1,2), 6.4 kb V_H luc 4 (hs4) and 12.4 kb V_H luc 3A 1,2 3B 4 (3'α) (Fig. 1A). Plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI) as previously described (Ong et al., 1998; Stevens et al., 2000). Mutation of the DRE located within the hs4 domain was performed using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). Briefly, alkaline denatured hs4

was hybridized with phosphorylated mutagenic (sequences below) and selection (specific for the ampicillin gene) oligonucleotides. Double-stranded circular mutant candidates were then generated with T4 DNA polymerase and T4 DNA ligase and screened by sequential transformation into BMH 71-18 mutS then JM109 competent cells and grown under dual antibiotic selection pressure (i.e., ampicillin and GeneEditor Antibiotic Selection Mix (Promega)). Finally, plasmids carrying the desired mutations in both strands were obtained and validated by sequencing (data not shown). The sequences of the mutagenic oligonucleotides (mutations in bold and italicized) used to generate the desired hs4 mutations are listed below.

DRE mutation:

5'-AGAGGGGGGGACTGG-***ATC***-GGAAAGCCCCATTCACCC-3'

κB mutation:

5'-CTGGCGTGGAAAG-***TTAT***-ATTCACCCATGGGAC-3'

DRE and κB mutation:

5'-AGGGGGGGACTGG-***ATC***-GGAAAG-***TTAT***-ATTCACCCATGGGAC-3'

Transient transfections were performed as follows. CH12.LX cells (1 X 10^7) were resuspended in 200 μl culture media with 10 μg of plasmid and transferred to a 2 mm gap electroporation cuvette. Cells were electroporated using an electro cell manipulator (ECM® 600, BTX, San Diego, CA) with the voltage at 150 volts, the capacitance at 1500 μF and the resistance at 72 ohms. For each plasmid, multiple transfections were pooled then cells were aliquoted into a 12-well plate at 5 X 10^5 cells per well.

Luciferase assay

Immediately following transfection, CH12.LX cells were treated with LPS and/or TCDD for 48 h. Treatments were either in triplicate or quadruplicate. Following the 48 h incubation period, cells

were washed with 1x PBS then lysed with 1x reporter lysis buffer (Promega, Madison, WI). Samples were immediately frozen at -80°C. To measure luciferase enzyme activity, samples were thawed at room temperature then 20 µl of sample lysate was mixed with 100 µl of luciferase assay reagent (Promega) using an auto injector. Luciferase activity or luminescence was measured by a Turner TD-20e luminometer and represented as relative light units. For determination of transfection efficiency, DNA was isolated from untreated samples following the 1x PBS wash. DNA isolation was performed using the GenElute Mammalian Genomic DNA miniprep kit (Sigma). Purified DNA was diluted 10-fold then analyzed by real-time PCR for the luciferase gene. Primers were generated using Primer Express (Applied Biosystems, Warrington, UK) and were specific for the luciferase gene encoded within the pGL3 luciferase vector series. Briefly, 2 µl of diluted DNA was mixed with 3 pmol of both forward and reverse primers and 1x SYBR Green master mix (Applied Biosystems) to a final concentration of 25 µl. SYBR green incorporation was measured for 40 cycles. The concentration (ng/µl) of plasmid DNA was calculated from a standard curve using known quantities of V_H plasmid DNA. The plasmid number per cell was calculated using the following equation: [(ng plasmid) x (molecules/ng of plasmid) / cell number] x 10. The factor of 10 adjusts for the 10-fold dilution of DNA used in the PCR reaction. To account for experimental variation, the transfection efficiency of each plasmid was calculated as fold difference relative to hs4. Transfection efficiencies for each plasmid were averaged and are represented in Figure 1B as the mean-fold difference relative to hs4. The transfection efficiency for each plasmid was not affected by LPS and/or TCDD treatment (data not shown).

Chromatin immunoprecipitation (ChIP) assay

Chromatin bound to the AhR was isolated through the employment of a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, CH12.LX cells

(1×10^6) were treated with vehicle (0.019% DMSO) or 30 nM TCDD for 1 h. Proteins were then cross-linked to chromatin by direct addition of formaldehyde to cell cultures at a final concentration of 1%. Following cell lysis, the DNA was sheared by sonication to lengths between 200 and 1000 bp. The AhR/histone complexes were immunoprecipitated overnight at 4°C with a polyclonal anti-AhR antibody (Santa Cruz, Santa Cruz, CA). Antibody/histone complexes were collected and washed leading to the isolation of AhR-bound DNA and the reversal of protein-DNA cross-links as outlined in the ChIP assay protocol (Upstate Biotechnology). The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Utilizing qualitative RT-PCR as described previously (Williams et al., 1996), DNA was analyzed for a 150 bp hs4 product containing the DRE motif. Total cellular DNA served as a positive control and a sample incubated without an antibody during the immunoprecipitation step served as the negative control.

Statistical analysis of data

The mean ± S.E. was determined for each treatment group of a given experiment. To determine significance between treatment groups and vehicle controls, the data were analyzed by a 1-way ANOVA followed by a Dunnett's two-tailed *t*-test. Comparisons between the hs4 plasmids containing a mutated or non-mutated DRE and/or κB were analyzed using a 2-way ANOVA followed by a Bonferroni's two-tailed *t*-test.

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

Results

LPS enhances the activity of the 3'α enhancer in CH12.LX cells. Few studies have directly addressed the effect of a B cell differentiation signal, such as LPS, on the activity of the 3'α enhancer. Presumably, LPS would activate the 3'α enhancer due to its proposed role in the regulation of Ig production and class switch. To test this hypothesis, we utilized several previously characterized reporter plasmids containing a V_H promoter 5' of a luciferase reporter gene and the 3'α enhancer (hs3A, hs1,2, hs3B and hs4 enhancer domains) or individual domains of the 3'α enhancer inserted 3' of the reporter gene (Ong et al., 1998; Stevens et al., 2000) (Fig. 1A). In the CH12.LX B cells LPS activated the 3'α enhancer (Fig. 2A). Activation was concentration-dependent and was induced by LPS concentrations as low as 0.03 μg/ml with maximal activity occurring at 0.3 μg/ml (Fig. 2A).

Previous analysis of each 3'α enhancer domain in mature B cell lines has demonstrated basal activity of hs4 and perhaps hs1,2 but not of hs3A and hs3B (Chauveau et al., 1998; Ong et al., 1998). In addition, a significant enhancement of hs1,2 activity has been demonstrated following LPS stimulation or crosslinking of the IgM receptor (Arulampalam et al., 1994; Grant et al., 1995). Therefore, we also characterized the effect of LPS stimulation on hs4 and hs1,2 activity in the CH12.LX cells. LPS produced a modest or sometimes no effect on basal activity of the hs4 domain (Fig. 2B, 3B and 4). Analysis of the hs1,2 enhancer domain consistently demonstrated low basal activity (averaging approximately 0.025 relative light units) that appeared to be induced by LPS (averaging approximately 0.1 relative light units). However, the low basal and LPS-induced activity of the hs1,2 domain suggests that, alone, hs1,2 has little biological activity in the CH12.LX cells. In addition, the V_H promoter showed little activity alone and was not activated by LPS even at concentrations as high as 30.0 μg/ml (Fig. 2C).

The transfection efficiency of each plasmid was directly determined as plasmid number per cell by quantitative real-time PCR and is represented as a fold difference relative to the hs4-containing plasmid (see Materials and Methods). We utilized a direct measure of transfection efficiency rather than co-transfecting a control plasmid such as β-gal because the basic assumption of identical transfection efficiencies between the 3'α enhancer plasmids and a control plasmid would be erroneous as illustrated in Figure 1B. In our hands, differences in transfection efficiencies due to plasmid size (i.e., the larger the plasmid size, the lower the transfection efficiency) could not be adjusted for by transfecting equal molar quantities of each plasmid. A second problem with co-transfecting a control plasmid such as β-gal concerns the fact that our studies required cellular activation. In addition to activating the 3'α enhancer and various hs4 reporter constructs, cellular activation also markedly increased the activity of the control plasmids whose promoters and enhancers are typically activated by LPS, making it impossible to standardize transfection results based on the activity of the control plasmid (unpublished observation). It is notable that treatment of the transfected cells by TCDD and/or LPS did not significantly alter the transfection efficiency of each plasmid (data not shown). Lastly, there was no need in this series of studies for normalizing plasmid activity based on transfection efficiency between the different plasmids due to the fact that comparisons were made between treatments for any given plasmid and not between plasmids.

TCDD modulates the activity of the 3'α enhancer and the hs4 domain in LPS-activated CH12.LX cells. A marked inhibition by TCDD of LPS-induced IgM secretion and μ heavy chain expression has been previously demonstrated in the CH12.LX cells (Sulentic et al., 1998; Sulentic et al., 2000). We have previously speculated that TCDD may mediate its effects on Ig expression, at least in part, through inhibition of LPS-induced 3'α enhancer activity. To test this premise, the effect of TCDD on LPS-induced activity of the 3'α enhancer and the hs4 domain was characterized. Interestingly,

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

co-treatment with 30 nM TCDD and various concentrations of LPS produced marked but contrasting effects on the 3'α enhancer and the hs4 domain. Consistent with the effect of TCDD on Ig expression, TCDD co-treatment, independent of LPS concentration, dramatically inhibited LPS-induced 3'α activity (Fig. 2A). In contrast, hs4 activity was significantly increased by TCDD in LPS-activated CH12.LX cells (Fig. 2B). Although LPS alone had no affect on basal hs4 activity, the magnitude of induction following TCDD and LPS co-treatment was clearly dependent on the LPS concentration (Fig. 2B). In addition, TCDD appeared to inhibit LPS-induced hs1,2 activity; though, as stated above, the luciferase activity was consistently low and at the level of detection (data not shown). TCDD, like LPS, did not alter V_H promoter activity in LPS-activated cells (Fig. 2C).

