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AH Receptor (AHR) Splice Variants in the Dioxin-Resistant Rat:

Tissue Expression and Transactivational Activity

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ABBREVIATIONS:

AHR, aryl hydrocarbon receptor;
AHRE, AH response element (also known as DRE or XRE-I);
AHRE-II, AH response element-II (also known as XRE-II);
ARNT, aryl hydrocarbon receptor nuclear translocator;
bHLH-PAS, basic-helix-loop-helix, Period-ARNT-Single-minded;
CYP1A1, cytochrome P4501A1;
DV, deletion variant form of the AHR from H/W(*Kuopio*) rats;
IVs, insertion variant forms of AHR-H/W;
L-E, Long-Evans rat strain;
LIV, long insertion variant form of AHR-H/W;
LnA, Line-A rat strain;
LnC, Line-C rat strain;
P-S-T-rich, proline-, serine-, threonine-rich subdomain of AHR;
Q-rich, glutamine-rich subdomain of AHR;
SIV, short insertion variant form of AHR-H/W;
TAD, transactivation domain;
TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin;
WT, wildtype AHR allele from dioxin sensitive rats.

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ABSTRACT

The *AHR* locus encodes the aryl hydrocarbon receptor (AHR), a transcriptional regulator of multiple drug-metabolizing enzymes and mediator of toxicity of dioxin-like chemicals. The Han/Wistar(*Kuopio*) rat strain (H/W) is remarkably resistant to lethal effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) due to a point mutation in the intron/exon 10 boundary in *AHR* genomic structure which leads to use of 3 alternative cryptic splice sites, potentially creating 3 alternative transcripts and 2 protein products. The deletion variant (DV), which lacks 43 amino acids in the transactivation domain, has the highest intrinsic transactivation activity *in vitro*; amino acids 766-783 suppress transactivation function. However, DV expression levels in H/W rats *in vivo* are low in liver, lung, thymus, kidney and testis; insertion-variant mRNAs (IVs) are the dominant mRNA forms in H/W rats where wildtype AHR mRNA is undetectable. In dioxin-sensitive rat strains and lines that are homozygous for wildtype *AHR* alleles, wildtype AHR mRNA is the most abundant transcript but some IV transcripts are detectable. TCDD treatment *in vivo* increases transcript levels for both the DV and IVs in H/W rats and increases wildtype transcript levels in dioxin-sensitive rats but does not alter which transcript forms are expressed. *In silico* modeling indicates that the DV mRNA has lost considerable secondary structure whereas at the protein level the transactivation domain of the IV in the dioxin-resistant H/W rat has greater α-helical content and a more hydrophobic terminus than wildtype AHR which may produce a protein conformation that is less amenable to interaction with other regulatory proteins.

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(Introduction)

The *AHR* locus encodes a ligand-activated transcription factor, the aryl hydrocarbon receptor which plays key roles in: 1) adaptive metabolism of xenobiotics; 2) developmental and physiological signaling; and 3) toxic responses to dioxin-like environmental pollutants (Fernandez-Salguero et al., 1996; Nebert et al., 2000; Okey et al., 2005; Walisser et al., 2004).

Agonist ligands such as TCDD convert the AHR into a heterodimeric complex with the ARNT protein which then regulates expression of specific genes by binding directly to AH responsive elements (AHREs) (Denison et al., 1988; Hankinson, 2005) or indirectly via binding to adaptor proteins at the AHRE-II site (Boutros et al., 2004; Sogawa et al., 2004). The AHR can either enhance gene transcription or inhibit it (Riddick et al., 2004; Tijet et al., 2006).

The AHR is a member of the bHLH-PAS protein family (Gu et al., 2000) and, like other transcriptional activators, is composed of modular domains that function independently. The highly conserved N-terminus contains the bHLH-PAS domain which mediates nuclear localization, heterodimerization with ARNT and AHRE site-recognition. The C-terminal domain contains a modular transactivation domain (TAD) comprised of three distinct subdomains, acidic, glutamine (Q)-rich, and proline-serine-threonine (P-S-T)-rich regions (Supplemental data Fig. S1).

Marked inter-species and intra-species differences exist in the AHR C-terminal mRNA and protein sequences but the sequence, per se, of the transactivation domain is not always a faithful predictor of sensitivity to dioxins (Korkalainen et al., 2000; Korkalainen et al., 2001). The Han/Wistar(*Kuopio*) (H/W) rat strain is extraordinarily

resistant to lethal effects of TCDD with an LD_{50} that is more than 1000-fold higher than in dioxin-sensitive rat strains such as Long-Evans (L-E) (reviewed in: (Pohjanvirta and Tuomisto, 1994)). Dioxin resistance in the H/W rat segregates genetically with the *AHR* locus and is a dominant trait (Pohjanvirta and Tuomisto, 1994; Tuomisto et al., 1999). Our molecular analysis (Pohjanvirta et al., 1998) of *AHR* gene structure in the dioxin-resistant H/W rat strain revealed a point mutation at the intron/exon-10 boundary which leads to use of 3 alternative cryptic splice sites, potentially creating 3 alternative transcripts and 2 protein products that are smaller than the wildtype (WT) AHR from dioxin-sensitive rat strains (Fig. 1).