The 3'α enhancer and the hs4 domain are sensitive targets of TCDD. To further characterize the effects of TCDD on the 3'α enhancer and the hs4 domain, a concentration response analysis in the absence or presence of LPS stimulation was performed. TCDD had little effect on the low basal activity of the 3'α enhancer; however, LPS-induced 3'α enhancer activity was particularly sensitive to the effects of TCDD, with a concentration of 0.3 nM completely abrogating activity (Fig. 3A). Unlike the 3'α enhancer, basal hs4 activity was modulated by TCDD and was significantly enhanced at 3.0 and 30.0 nM TCDD treatment (Fig. 3B). LPS-stimulation resulted in a synergistic effect with a marked enhancement of TCDD-induced hs4 activity at 0.3, 3.0 and 30.0 nM TCDD (Fig. 3B). The maximal induction of hs4 activity produced by the synergistic interaction of TCDD and LPS was concentration dependent (Fig. 2B and Fig. 3B). In addition, TCDD did not alter the low basal activity of the V_H promoter in the absence or presence of LPS (Fig. 3C).

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

The AhR binds within the hs4 domain and the hs4 DRE and κB motifs coordinately mediate the effect of TCDD on hs4 activity. Previous electrophoretic mobility shift assays (EMSA) have demonstrated binding of the AhR and ARNT protein to a 50 bp oligomer from the hs4 domain which contained a DRE-like site (Sulentic et al., 2000). However, EMSA analysis creates an artificial environment where the conditions (i.e., the concentration of linear DNA probe, nuclear proteins and salt) have been optimized to facilitate binding of transcription factors to DNA and therefore, may not accurately reflect the actual binding conditions within the intact cell. To determine if the AhR binds the hs4 domain in CH12.LX cells after a 1 h treatment with 30 nM TCDD, a chromatin immunoprecipitation (ChIP) assay was performed in which protein-DNA complexes were crosslinked in the intact cell followed by cell lysis and immunoprecipitation of AhR-DNA complexes with an anti-AhR antibody. PCR analysis of immunoprecipitated DNA confirmed AhR-binding to the hs4 domain (Fig. 4).

To evaluate the functional consequence of DRE binding within the hs4 domain, three core nucleotides of the DRE were mutated in the hs4 luciferase reporter construct by site-directed mutagenesis. Mutation of the DRE significantly decreased the effect of TCDD on LPS-induced hs4 activity (Fig. 5). Since the hs4 DRE overlaps a κB binding motif which has previously been shown to be essential to basal hs4 activity (Michaelson et al., 1996; Kanda et al., 2000), three core nucleotides of the κB were mutated in the hs4 luciferase reporter construct. In agreement with previous results, mutation of the κB results in a significant and dramatic decrease in basal hs4 activity and the co-treatment of TCDD and LPS had no effect (Fig. 5). Double mutation of both the DRE and κB also resulted in a significant decrease in basal activity as well as a significantly decreased effect of TCDD on LPS-induced hs4 activity (Fig. 5). It is interesting, however, that compared to the hs4 reporter with a mutated κB, TCDD and LPS modestly activated the hs4 reporter with a double mutation of the DRE and κB (Fig. 5). In addition, the transfection

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

efficiencies of the hs4 control and mutant reporter plasmids were not significantly different as determined directly by PCR (data not shown).

Discussion

Past studies of the 3'α enhancer have primarily focused on characterizing the basal activity of the individual enhancer domains (i.e., hs3A, hs1,2, hs3B or hs4) and/or combinations of these domains using various cell lines which represent different stages of B cell maturation. In contrast, the present studies have focused on a surface Ig-expressing B cell line, CH12.LX, and have characterized the effect of LPS-stimulation and of TCDD, a known disrupter of B cell function, on transcriptional activity of the 3'α enhancer and its domains. In general agreement with previous studies in murine and human Ig-expressing B cell lines (Chauveau et al., 1998; Ong et al., 1998) and in primary murine B cells (Stevens et al., 2000), the 3'α enhancer and the hs4 domain, but not the hs1,2 domain, were active in LPS-unstimulated CH12.LX cells. Low hs1,2 activity in the CH12.LX cells was previously identified and attributed to repression by a B cell specific transcription factor, BSAP, which is a negative regulator of B cell differentiation (Neurath et al., 1994; Usui et al., 1997). BSAP and other transcriptional regulators such as Oct, OCA-B and NF-κB have been shown to influence 3'α enhancer activity. Michaelson and coworkers have suggested a "concerted repression" of basal hs1,2 activity in B cells where mutation of the binding site for BSAP or Oct-1/Oct-2 or κB proteins results in activation of hs1,2; in contrast to "concerted activation" of basal hs4 where mutation of the binding site for BSAP, Oct-1 or NF-κB results in inhibition of hs4 activity in B cells (Michaelson et al., 1996). The hs4 domain is the only 3'α domain that may be active in pre-B cells which has lead to the notion that the hs4 domain may also be involved in B cell development (Giannini et al., 1993; Madisen and Groudine, 1994; Michaelson et al., 1995; Chauveau et al., 1998). Distinct from its contribution to 3'α enhancer activity, a role for the hs4 domain in B cell development would seem to necessitate the above variations in

regulation of the hs4 and hs1,2 domains due to likely differences in the expression and activity of regulatory proteins during B cell development and differentiation.

Presumably, activation of B cells will alter the binding profile of transcriptional regulators and therefore the activity of the 3'α enhancer. Indeed, OCA-B knockout studies have suggested a role of OCA-B in the enhancement of 3'α activity following IL-4 and anti-CD40 stimulation of primary B cells (Stevens et al., 2000). In addition, LPS stimulation of primary murine B cells activates hs1,2 which was enhanced further by TPA co-treatment (Arulampalam et al., 1994). Due to the proposed role of the 3'α enhancer in the regulation of Ig production and class switch, it is reasonable to speculate that stimulators of B cell differentiation will activate the 3'α enhancer. The converse may also be true in that disruptors of B cell differentiation may repress the 3'α enhancer. Since the effects of LPS, a stimulator of B cell differentiation, and TCDD, a disrupter of B cell differentiation, have been well characterized in the CH12.LX B cell line, the CH12.LX cells offer a unique model to study the 3'α enhancer and its role in B cell differentiation.

In the CH12.LX cells, LPS stimulation induces μ heavy chain expression and IgM secretion (Sulentic et al., 1998; Sulentic et al., 2000). However, TCDD co-treatment disrupts LPS-induced differentiation and abrogates induction of μ heavy chain expression and IgM secretion. TCDD appears to mediate these effects through the AhR signaling pathway (Sulentic et al., 1998; Sulentic et al., 2000). Here, we examined the effects of LPS and TCDD on the activity of the 3'α enhancer in the CH12.LX cells. Similar to the effects of LPS on μ heavy chain expression and IgM secretion, LPS activated the 3'α enhancer; and consistent with its disruption of B cell differentiation, TCDD repressed LPS-induced activity of the 3'α enhancer. In addition, LPS also appeared to induce the hs1,2 domain and TCDD inhibited this induction; however, the overall activity of this enhancer is very low in the CH12.LX cells as previously identified (Neurath et al., 1994). Unlike the effect of

TCDD on LPS-induced activation of the 3'α enhancer and of the hs1,2 domain, TCDD and LPS co-treatment resulted in a synergistic activation of the hs4 domain.

Transcription factors such as κB binding proteins and the AhR may mediate the effects of LPS and TCDD on the activity of the 3'α enhancer and its domains. EMSA-Western experiments have previously demonstrated TCDD-induced binding of the AhR nuclear complex to DRE sites located within the hs4 and hs1,2 domains (Sulentic et al., 2000). Here, binding of the AhR to the hs4 domain was confirmed through a chromatin immunoprecipiation assay with AhR binding occurring in a region of the hs4 domain that contained the DRE. Furthermore, site-directed mutation of this DRE significantly reduced the effect of TCDD on LPS-induced hs4 activity. These results further support an AhR/DRE mechanism in the effects of TCDD on hs4 activity. It is notable that mutation of the DRE did not completely abrogate the effects of TCDD and LPS. Moreover, a κB binding motif overlaps the DRE flanking region in the hs4 domain and TCDD induces κB binding which appears to be independent of the AhR (Sulentic et al., 2000) and perhaps may account for the residual effect of TCDD and LPS on hs4 activity following mutation of the DRE. Alternatively, mutation of the κB site markedly diminished basal activity of the hs4 domain as previously demonstrated (Michaelson et al., 1996; Kanda et al., 2000) and the co-treatment of TCDD and LPS had no affect on basal activity. Since the DRE mutation primarily affects the ARNT binding site and previous studies have demonstrated an interaction between the AhR and RelA (Tian et al., 1999; Kim et al., 2000), an additional possibility for residual activation of the mutant DRE is an interaction between the AhR and NF-κB proteins. However, the AhR and Rel proteins do not appear to dimerize as evidenced by EMSA-Western analysis in which the AhR/ARNT bound DNA migrated differently than NF-κB/Rel bound DNA (Sulentic et al., 2000). The implications of inducing transcription factor binding to overlapping DRE and κB elements are difficult to predict. Clearly, TCDD and LPS synergistically activate the hs4 domain. Perhaps

binding to either the DRE or the κB facilitates or stabilizes binding to the other motif. Studies utilizing a luciferase reporter construct with only 50 bp of the 1.4 kb hs4 domain which contained the overlapping DRE and κB have demonstrated that alone TCDD or LPS can modestly activate this reporter but, as seen in this manuscript, co-treatment of TCDD and LPS markedly and synergistically enhanced activity of the 50 bp hs4 enhancer. Furthermore, mutational analysis of the DRE and/or κB has determined that activity of the 50 bp enhancer is dependent on both the DRE and the κB supporting a positive interaction between proteins that bind to these sites, presumably the AhR and ARNT and NF-κB proteins (manuscript submitted for publication).

Like the single κB mutation, double mutation of the DRE and κB also resulted in a decrease in hs4 basal activity. However, contrary to the single κB mutation, TCDD and LPS co-treatment enhanced the activity of the double mutant, though to a lesser degree than with the single DRE mutation. This result implies that the TCDD and LPS co-treatment activates protein binding to other sites within the hs4 domain. As illustrated in Figure 1C, the DRE and κB are closely flanked by two octamer and two BSAP sites and one PU.1 site. Removal of protein binding to the DRE and κB sites may alter DNA binding or protein:protein interactions of transcription factors with affinity for these flanking DNA motifs. Additionally, TCDD has been shown to alter the regulation and expression of Pax5/BSAP in LPS-stimulated CH12.LX cells (Yoo et al., 2003). Specifically, TCDD maintains the expression of BSAP which is typically downregulated during B cell differentiation into plasma cells. Therefore, removing protein binding to the DRE and κB may open the 5' low affinity BSAP site to the TCDD-induced and sustained elevation of BSAP (Fig. 1C). Furthermore, BSAP is a positive regulator of hs4 activity in a mature B cell line but a negative regulator of hs1,2 activity as demonstrated previously by Michaelson and coworkers (Michaelson et al., 1996) which correlates well with the observed TCDD-induced upregulation of hs4 activity and inhibition of 3'α activity. Additionally, mutating the DRE and κB motifs may alter the repertoire of

DNA binding proteins, influencing the recruitment of co-activators and co-repressors and the modulation of hs4 activity. Alternatively, though highly unlikely, double mutation of the DRE and κB may have created a new binding site. Computer analysis of this sequence did not identify a new binding motif; however, this possibility can not be excluded since a motif search will not identify uncharacterized binding sites or motifs that are variants of the consensus.

As mentioned previously, mutation of the κB, Oct or BSAP binding sites appears to dramatically inhibit basal activity of the hs4 domain whereas basal activity of the hs1,2 domain is enhanced (Michaelson et al., 1996; Kanda et al., 2000). Perhaps a similar dichotomy in AhR/DRE regulation also occurs and paired with the effects of TCDD on κB (Sulentic et al., 2000) and BSAP binding may conceivably explain the contrasting effects of TCDD on the 3'α enhancer versus the hs4 domain. Moreover, in addition to the hs4 and hs1,2 DRE sites we have identified, but have not characterized, a DRE core motif (GCGTG) within both the hs3A and hs3B domains, which may also contribute to the effects of TCDD on the 3'α enhancer. A limitation of studying the regulation of the individual 3'α enhancer domains (hs3A or hs1,2 or hs3B or hs4) is that it may not reflect the overall interaction of the various regulatory sites within the 3'α enhancer as demonstrated here. This should not, however, imply that studying the individual domains is inappropriate especially considering that the hs4 domain may have a distinct function in the developing B cell. Indeed, TCDD has been shown to affect B cell maturation, mostly identified as alterations in B cell phenotype (Thurmond et al., 2000a; Thurmond et al., 2000b; Wyman et al., 2002) which may be a consequence of inappropriate maturation signals mediated through TCDD-induced activation of the hs4 domain.

Our studies demonstrate inhibition of LPS-induced 3'α activity by TCDD, which correlates well with the effects of TCDD on μ heavy chain expression and IgM secretion. Although regulation of μ expression by the 3'α enhancer has not been directly examined, we propose that the

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

AhR is a novel transcriptional regulator of the 3'α enhancer, which may mediate, at least in part, the effects of TCDD on the 3'α enhancer and its domains, ultimately contributing to a marked suppression of μ expression and IgM production.

Acknowledgements

We thank Dr. Robert Roeder for generously providing the 3'α enhancer plasmid constructs, Dr. Geoffrey Haughton for the CH12.LX cells and Kimberly Townsend for her assistance in preparing this manuscript for publication.

References

Arnold LW, LoCascio NJ, Lutz PM, Pennell CA, Klapper D and Haughton G (1983) Antigen-induced lymphomagenesis: identification of a murine B cell lymphoma with known antigen specificity. *J Immunol* **131**:2064-2068.

Arulampalam V, Grant PA, Samuelsson A, Lendahl U and Pettersson S (1994) Lipopolysaccharide-dependent transactivation of the temporally regulated immunoglobulin heavy chain 3' enhancer. *Eur J Immunol* **24**:1671-1677.

Birnbaum LS and Tuomisto J (2000) Non-carcinogenic effects of TCDD in animals. *Food Addit Contam* **17**:275-288.

Bishop GA and Haughton G (1986) Induced differentiation of a transformed clone of Ly-1+ B cells by clonal T cells and antigen. *Proc Natl Acad Sci U S A* **83**:7410-7414.

Calame K and Eaton S (1988) Transcriptional controlling elements in the immunoglobulin and T cell receptor loci. *Adv Immunol* **43**:235-275.

Chauveau C, Pinaud E and Cogne M (1998) Synergies between regulatory elements of the immunoglobulin heavy chain locus and its palindromic 3' locus control region. *Eur J Immunol* **28**:3048-3056.

Chen J, Young F, Bottaro A, Stewart V, Smith RK and Alt FW (1993) Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. *Embo J* **12**:4635-4645.

Dooley RK and Holsapple MP (1988) Elucidation of cellular targets responsible for tetrachlorodibenzo-p- dioxin (TCDD)-induced suppression of antibody responses: I. The role of the B lymphocyte. *Immunopharmacology* **16**:167-180.

Giannini SL, Singh M, Calvo CF, Ding G and Birshtein BK (1993) DNA regions flanking the mouse Ig 3' alpha enhancer are differentially methylated and DNAase I hypersensitive during B cell differentiation. *J Immunol* **150**:1772-1780.

Grant PA, Thompson CB and Pettersson S (1995) IgM receptor-mediated transactivation of the IgH 3' enhancer couples a novel Elf-1-AP-1 protein complex to the developmental control of enhancer function. *Embo J* **14**:4501-4513.

Holsapple MP, Snyder NK, Wood SC and Morris DL (1991) A review of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology* **69**:219-255.

Kanda K, Hu HM, Zhang L, Grandchamps J and Boxer LM (2000) NF-kappa B activity is required for the deregulation of c-myc expression by the immunoglobulin heavy chain enhancer. *J Biol Chem* **275**:32338-32346.

Kim DW, Gazourian L, Quadri SA, Romieu-Mourez R, Sherr DH and Sonenshein GE (2000) The RelA NF-kappaB subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-myc promoter in mammary cells. *Oncogene* **19**:5498-5506.

Klein S, Sablitzky F and Radbruch A (1984) Deletion of the IgH enhancer does not reduce immunoglobulin heavy chain production of a hybridoma IgD class switch variant. *Embo J* **3**:2473-2476.

Luster MI, Germolec DR, Clark G, Wiegand G and Rosenthal GJ (1988) Selective effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and corticosteroid on in vitro lymphocyte maturation. *J Immunol* **140**:928-935.

Madisen L and Groudine M (1994) Identification of a locus control region in the immunoglobulin heavy- chain locus that deregulates c-myc expression in plasmacytoma and Burkitt's lymphoma cells. *Genes Dev* **8**:2212-2226.

Michaelson JS, Giannini SL and Birshtein BK (1995) Identification of 3' alpha-hs4, a novel Ig heavy chain enhancer element regulated at multiple stages of B cell differentiation. *Nucleic Acids Res* **23**:975-981.

Michaelson JS, Singh M, Snapper CM, Sha WC, Baltimore D and Birshtein BK (1996) Regulation of 3' IgH enhancers by a common set of factors, including kappa B-binding proteins. *J Immunol* **156**:2828-2839.

Morris DL and Holsapple MP (1991) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on humoral immunity: II. B cell activation. *Immunopharmacology* **21**:171-181.

Morris DL, Karras JG and Holsapple MP (1993) Direct effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on responses to lipopolysaccharide (LPS) by isolated murine B-cells. *Immunopharmacology* **26**:105-112.

Neurath MF, Strober W and Wakatsuki Y (1994) The murine Ig 3' alpha enhancer is a target site with repressor function for the B cell lineage-specific transcription factor BSAP (NF- HB, S alpha-BP). *J Immunol* **153**:730-742.

Ong J, Stevens S, Roeder RG and Eckhardt LA (1998) 3' IgH enhancer elements shift synergistic interactions during B cell development. *J Immunol* **160**:4896-4903.

Saleque S, Singh M and Birshtein BK (1999) Ig heavy chain expression and class switching in vitro from an allele lacking the 3' enhancers DNase I-hypersensitive hs3A and hs1,2. *J Immunol* **162**:2791-2803.

Saleque S, Singh M, Little RD, Giannini SL, Michaelson JS and Birshtein BK (1997) Dyad symmetry within the mouse 3' IgH regulatory region includes two virtually identical enhancers (C alpha3'E and hs3). *J Immunol* **158**:4780-4787.

Serwe M and Sablitzky F (1993) V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer. *Embo J* **12**:2321-2327.

Singh M and Birshtein BK (1993) NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3' alpha enhancer at early stages of B-cell differentiation. *Mol Cell Biol* **13**:3611-3622.

Stevens S, Ong J, Kim U, Eckhardt LA and Roeder RG (2000) Role of OCA-B in 3'-IgH enhancer function. *J Immunol* **164**:5306-5312.

Sulentic CE, Holsapple MP and Kaminski NE (1998) Aryl hydrocarbon receptor-dependent suppression by 2,3,7, 8- tetrachlorodibenzo-p-dioxin of IgM secretion in activated B cells. *Mol Pharmacol* **53**:623-629.