The restructured TAD C-terminus in H/W rats appears to exert selective effects on gene transcription. Despite the large deletion in the TAD of H/W rats, CYP1A1 and several other members of the conventional AH gene battery continue to respond normally to induction by TCDD (Okey et al., 2005). However, expression array experiments in our laboratories indicate that several other genes respond differently in rats with the H/W *AHR* genotype than in rats with WT *AHR* (Franc et al. and Moffat et al., in preparation). Potentially those genes whose response to TCDD differs between sensitive strains and resistant strains are central to the mechanism of dioxin toxicity.

The dramatic difference in dioxin susceptibility and the variant *AHR* gene structure between rats with WT AHR versus H/W rats, offers a unique opportunity to better characterize the impact of the AHR's TAD structure in regulating gene expression and dioxin toxicity. Our overall hypothesis is that H/W rats are resistant to lethality from TCDD because the TAD deletion prevents the AHR from dysregulating specific genes that are essential in the mechanism of toxicity. Our goals in the current study were to:

(1) determine which AHR splice variants are expressed in tissues of dioxin-sensitive and dioxin-resistant rats; (2) determine if TCDD affects expression of the AHR splice variants in a way that differs among the variant mRNAs; (3) determine if the altered TAD structure affects transactivation function.

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Materials and Methods

Animal Treatments and Isolation of Total RNA.

We measured expression of each AHR splice variant and the effect of TCDD on expression of the variants in two dioxin-sensitive rat strains/lines (L-E and Line-C), two dioxin-resistant rat strains/lines (H/W and Line-A) and in F1 offspring from the L-E x H/W cross. L-E is the prototype dioxin-sensitive strain homozygous for AHR^{WT} and H/W is the prototype dioxin-resistant strain homozygous for $AHR^{H/W}$ (Okey et al., 2005; Pohjanvirta and Tuomisto, 1994). Line-A (LnA, resistant) and Line-C (LnC, sensitive) lines were produced by multiple generations of crosses beginning with L-E and H/W rats, combined with phenotyping for dioxin sensitivity/resistance by TCDD challenge (Tuomisto et al., 1999). All animals were from the breeding colony of the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. At age 10-12 weeks male rats were given 100 µg/kg TCDD or the corn oil vehicle by gavage, then euthanized by decapitation after 19 or 96 h. There were 4 rats per treatment group. Total RNA was extracted from liver using Qiagen RNeasy kits according to the manufacturer's instructions. For kidney, lung, testis, and thymus, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and subsequently treated with DNase (MBI Fermentas, Burlington, Canada). RNA quality was assessed with an Agilent Bioanalyzer prior to further experiments.

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Real-Time Quantitative RT-PCR.

Three allele-specific primer/probe sets were designed using IDT SciTools Primer Quest software (http://scitools.idtdna.com/Primerquest/) to amplify mRNA encoding the 2 variant forms of the H/W receptor protein in addition to the WT receptor protein. Since the identical stop codon is used in SIV mRNA and LIV mRNA, both mRNA variants encode the identical protein product. Thus a single PCR primer set was used to quantitate SIV mRNA + LIV mRNA and simply termed IVs. See Table 1 for primer and probe sequences. The PUNS (Primer-UniGene Selectivity Testing) program (http://okeylabimac.med.utoronto.ca/PUNS; (Boutros and Okey, 2004)) was applied to ensure that primers were specific to the intended transcript sequence. Probes were labeled with a reporter fluorescent dye FAM (6-carboxyfluorescein) at the 5′-end and a quencher-fluorescent dye Iowa Black FQ™ at the 3′-end. Primers and probes were synthesized by IDT (Coralville, IA). Specificity of each primer/probe set was confirmed by: (1) sequencing of purified PCR products (MinElute, Qiagen, Mississauga, Canada) amplified from liver by real-time PCR; (2) positive real-time PCR amplification from a construct containing the specific cDNA of one variant; and (3) negative real-time PCR amplification from constructs containing the other AHR cDNAs. Total RNA (2 µg) was reverse transcribed using oligo-dT primer, $p(dT)_{15}$ (Roche Applied Science, Laval, Canada) and Superscript II RNA polymerase (Invitrogen), according to the manufacturer's instructions. Real-time PCR was performed on a Stratagene MX4000 real-time PCR system using primers, probe, and Brilliant QPCR Mastermix (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. PCR conditions were established as follows: after 10 min at 95°C, 40 cycles were performed at 95°C for 30

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sec/each and 59°C for 1 min. A 10-fold dilution series of each purified PCR product (WT or IVs or DV) was used as an external standard to assess transcript levels for each of the 3 forms of the AHR mRNA. All dilution series were performed in triplicate. Total levels of AHR receptor transcript were compared between strains/lines by a one-way analysis of variance followed by Bonferroni *post hoc* analysis. Differences between total transcript levels were considered significant when $p<0.05$. For variables with two factors (treatment and AHR-variant type) comparisons were made within each strain by two-way ANOVA. For significant comparisons ($p<0.05$), Bonferroni *post hoc* analysis for detecting deviating groups was employed.

Expression Constructs Containing the AHR TAD and Reporter-Gene Constructs for Assessment of Transactivation Function.

We generated expression constructs for the TADs of WT and the variant receptor forms, IV and DV. The TAD of each AHR variant was cloned from its respective full-length AHR expression construct and inserted into pFA-CMV (Stratagene) in-frame with the Gal4-DNA binding domain to create Gal4-AHRTAD chimeras. Deletion constructs were created in a two-step process using Gal4-AHRTAD-WT as a template. For example, to create Gal4-AHRTAD-WTΔaa766-773: Step 1: PCR was used to amplify a region representing nucleotides 1270-2297 (where nucleotide 1 is the A of the ATG translation start site of WT rat AHR). In this fragment an artificial *Bam*H I site was added immediately prior to nt 1270 and an artificial *Nco* I site was added immediately after nt 2297. Step 2: PCR was used to amplify a region representing nt 2314-2563. In this fragment, an artificial *Nco* I site was introduced immediately prior to nt 2314 and an

artificial *Hin*d III site immediately after nt 2563. The fragments from step one and step two were digested with *Bam*H I/*Nco* I or *Nco* I/*Hin*d III, respectively, ligated together at the *Nco* I site and inserted into pFA-CMV in-frame with the Gal4-DNA binding domain. A similar process was used to create Gal4-AHRTAD-WTΔaa766-783, where the *Nco* I site was introduced at nt 2344 and for Gal4-AHRTAD-WTΔaa766-800, where the *Nco* I site was introduced at nt 2386. pFR-LUC (Stratagene) was used as a reporter of transactivation activity of the Gal4-AHRTAD chimera. pFR-LUC consists of five Gal4-DNA binding elements upstream of a basic TATA transcriptional promoter which drives the transcription of a firefly luciferase gene. The plasmid pRL-TK (Promega, Madison, WI) encoding renilla luciferase was used as a control for transfection efficiency.

In vitro Assay for Intrinsic TAD Activity.

Rat hepatoma cells (5L) were seeded in 12-well plates (1.0×10^5 cells/well) in αMEM medium supplemented with 10% FBS and incubated for 24 h. Cells were transfected with expression constructs and reporter constructs using Lipofectamine Plus reagent (Invitrogen). Transfection conditions were as follows (per well): 2 µl Lipofectamine, 2 µl Plus reagent, 0.15 µg of one of the three Gal4-AHRTAD chimeras, 0.115 µg pFR-LUC and 0.035 µg pRL-TK in 900 µl of αMEM. Cells were exposed to transfection complex for 4 h; medium then was changed to FBS-supplemented growth medium and incubated for 20 h. Cells were harvested and assayed for both firefly and renilla luciferase activity using the Dual Luciferase Assay (Promega). Significant differences were determined by t-test.

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Measurement of TAD Chimeric Protein Levels by Immunoblotting.

Rat hepatoma cells (5L) were seeded in 60-mm plates (4.0 x 10^5 cells/plate) in αMEM medium supplemented with 10% FBS and incubated for 24 h. Cells were transfected (per plate) with 5 µl Lipofectamine and 5 µl Plus reagent (Invitrogen) and mixed with 0.875 µg of one of the three Gal4-AHRTAD chimeras in 1500 µl of αMEM. Cells were exposed to transfection complex for 4 h; medium then was changed to FBS-supplemented growth medium and incubated for 20 h. Cells were scraped from the plate in 1.5 ml of cold PBS and centrifuged at 3000 x g for 1 min. Pelleted cells were lysed in 300 ml of cold RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario) and incubated for 45 min with end-over-end mixing. Lysate was then centrifuged at 15,000 x g for 15 min and supernatant retained. Protein concentration in the supernatant was determined by the Bradford assay (Biorad, Hercules, CA). Fifty µg of total protein was added to LDS sample buffer containing reducing agent (Invitrogen). Proteins were separated via SDS-PAGE on pre-cast 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen) then transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA). After transfer, the membrane was washed twice in TRIS buffered saline containing 0.1% Tween (TBS-T), then incubated in TBS-T containing 5% milk (TBST+M) for 1 h at room temperature. Anti-Gal4 antibody and anti-β-actin (Santa Cruz, Santa Cruz, CA) antibody were diluted in TBST+M and incubations were performed in TBST+M at 4°C overnight. Membranes were washed in 5 changes of TBS-T buffer over 30 min. Membranes were exposed to appropriate alkaline phosphatase-

conjugated secondary antibody (Santa Cruz) for 1 h in TBST+M, then washed as described above. Antibody conjugates were visualized by ECF (enhanced chemofluorescent) substrate assay as described in the manufacturer's protocol (Amersham Biosciences, Pittsburgh, PA). Band intensities were determined using a Molecular Dynamics scanner and ImageQuant software (Amersham Biosciences, Pittsburgh, PA).

In Silico Prediction of AHR Structure and Comparison Among Species and Strains.

The minimum free energy of RNA formation (Δ G kcal/mol) and 20 suboptimal secondary RNA structures were predicted for each full-length AHR (WT, SIV, DV) using a modified thermodynamics-based Zucker algorithm and the nearest neighbor parameters implemented in RNASTRUCTURE (version 4.4, default parameters (Mathews et al., 2004)). Next, base pairing probabilities within each AHR sequence were predicted using the partition function algorithm within RNASTRUCTURE. The color-annotated base pairing probabilities for each sequence were overlaid on each of the 20 suboptimal structures. Finally, an optimal AHR structure was selected for each AHR type based on two criteria: (1) conserved predicted N-terminal TAD secondary RNA structure among all AHR-types since differences in primary RNA structures occur within the C-terminus; and (2) predicted structure with the greatest abundance of highly-probable base pairings.