Sulentic CE, Holsapple MP and Kaminski NE (2000) Putative link between transcriptional regulation of IgM expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin and the aryl hydrocarbon receptor/dioxin-responsive enhancer signaling pathway. *J Pharmacol Exp Ther* **295**:705-716.

Swanson HI (2002) DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. *Chem Biol Interact* **141**:63-76.

Thurmond TS, Murante FG, Staples JE, Silverstone AE, Korach KS and Gasiewicz TA (2000a) Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. *Endocrinology* **141**:2309-2318.

Thurmond TS, Staples JE, Silverstone AE and Gasiewicz TA (2000b) The aryl hydrocarbon receptor has a role in the in vivo maturation of murine bone marrow B lymphocytes and their response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Appl Pharmacol* **165**:227-236.

Tian Y, Ke S, Denison MS, Rabson AB and Gallo MA (1999) Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem* **274**:510-515.

Tucker AN, Vore SJ and Luster MI (1986) Suppression of B cell differentiation by 2,3,7,8-tetrachlorodibenzo-p- dioxin. *Mol Pharmacol* **29**:372-377.

Usui T, Wakatsuki Y, Matsunaga Y, Kaneko S, Koseki H, Kita T and Kosek H (1997) Overexpression of B cell-specific activator protein (BSAP/Pax-5) in a late B cell is sufficient to suppress differentiation to an Ig high producer cell with plasma cell phenotype. *J Immunol* **158**:3197-3204.

Vorderstrasse BA, Steppan LB, Silverstone AE and Kerkvliet NI (2001) Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. *Toxicol Appl Pharmacol* **171**:157-164.

Wabl MR and Burrows PD (1984) Expression of immunoglobulin heavy chain at a high level in the absence of a proposed immunoglobulin enhancer element in cis. *Proc Natl Acad Sci U S A* **81**:2452-2455.

Williams CE, Crawford RB, Holsapple MP and Kaminski NE (1996) Identification of functional aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator in murine splenocytes. *Biochem Pharmacol* **52**:771-780.

Wyman A, Lavin AL, Wilding GE and Gasiewicz TA (2002) 2,3,7,8-tetrachlorodibenzo-p-dioxin does not directly alter the phenotype of maturing B cells in a murine coculture system. *Toxicol Appl Pharmacol* **180**:164-177.

Yoo BS, Boverhof DR, Shnaider D, Crawford RB, Zacharewski TR and Kaminski NE (2003) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Alters the Regulation of Pax5 in Lipopolysaccharide-Activated B Cells. *Toxicol Sci*.

Footnotes

*This work was supported in part by funds from NIEHS Grants ES02520 and ES05914.

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1. Schematic of the IgH locus and the 3'α plasmid constructs. A) A simplified diagram of a rearranged murine IgH locus which includes the four domains (hs3A, hs1,2, hs3B and hs4) of the 3'α enhancer. All luciferase reporter plasmids originated from pGL3 basic (Promega) and contained a V_H promoter. Similar to the murine IgH locus, the 3'α enhancer or specific domains of the 3'α enhancer were inserted 3' of the luciferase gene. B) The averaged transfection efficiency for each reporter construct represented as mean-fold difference relative to hs4. C) A schematic of specific binding motifs within the hs4 domain of the 3'α enhancer. Octamer (Oct), NF-κB and BSAP binding sites were previously identified by Michaelson and coworkers (Michaelson et al., 1996). The 5' Oct site has weaker binding activity as compared to the 3' Oct site (Michaelson et al., 1996). The boxed BSAP site has relatively high affinity and the broken arrow indicates the presence of a weaker BSAP binding site that has not been precisely mapped (Michaelson et al., 1996).

Figure 2. LPS enhances the activity of the 3'α enhancer and the hs4 domain in CH12.LX cells. The CH12.LX cells were transiently transfected with the 3'α (A), hs4 (B) or V_H (C) reporter plasmids. Transfected cells were activated with varying concentrations of LPS (0.0-30.0 μg/ml) and then cultured in the absence of additional treatment (naïve, NA) or simultaneously treated with vehicle (VH, 0.019% DMSO) or 30 nM TCDD for 48 h. Luciferase enzyme activity is represented on the y-axis as relative light units. n=3 for each treatment. Comparisons between the treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's two-tailed *t*-test. Asterisk "*" denotes significance compared to the corresponding vehicle control at $p<0.05$. Results are representative of three experiments.

JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.059493
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. The 3'α enhancer and the hs4 domain are sensitive targets of TCDD. The CH12.LX cells were transiently transfected with the 3'α (A), hs4 (B) or V_H (C) reporter plasmids. Concentration-dependent effect of TCDD on 3'α activity in LPS-activated CH12.LX cells. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 48 h with vehicle (VH, 0.019% DMSO) or varying concentrations of TCDD (0.03-30.0 nM) in the absence or presence of LPS (3 μg/ml) stimulation. Luciferase enzyme activity is represented on the y-axis as relative light units. n=3 for each treatment. Comparisons between the treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's two-tailed *t*-test. Asterisk "*" denotes significance compared to the corresponding vehicle control at $p<0.05$. Results are representative of three experiments.

Figure 4. AhR binds to an hs4 oligomer containing DRE and κB motifs. CH12.LX cells were cultured in the absence of treatment (naïve, NA) or treated with vehicle (VH, 0.019% DMSO) or 30 nM TCDD for 1 h. DNA bound by the AhR was immunoprecipitated, purified and analyzed by PCR for a 150 bp hs4 product containing the DRE motif. Total cellular DNA served as a positive control and a sample incubated without the anti-AhR antibody during the immunoprecipitation step served as a negative control. Results are representative of three experiments.

Figure 5. Mutation of the DRE and κB motifs diminishes the effect of TCDD and LPS on hs4 activity. Three nucleotides of the DRE sequence and/or three nucleotides of the κB sequence were mutated by site-directed mutagenesis. The CH12.LX cells were then transiently transfected with the control (hs4), mutated DRE (hs4 mDRE), mutated κB (hs4 mκB), or double mutated DRE and κB (hs4 mDRE/mκB) hs4 reporter plasmids and cultured in the absence of additional treatment (naïve, NA) or treated for 48 h with either vehicle (VH, 0.019% DMSO) or 30.0 nM TCDD in the presence of LPS (3 μg/ml) stimulation. Luciferase enzyme activity is represented on the y-axis as relative light units. n=3 for each treatment. Comparisons between the treatment groups for each plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's two-tailed *t*-test. Asterisk

"*" denotes significance compared to the corresponding vehicle (VH) control at $p<0.05$. Comparisons between the hs4 plasmids containing a mutated or non-mutated DRE and/or κB were analyzed using a 2-way ANOVA followed by a Bonferroni's two-tailed *t*-test. "†" and "††" denote significance compared to the hs4 NA at $p<0.05$ and $p<0.01$, respectively. Results are representative of three experiments.

A.

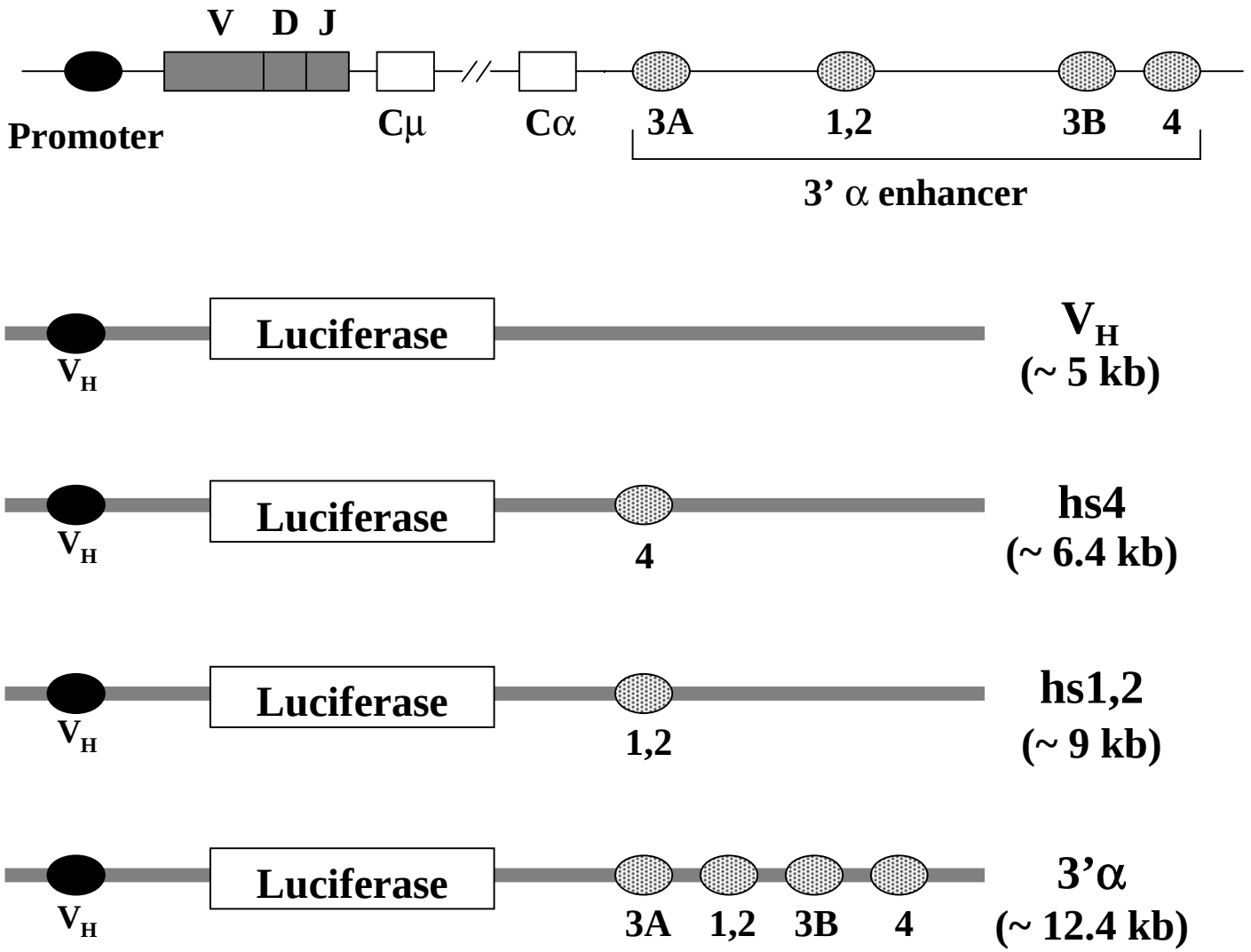

Figure 1A

B.