BioEdit software (version 7.0.5.3 (Hall, 1999)) was used to align AHR protein sequences from L-E rat (P41738), H/W rat (H/W-IV AAC35170/AAC35169, H/W-DV AAC35168), C57BL/6J mouse (P30561), DBA/2J mouse (AAL89732), guinea pig (AAR27312), hamster (AF275721), and human (P35869). Mean hydrophobicity profiles

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were generated using the Cornette scale mean method (Cornette et al., 1987) implemented in BioEdit. Cornette method assigns hydrophobicity values to the 20 amino acids based upon a compilation of experimental data from the literature. A window of 9 amino acids was moved along each TAD sequence, the hydropathy scores were summed along the window, and the average (the sum divided by the window size) was taken for each position in the sequence. The mean hydrophobicity value was plotted for the middle residue of the window. The consensus secondary protein structures obtained for these proteins were predicted using NPS@ (MLRC, DSC, and PHD algorithms using default parameters, Network Protein Sequence Analysis, (Combet et al., 2000)). NPS takes into account multiple predictions; its alignments are 70% correct for a three-state description of secondary structure. This quality is obtained by a 'leave-one out' procedure on a reference database of proteins sharing less than 25% identity. Differences in post-translational modifications of the three TADs were predicted using PhosphoMotif Finder (Peri et al., 2003) which contains known kinase/phosphatase substrates and binding motifs curated from published literature. It reports the presence of any literature-derived motif in the query sequence.

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Results

Insertion Variants are the Predominant AHR Splice-Variant Transcripts Constitutively Expressed in Dioxin-Resistant Rats.

In untreated dioxin-resistant H/W and LnA rats, the IVs were by far the predominant AHR transcripts constitutively expressed in all tissues examined – liver, lung, thymus, kidney and testis (Fig. 2). A low level of DV transcript was present in each tissue but WT transcript was undetectable in any tissue from dioxin-resistant H/W or LnA rats. Virtually all AHR transcripts in dioxin-sensitive L-E and LnC rats represent the WT receptor. Some IVs mRNAs were present in L-E and LnC rats but at very low levels (Fig. 2).

In liver of F1 offspring from the L-E x H/W cross, each of the three transcripts was expressed (Fig. 2); IV transcript levels were equal to WT transcript levels and a very low amount of DV was detected. Phenotypically, F1 rats are highly dioxin-resistant, but not as resistant as the H/W parent (Pohjanvirta and Tuomisto, 1994). The lower level of expression of the WT AHR in F1 rat livers compared with L-E rat livers cannot account for the sensitivity difference because the AHR expression level also is low in LnC rats (Fig. 2) but their sensitivity is almost equal to that of L-E rats.

Sensitivity to dioxin toxicity probably is not related to differing levels of total AHR transcripts between sensitive rats and resistant rats. Liver, kidney, and testis from sensitive L-E rats did express ~2.7 to 8.7-fold higher total AHR transcript levels than resistant rats but these higher levels were not observed in the sensitive LnC rats. In lung and thymus, total AHR transcript levels did not differ between sensitive L-E rats and

resistant H/W or LnA rats; however, sensitive LnC rats expressed slightly higher transcript levels in lung and thymus than did any of the other strains/lines (Fig. 2).

We attempted to quantitate the specific protein products encoded by each splice variant. However, because the structures are highly similar between the DV and the IVs, we were not able to discriminate between these variant proteins either by 2D gel electrophoresis or by immunoblotting with antibodies raised against peptide sequences that differ between the DV and IV (data not shown).

Dioxin Treatment Increases Expression of AHR Splice-Variant Transcripts.

In livers from dioxin-resistant H/W rats, both the IVs and the DV transcripts were significantly increased by treatment with TCDD. The magnitude of upregulation was higher for the DV than for the IVs; however, the IVs remained the most abundant transcripts in H/W liver after TCDD treatment (Fig. 3).

Wildtype AHR transcript levels in dioxin-sensitive L-E rat livers also were significantly increased at 19 h or 96 h post-TCDD treatment. In L-E rats there was no significant effect of TCDD on transcript levels for the IVs which remained at very low levels. The DV transcript was not detectable in control L-E rats nor after TCDD treatment (Fig. 3).

Feed-restricted-control L-E rats were included to ensure that changes in AHR transcript levels were due to TCDD-treatment per se and not the result of decreased feed intake which occurs in dioxin-sensitive strains within 96 h after TCDD exposure. AHR transcript levels did not differ between feed-restricted controls and corn-oil controls.

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After TCDD treatment, total hepatic AHR transcript levels increased 2-fold in dioxin-sensitive L-E rats and 3.6-fold in the resistant H/W strain (Fig. 3). Despite the larger TCDD-induced increase in AHR levels in H/W rats compared with L-E rats, the absolute total mRNA levels were 2-fold lower in resistant H/W rats than in sensitive L-E rats (Fig. 3).

Functional Differences in Intrinsic Transactivation Activity Among the Splice Variants

We thought it important to determine whether the variant forms of AHR that contain different structures in their transactivation domains have different abilities to transactivate gene expression. To remove the influence of other AHR domains we created chimeric constructs in which the isolated AHR TAD is linked to the Gal4 DNA-binding domain so that the Gal4-AHRTAD construct drives expression of a firefly luciferase reporter gene. The Gal4 system tests the intrinsic transactivation ability of the TAD rather than the response of the full-length receptor to ligand-induced activation.

We compared intrinsic activities of the TAD from the three rat AHRs: WT, DV and IV. Immunoblotting confirmed that expression levels for the Gal4-AHRTAD proteins were equivalent for all three constructs when transfected into rat 5L hepatoma cells (Fig. 4; left panel). The intrinsic transactivation activity was significantly higher for the DV than for the WT or IV; the intrinsic activity of the IV TAD was slightly lower than that of the WT TAD (Fig. 4; right panel). Since intrinsic transactivation activity was higher for the TAD derived from the DV than from the wildtype rat AHR, we sequentially deleted portions of the TAD from the WT AHR to determine which specific regions were

responsible for the difference. Deletion of amino acids 766-773 significantly increased activity above that of the WT receptor. Deletion of a further segment up to aa783 brought the activity up to the level of the DV. Extending the deletion up to aa800 did not result in a further increase in transactivation activity (Fig. 5).

AHR Structures Predicted In Silico: Comparison Between Species and Within Species.

Secondary structure of mRNA is, itself, a potential mechanism of translational regulation by cis-acting factors since secondary structure can affect mRNA localization and degradation. To determine if the alternatively-spliced AHR variants possess altered secondary mRNA structures, we compared predicted secondary RNA structures for the TADs of the SIV and DV variants to that of WT. The least energetically-favorable TAD structure is the DV which has a predicted free energy of minus 274 kcal/mol compared with minus 296 for the SIV and minus 327 for the WT. The loss of 129 nucleotides from the DV TAD leads to a significant loss of secondary structure such as hairpin loops and bulges (Supplemental Data Fig. S2A). The SIV has acquired 29 nucleotides from intron-10 and this is predicted to create a small hairpin loop (Supplemental Data Fig. S2B). Moreover, in the IVs, 138 nucleotides are deleted from the carboxy terminus which is predicted to result in considerable loss of structure (Supplemental Data Fig. S2C).

In addition to examining the predicted secondary structure of mRNAs for the splice variants we examined how variations in mRNA sequence might affect subsequent protein structure in the TAD of multiple species and strains. As shown in Figure 6, predicted secondary structures are similar for the TADs of rat, mouse, guinea pig and human

whereas the TAD from a highly dioxin-resistant species, hamster, contains a large additional segment of amino acids predicted to be involved in formation of α-helices (Fig. 6A). Our *in silico* analysis (Fig. 6) reveals that the TAD of the rat AHR protein has abundant amino acids that favor formation of α-helices in the acidic region and in the amino-terminal end of the Q-rich region of the WT AHR as well as each H/W splice variant (Fig. 6). In contrast, the carboxyl-terminal half of the Q-rich subdomain is relatively devoid of secondary structure (Fig. 6). There is a conservative valine-to-alanine amino acid substitution at position 497 of the H/W AHR but this has no impact on predicted secondary protein structure (data not shown).

The DV protein is predicted to lack most of the C-terminal α-helix (Fig. 6B). Inter-species comparisons indicate that this predicted α-helical region is conserved among rat, mouse, human, guinea pig and hamster. The insertion of seven amino acids into the IV TAD is predicted to lead to a small increase the α-helical content as can be seen in Figure 6B while the deletion of the last 45 amino acids is predicted not to result in any loss of secondary protein structure. The predicted TAD protein structures of some highly-resistant species/strains such as H/W rat and hamster appear to be more ordered and to form fewer ambiguous states than AHRs of dioxin-sensitive species (Table 2). While dioxin-resistant DBA/2J mice have predicted secondary TAD protein structure that is virtually identical to that of dioxin-sensitive C57/BL6J mice, dioxin resistance in DBA/2 mice is primarily attributable to structural variation in the ligand-binding domain (rather than the TAD) which leads to lowered affinity for TCDD (Okey et al., 2005). Thus, lack of homogeneity in predicted TAD structure between mouse strains does not negate possible involvement of the TAD in resistance to TCDD.

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When compared with the WT protein, hydrophobicity profiles (Supplemental data Fig. S3) revealed deletion of hydrophobic as well as hydrophilic regions from the TAD domain of the DV protein. While the terminal hydrophobic region predicted to exist in the WT TAD protein is lost from the IV TAD protein, a region of greater hydrophobicity is inserted at the terminus (Supplemental data Fig. S3). Aside from 2 hydrophilic regions inserted within the hamster TAD and 3 small hydrophobic regions within the guinea pig TAD, the hydrophobicity profiles of rat, mouse, guinea pig, human, hamster are very similar (Supplemental data Fig. S3).