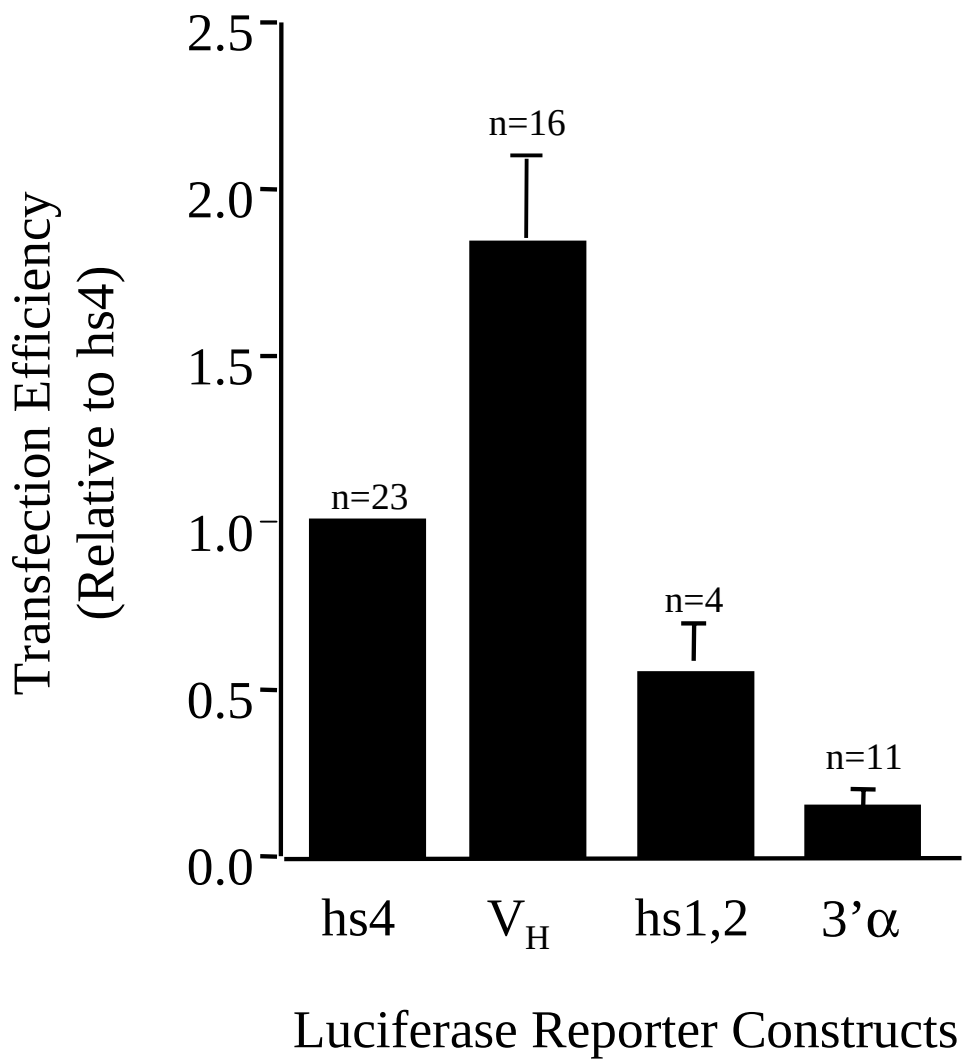

Figure 1B

C.

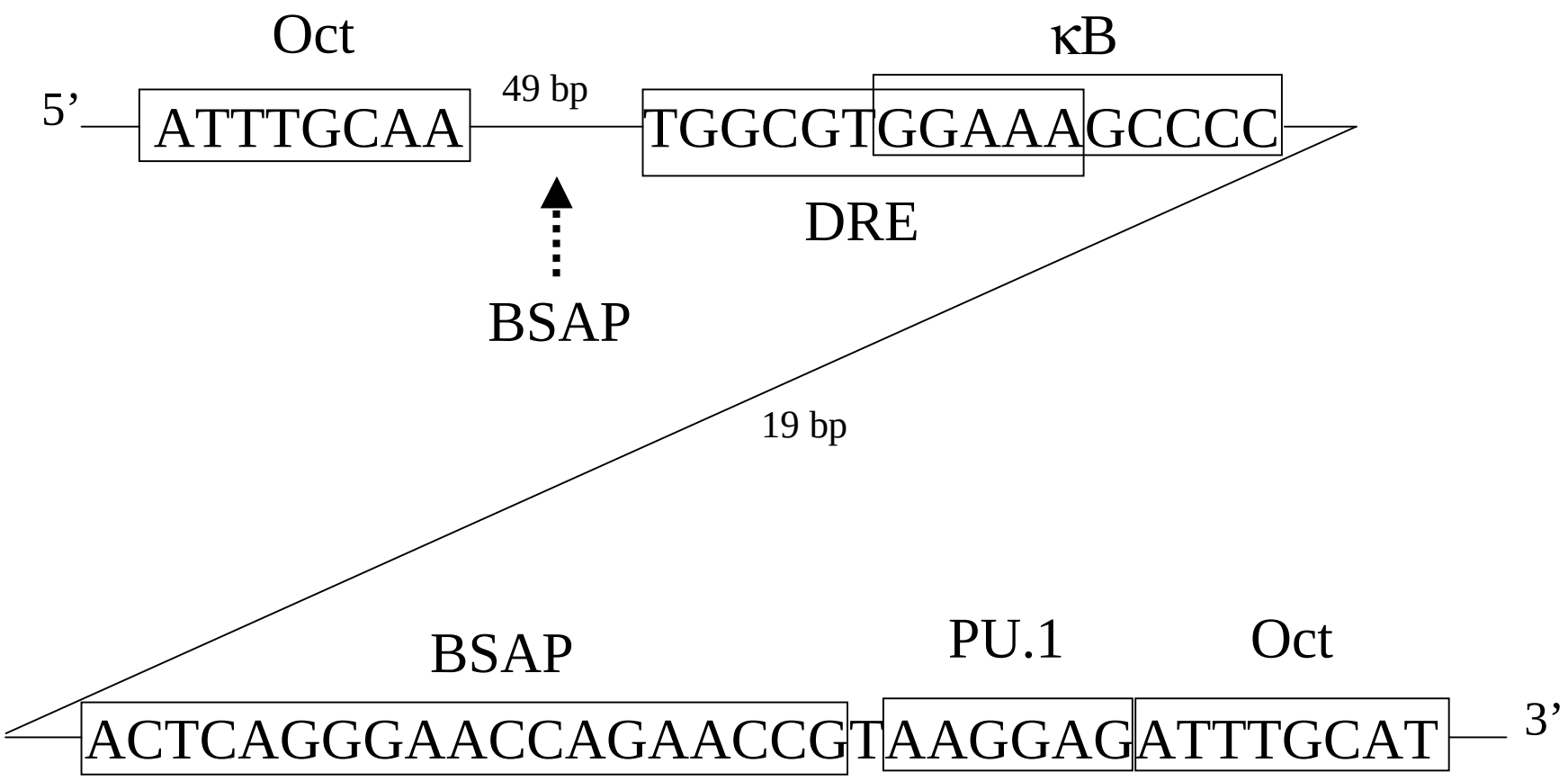

Figure 1C

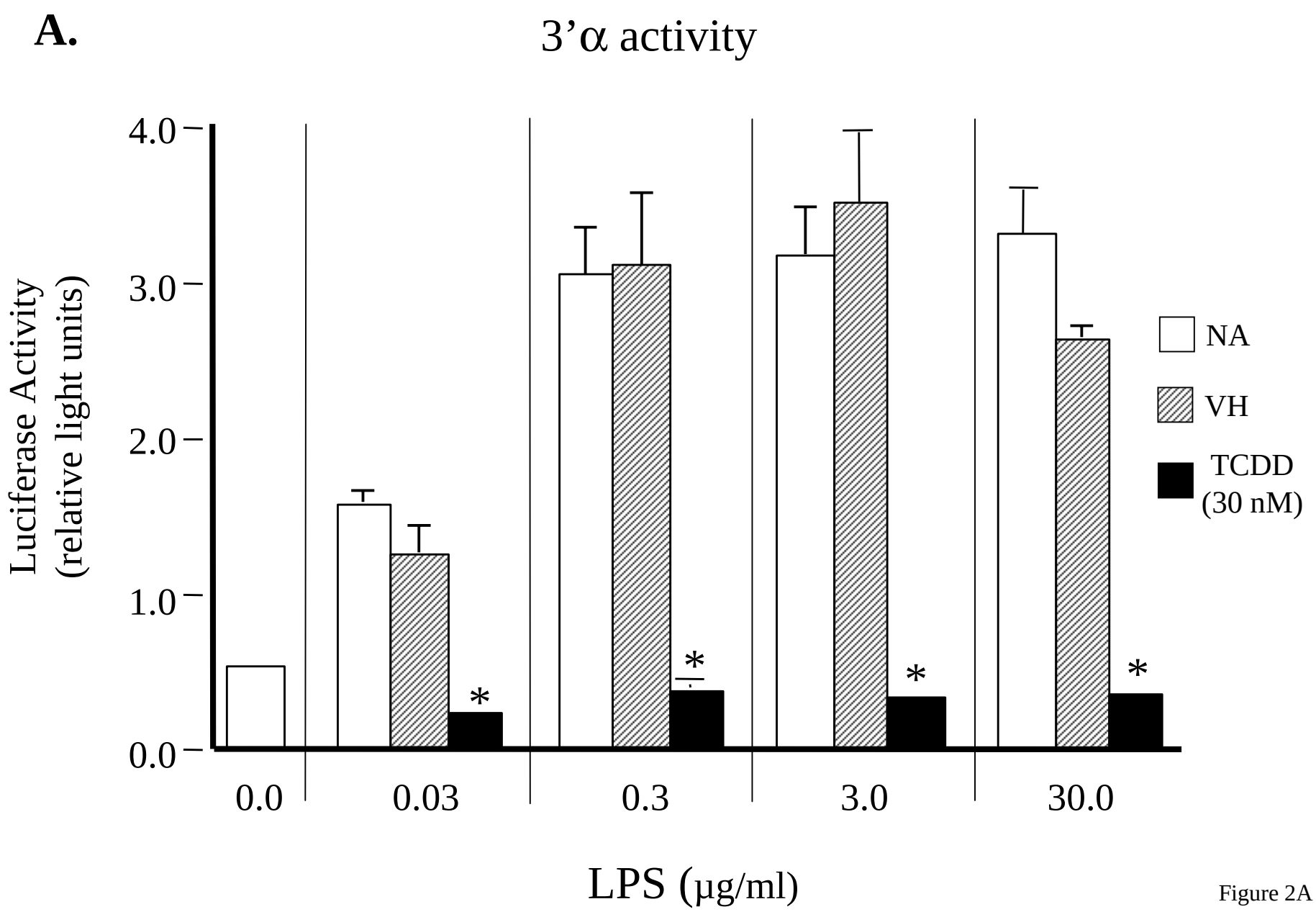

Figure 2A

B.

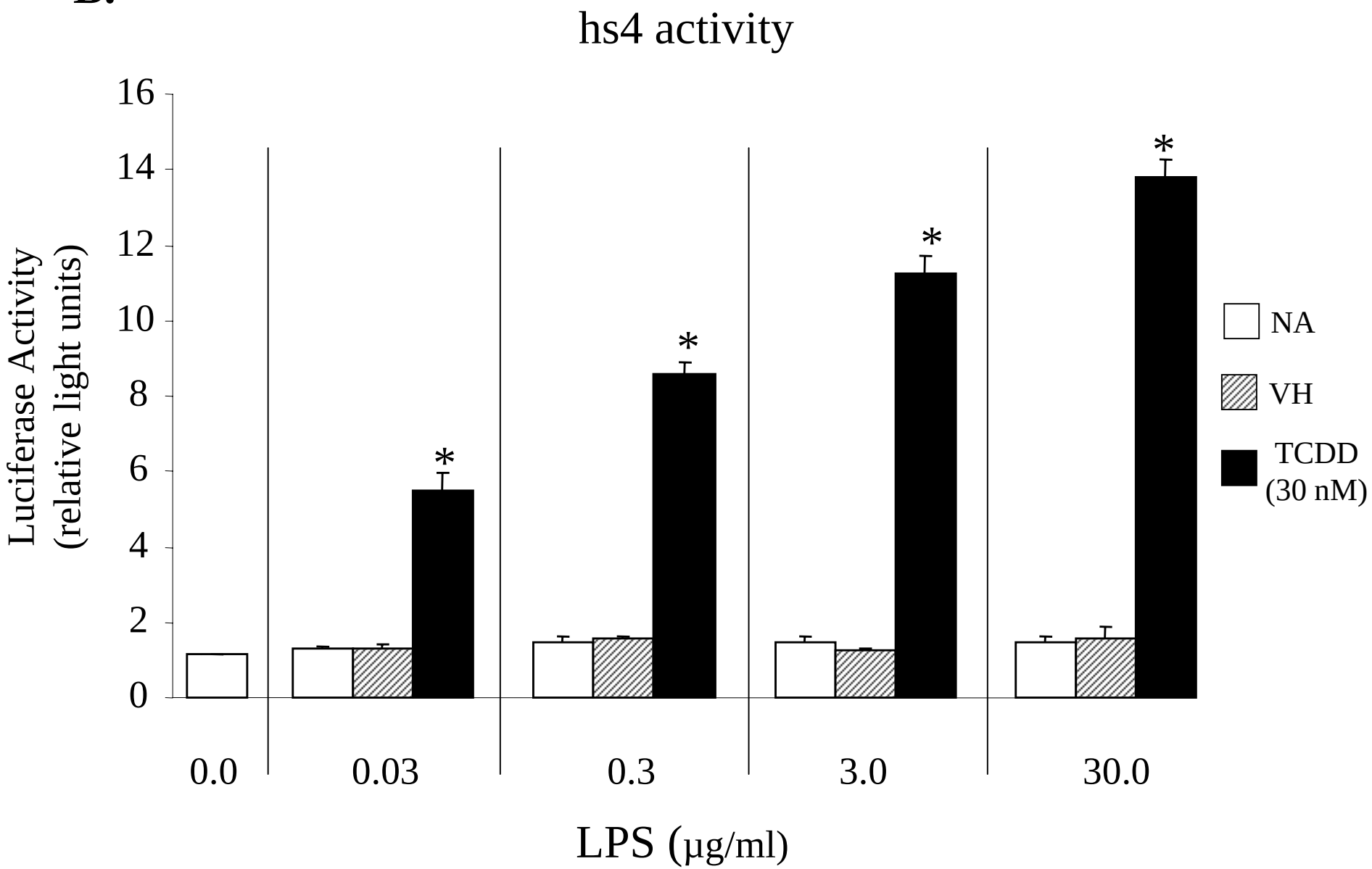

Figure 2B

C.

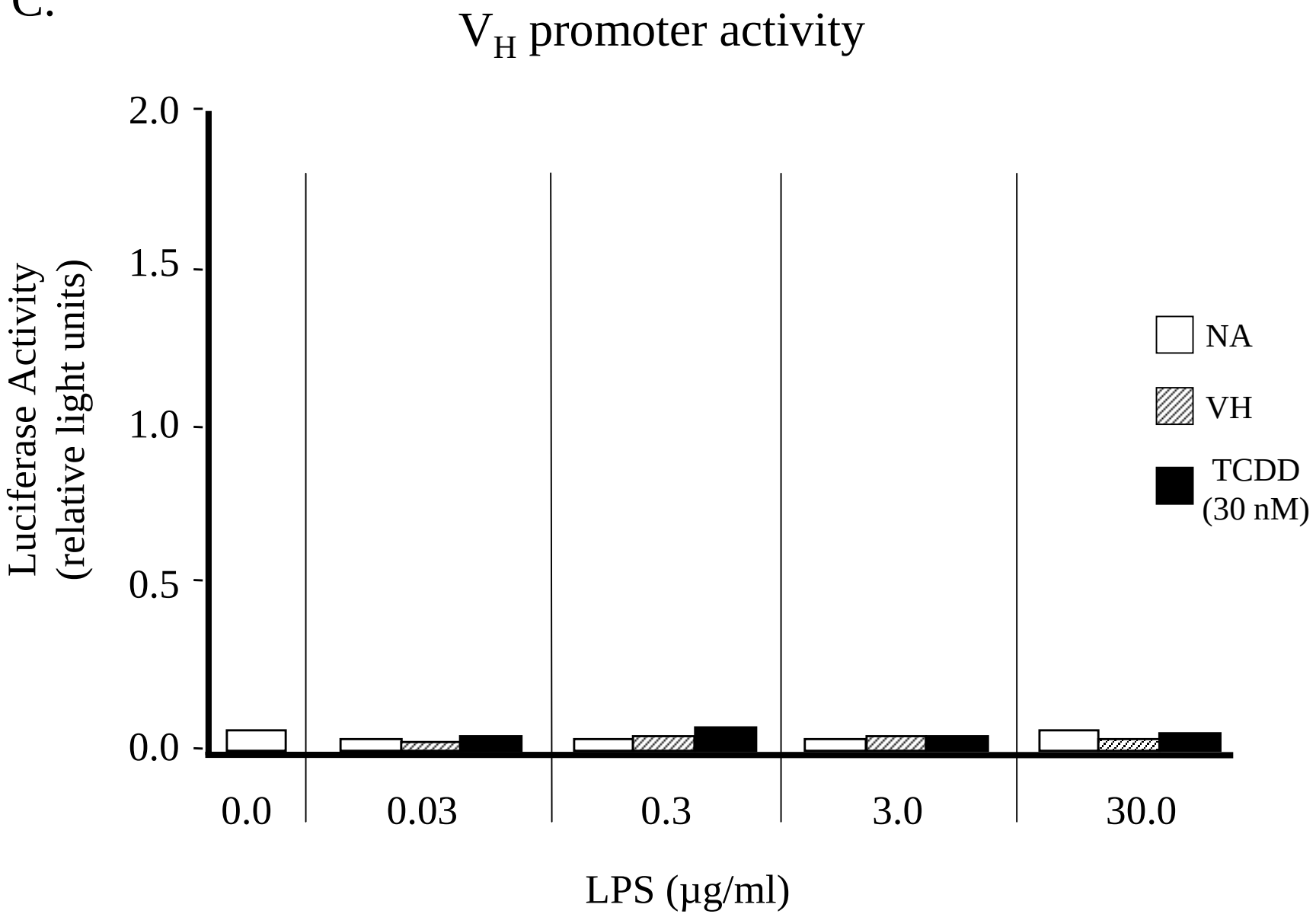

Figure 2C

A.