Post-translational modifications, particularly phosphorylation status, are known to affect AHR function (Long and Perdew, 1999; Mahon and Gasiewicz, 1995). Therefore we searched all TAD protein domains derived from each splice variant for amino acid motifs known (from curated literature) to be targets for kinase/phosphatase activities. In the DV, 15 potential post-translational-modification motifs are deleted from the TAD whereas 20 potential motifs are deleted in the IV (Fig. 7; Supplemental Table S1). The 7 amino acids inserted from former intronic sequence into the IV do not add any unique post-translational modification sites. No sumoylation sites are predicted to exist within the TAD for IV, DV, or WT receptors.

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Discussion

The deletion in the TAD of the H/W rat AHR provides a natural window onto the relationship between AHR structure and AHR function. To better understand how changes in the TAD impact the H/W rat's responsiveness to dioxin we compared IV and DV AHR variants to WT receptor for constitutive *in vivo* expression levels, *in vivo* expression after dioxin treatment, intrinsic transactivation ability, and predicted mRNA and protein structures.

Despite the substantial alteration in AHR structure in H/W rats, genes in the conventional AH gene battery such as *CYP1A1*, *CYP1A2*, *CYP1B1*, *ALDH3A1*, *NQO1* and *UGT1A1* continue to be highly responsive to induction by TCDD (Okey et al., 2005). The TAD deletion in H/W rats appears to selectively prevent TCDD from dysregulating genes that are essential to dioxin toxicity rather than causing a blanket failure of transactivation.

Constitutive Expression of Splice Variants

Our experiments show that the DV of the H/W AHR has, in fact, higher intrinsic transactivation activity than either the WT or the IVs. However, DV transcript levels are very low in dioxin-resistant H/W and LnA rats and are undetectable in L-E and LnC rats. Thus, despite high intrinsic transactivation function, the DV is unlikely to be a significant mediator of responses to TCDD *in vivo*.

Dioxin resistance behaves as a dominant trait in crosses between dioxin-sensitive strains and dioxin-resistant strains. F_1 offspring are resistant to TCDD lethality and they express both the WT AHR protein and the smaller proteins that result from the TAD

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deletion (Pohjanvirta et al., 1999). Our current experiments show that F1 rats express all three mRNA splice variants. However, expression from the single copy of the WT *AHR* allele from the sensitive parent is not sufficient to confer dioxin sensitivity in F1 offspring. Rather, the AHR from the resistant H/W parent appears to act in a "dominant negative" fashion to create resistance to TCDD lethality (Pohjanvirta and Tuomisto, 1994; Tuomisto et al., 1999).

Our current experiments reveal virtually no DV transcript in tissues of homozygous AHR^{WT}/AHR^{WT} L-E or LnC rats and very low levels of IV transcripts; therefore these animals receive no "protection" from the potential dominant-negative influence of the DV or IV. Conversely, H/W rats and LnA rats (homozygous for $AHR^{H/W}/AHR^{H/W}$), predominantly express IV transcripts; no WT AHR is detectable in any tissues examined in H/W or LnC indicating that the mutation at the intron/exon-10 boundary completely disrupts the normal splice site in these dioxin-resistant animals.

Total AHR abundance might affect susceptibility to TCDD. Our earlier studies indicated that AHR mRNA and protein levels are higher in lung and liver of dioxin-sensitive L-E rats than in dioxin-resistant H/W rats (Franc et al., 2001; Pohjanvirta et al., 1999; Viluksela et al., 2000). Our current study confirms that total constitutive AHR mRNA levels are higher in liver of L-E rats than in H/W or LnA rats. Mice that are hypomorphic for AHR expression are resistant to TCDD toxicity (Walisser et al., 2004). However, the dioxin-sensitive LnC rat expresses total AHR mRNA levels that are no higher than in dioxin-resistant H/W rats, indicating that higher AHR levels are not invariably associated with high susceptibility to TCDD toxicity.

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Because the biochemical and toxic actions of dioxin-like compounds affect many tissues other than liver, we measured splice variant levels in lung, thymus, kidney and testis. Tissue-dependent regulation of alternative splicing is a common occurrence in transcriptional regulation (Le et al., 2004; Nakahata and Kawamoto, 2005) and is employed by members of the bHLH-PAS family such as ARNT2 (Korkalainen et al., 2003) and HIF-1α (Drutel et al., 2000). We found that the expression pattern in liver for WT and H/W variants was recapitulated among other tissues although total mRNA levels varied among tissues.

TCDD Effect on Expression of Splice Variants

Previous reports on the effect of TCDD on AHR expression levels in rodent tissues *in vivo* have been contradictory. TCDD increases in AHR protein in liver (Sloop and Lucier, 1987) but there also are reports of AHR depletion in multiple rat tissues (Pollenz et al., 1998; Roman and Peterson, 1998; Sommer et al., 1999). We previously found that exposing H/W and L-E rats to 5 μg/kg TCDD produced a 2- to 3-fold increase in total cytosolic AHR protein levels whereas 50 μg/kg TCDD led to depletion at one day post-TCDD followed by recovery in H/W but not L-E rats (Franc et al., 2001). Our current study had the capability to quantitatively differentiate variant forms of AHR transcripts. There was no difference in which splicing products were generated constitutively versus the splicing products formed in response to TCDD. After TCDD treatment, IV transcripts remained predominant in resistant rats while the WT transcript was dominant in livers of dioxin-sensitive L-E rats. In our current study, exposure to 100 μg/kg TCDD for 19 h or for 96 h significantly increased total AHR mRNA levels in both dioxin-

sensitive (2-fold) and dioxin-resistant (3.6-fold) rat livers. The extraordinary resistance of the H/W rat to lethal effects of TCDD cannot be attributed to alteration of the level of any individual splice variant nor to a failure to up-regulate the total pool of AHR transcripts. However, after TCDD treatment, total AHR transcript levels in H/W rats were ~2-fold lower than in L-E rats. In agreement with our previous study (Franc et al., 2001), sensitivity to dioxin toxicity is not likely to be attributable to differential regulation of AHR levels by the potent agonist ligand, TCDD.