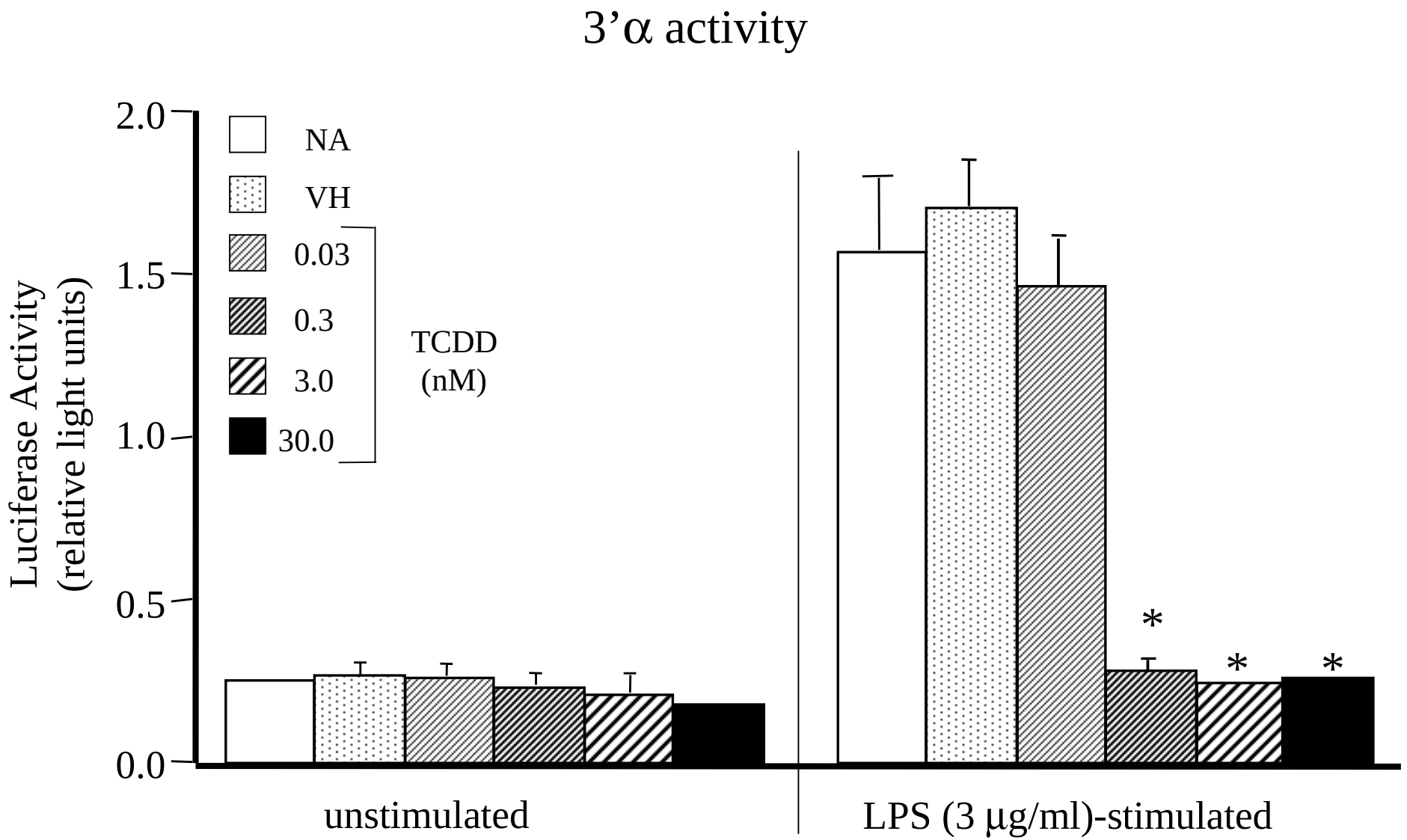

Figure 3A

B.

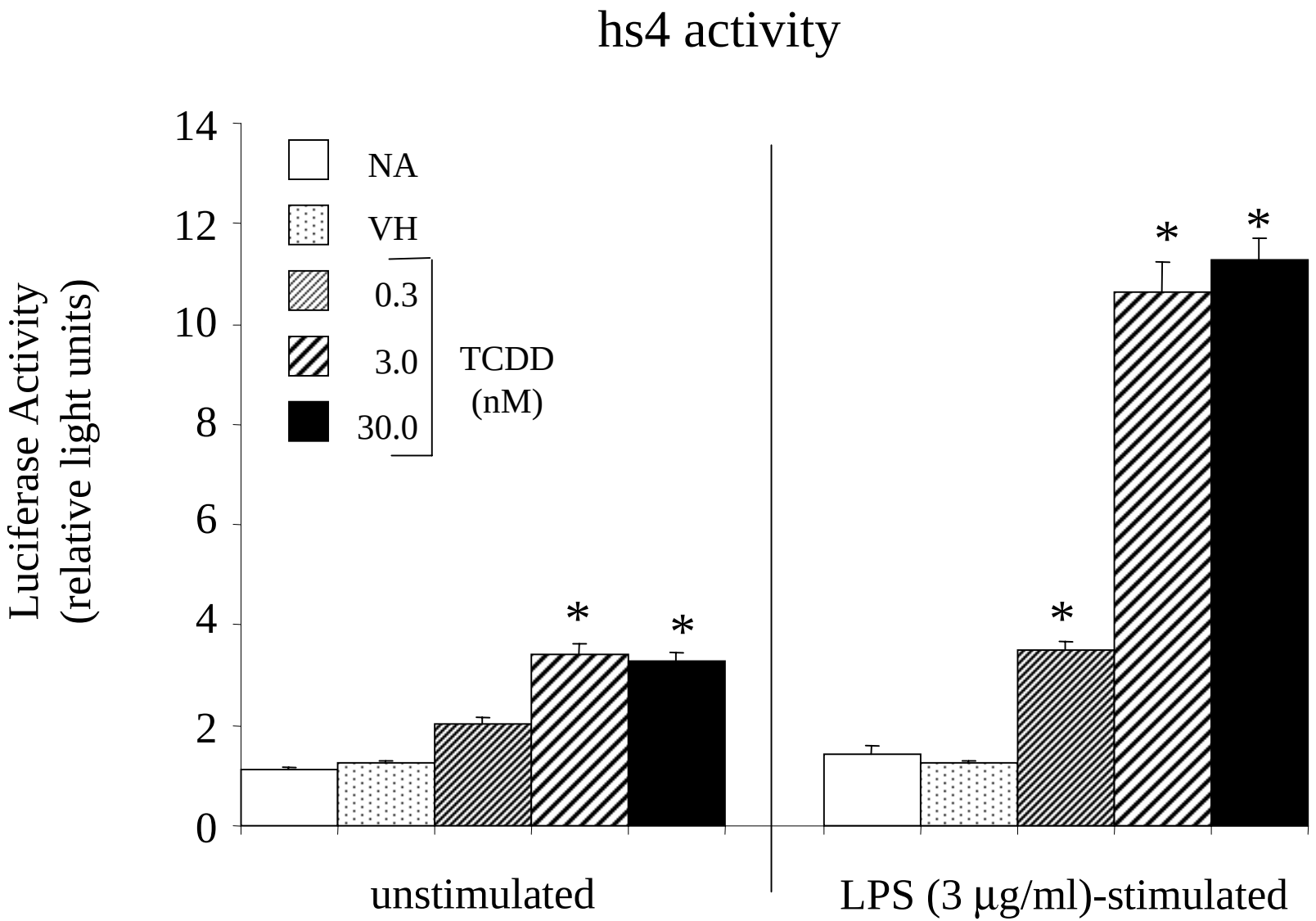

Figure 3B

C.