TAD Structure in Relation to AHR Function

We used deletion analysis and reporter-gene assays to identify which amino acids in the AHR TAD account for the higher intrinsic activity of the DV. This was supplemented by modeling *in silico* to predict secondary structures of mRNA and protein products for each splice variant with the goal of identifying features that might be responsible for differences in transactivation function among the variants from dioxin-sensitive vs dioxin-resistant rats.

Our systematic deletion of the region that is divergent between WT AHR and the DV indicates that amino acids 766-783 of the WT AHR are responsible for its lower intrinsic transactivation activity. Previously Kumar et al. showed that deletion of the entire P-S-T-rich subdomain of human AHR TAD enhances transcriptional activity (Kumar et al., 2001), suggesting that the elevated intrinsic transactivation ability of the DV in our *in vitro* experiments is due to deletion of amino acids that suppress TAD function.

There are many ways in which alteration of TAD structure might affect levels or function of mRNA or protein. At the mRNA level, loss of structure may flag mRNA for

degradation leading to the low cellular levels that we observed for the DV mRNA. At the protein level, altered TAD structure, encoded by different splice-variant mRNAs may impair the receptor's ability to interact with essential regulatory proteins such as co-regulators or phosphatases. Hydrophobicity content and secondary structure are indices of a protein's accessibility to interact with other proteins. Hydrophobic regions have a low probability of being on the surface of a protein and amino acids that lie within a structured region often are not accessible. Previous studies indicated that an increased content of hydrophobic amino acids and/or α-helical secondary structures in the AHR TAD impair AHR protein-protein interactions (Watt et al., 2005) and enhance transactivation (Jones and Whitlock, 2001), perhaps interrupting repressor protein binding. In the IV protein, our modeling predicts that the altered amino acid sequence increases both α-helical content and hydrophobicity of the TAD terminus compared to WT AHR. Overall, modeling suggests that the TAD terminus of the IV protein adopts a conformation that is less accessible to interactions with other proteins. Noteworthy, in the IV TAD there is loss of a predicted Src kinase-substrate motif and a second such motif adjacent to the inserted α-helix may be obstructed. Mice lacking *c-Src* kinase activity are resistant to lethality of TCDD but are fully responsive to CYP1A1 induction, as are H/W rats (Dunlap et al., 2002; Matsumura et al., 1997). Loss of AHR phosphorylation by Src kinase may inhibit particular AHR functions relating to dioxin lethality but not alter the AHR's ability to regulate CYP1A1.

In summary, we found that IV transcripts are the dominant AHR splice variants expressed in tissues of dioxin-resistant rats and remain the dominant transcripts after TCDD treatment. Since very little DV mRNA is present *in vivo* and since its structure is

the energetically least-favorable form, it is unlikely that a significant amount of DV protein is synthesized or that the DV plays a role in biochemical or toxic responses. Our deletion analysis indicates that amino acids 766-783 within the AHR's TAD are critical for suppression of TAD function. Modeling *in silico* predicts that insertion of 7 aa. combined with deletion of 45 aa. from the TAD terminus results in increased secondary structuring that selectively impairs the receptor's ability to interact with other proteins, subsequently attenuating transcription of genes that are involved in dioxin toxicities while leaving transcription of non-lethal AHR-mediated genes unaltered.

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Figure Legends

Fig. 1. Domain structure of the AHR and the region deleted in the transactivation domain of the H/W(*Kuopio*) rat due to alternative splicing. The ligand-binding domain (LBD), dimerization domain and AHRE-binding domain are near the N-terminus, in a conserved region that does not differ between H/W rats and rats with WT AHR. An exonic mutation at nucleotide 1520 from the start codon causes a conservative amino acid substitution that is not believed to significantly affect receptor function. A second mutation at nucleotide 2454 in the intron-10/exon-10 boundary disrupts the normal splice site, leading to potential use of the 3 cryptic splice sites "a", "b", and "c" and giving rise to three possible mRNAs and 2 possible protein products. The alternative splice variant termed **DV** results from use of splice site "a" which leads to a deletion of 129 nucleotides from exon-10 producing a protein lacking 43 amino acids. The **IV** splice variant results from use of splice sites "b" or "c". These splice sites lead to an addition of 29 nt (SIV, short insertion variant) or 134 nt (LIV, long insertion variant) from intron-10; however, identical addition of a translation termination site produces identical protein products. The IV protein has a net loss of 38 amino acids (insertion of 7 amino acids from the intron and deletion of 45 amino acids encoded by the last exon). (Modified from: (Okey et al., 2005; Pohjanvirta et al., 1998)).