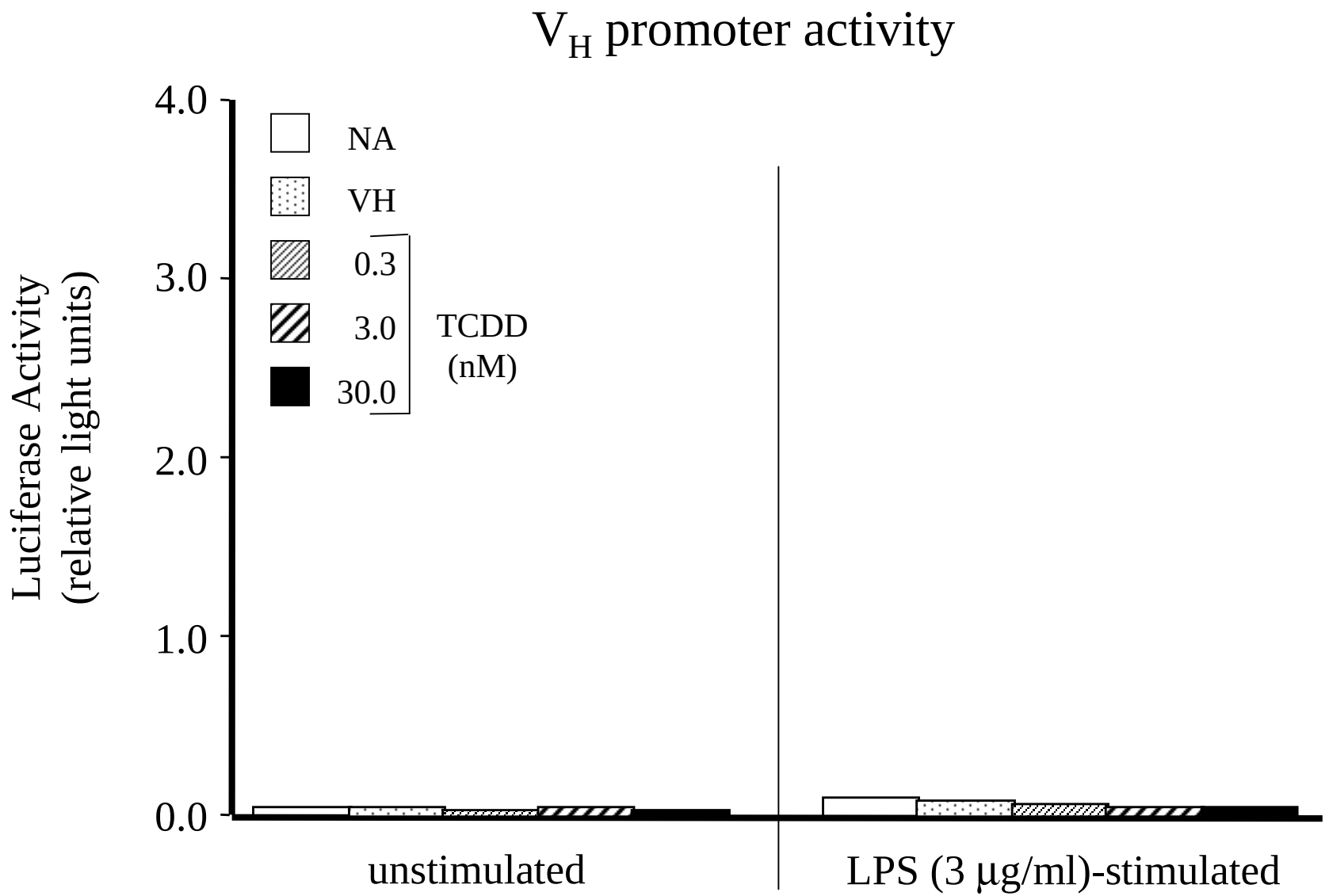

Figure 3C

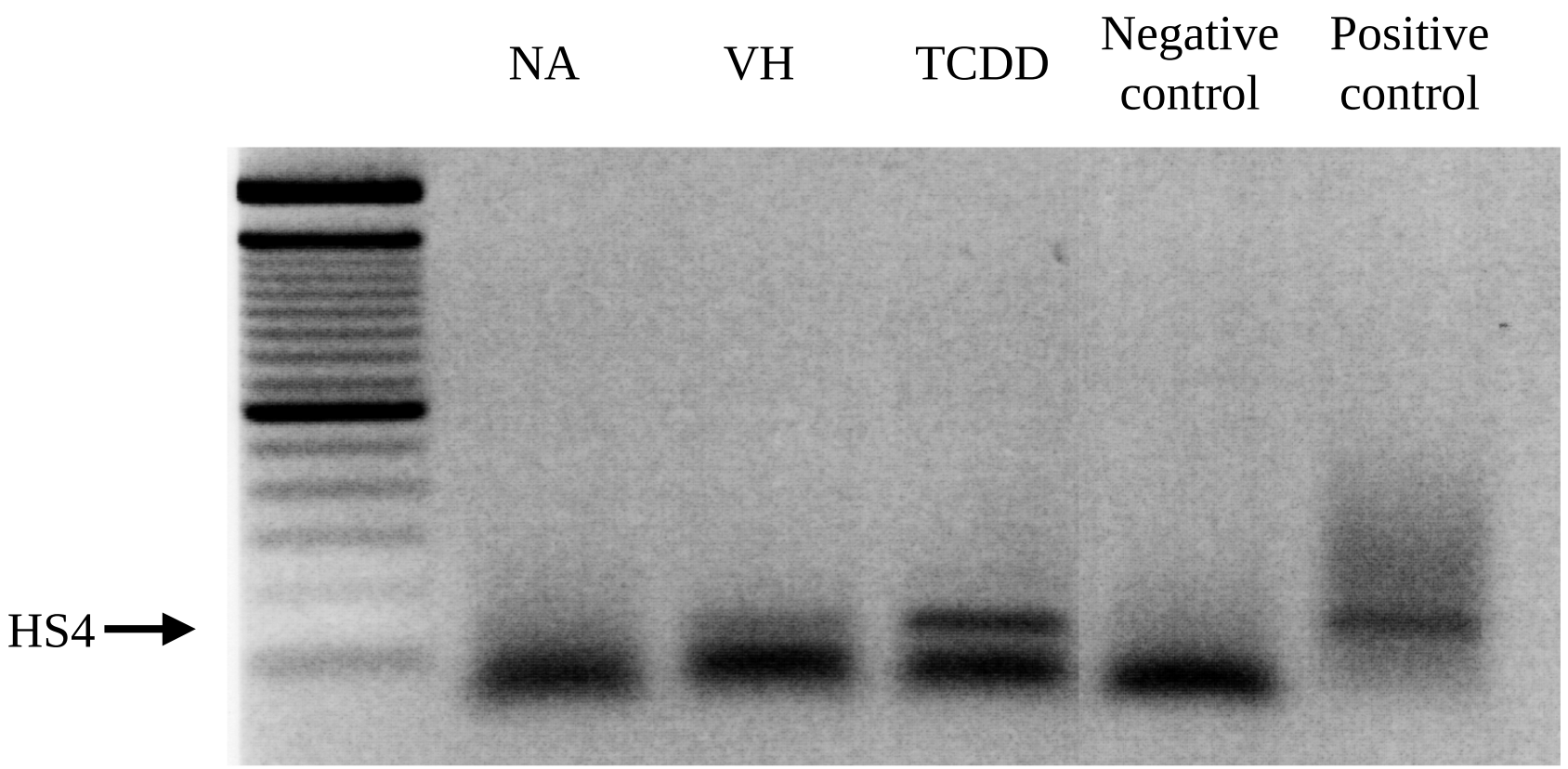

Figure 4

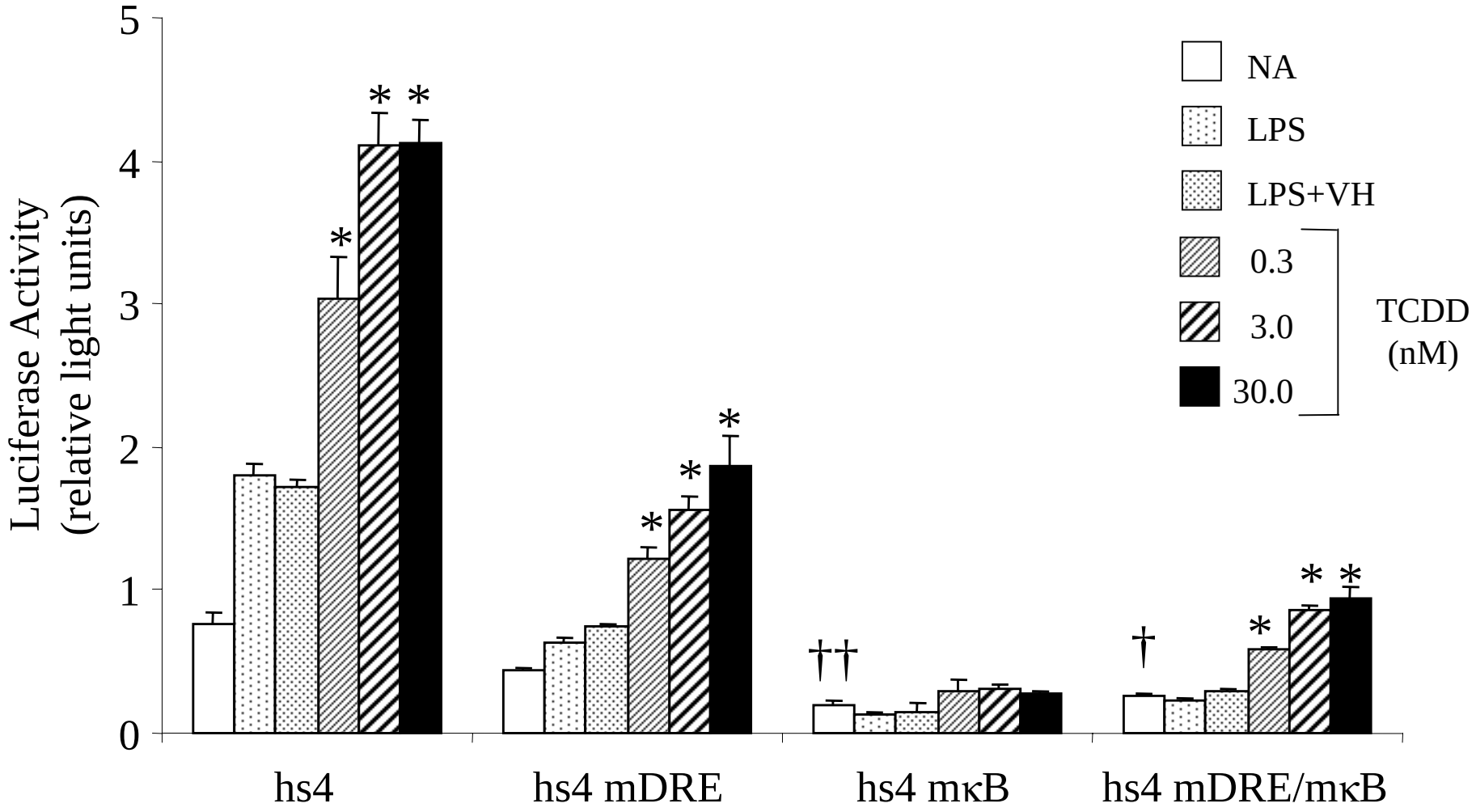

Figure 5