Fig. 2. Constitutive AHR transcript expression levels in 5 tissues from dioxin-resistant (H/W, LnA and F1) and dioxin-sensitive (L-E and LnC) rats. Transcript levels for WT, IVs and DV were measured by real-time RT-PCR with primers specific to

each variant as described in Materials and Methods (mean ± S.D., n=4). F1 rats are the offspring of a H/W x L-E mating.

Fig. 3. Expression levels of AHR splice-variant transcripts in rat liver after treatment with TCDD. Liver was taken from H/W and L-E rats at 19 h or 96 h after TCDD treatment and transcript levels measured as described in Materials and Methods. Since the identical stop codon is used in H/W's SIV mRNA and LIV mRNA both mRNA variants encode the identical protein product. Thus PCR primers common to SIV mRNA and LIV mRNA were designed and simply termed IVs. Control animals received corn oil. The L-E data set includes an additional control group (FRC) whose food intake was restricted to the same level as TCDD-treated animals to determine if any changes in transcript expression were due to TCDD treatment per se versus the possibility that the changes were due to reduced food intake caused by TCDD treatment in the dioxin-sensitive L-E rats. Bars (mean ± S.D., n=4) with non-identical letters significantly differ from one another (ANOVA, Bonferroni post-hoc analysis $p<0.05$).

Fig. 4. Effect of rat AHR TAD polymorphism on intrinsic transactivation activity. The left-hand panel shows protein expression levels of Gal4-AHRTAD chimeras in the transfected 5L hepatoma cell line. Total protein was separated via SDS-PAGE. Immunoblots were performed for AHR (upper band) and β-actin (lower band) and densities measured with ImageQuant software. The slower migration of the band for the WT chimera is due to the fact that the size of the TAD protein domain is larger for the WT AHR than for either the IV or DV. Expression values are represented as a fraction of

expression in cells transfected with the Gal4-AHRTAD-WT normalized to corresponding β-actin (n=3; significance calculated by one-way ANOVA, Bonferroni post-hoc analysis; error bars represent SD). The right-hand panel shows intrinsic activity of the Gal4-AHRTAD chimera from WT AHR versus the alternative AHR splice variants DV or IV in rat 5L hepatoma cells. Firefly luciferase was first normalized to renilla luciferase for each sample. Luciferase activity is represented relative to activity in cells transfected with Gal4-AHRTAD-WT set to 1.0 (mean ± S.D., n=3; unpaired t-test, *=p<0.01, **=p<0.001, ***=p<0.0001).

Fig. 5. Sequential deletion of rat WT AHR TAD increases intrinsic activity to levels equivalent to that of the DV TAD. Deletions of increasing size were created in the TAD of the WT AHR as described in Materials and Methods and illustrated in the bottom panel. The resulting TAD constructs were assayed for intrinsic transactivation activity in rat 5L hepatoma cells as described in the legend to Figure 4. The top-left panel indicates that the level of protein expressed was equivalent for all TAD constructs. In the top-right panel luciferase activity is represented relative to activity in cells transfected with Gal4-AHRTAD-WT as in the legend to Fig. 4.

Fig. 6. Inter- and intra-species comparison of predicted secondary protein structure of the TAD. Consensus secondary AHR protein structures for transactivation domains were predicted using Network Protein Sequence Analysis (NPS@, (Combet et al., 2000)). Tall bars represent amino acids contributing to α-helix structure(s). Short bars represent amino acids contributing to β-strand(s). **A:** Hamster is predicted to have a high

abundance of α-helices in its TAD. **B:** Restructured region of the H/W rat. The approximate boundaries for the major TAD subdomains, based on human nomenclature, are displayed. Note that first amino acid number marks the beginning of the TAD for each species. Subsequent numbering of amino acids refers only to the position within the TAD domain, not the full-length AHR protein.

Fig. 7. Schematic representation of differences in predicted TAD protein structures between rat WT AHR and the rat splice-variants. Predicted post-translational kinase/phosphatase substrates (represented as asterisks) curated from published literature were identified using PhosphoMotif Finder (Peri et al., 2003). Note: the segment inserted into the IV had no unique posttranslational modifications. See Supplemental Table S1 for specific binding motifs and their substrates. Consensus secondary AHR protein structure was predicted using NPS@ as described in Materials and Methods. The unshaded box represents β-strands and the shaded box represents α-helices.

TABLE 1
Primer and probe sequences for real-time quantitative RT-PCR measurements of rat WT AHR and rat splice-variant transcripts.

	Wildtype AHR	Insertion-Variant AHR	Deletion-Variant AHR
Sequence	NM_013149	AF082125[a]/AF082126[b]	AF082124
Forward	ATGGTCAGTCCTCAGGCGTACTA	ATGGTCAGTCCTCAGGCGTACTA	AAC TCA CAG TCA GCC ATG TTT CAG
Reverse	AAT GCT CGG ACT CTG AAA CTT GC	TCC CTG TAG AAA GCC CTT ATC TTG C	ATATCAGGAAGAGGCTGGGCTTC
Probe	CCATGTCCATGTACCAGTGCCAGGCAGG	CCATGTCCATGTACCAGTGCCAGGCAGG	CCAGGCGAGGGAGGTGAGCAGCAGTC
Product size	141	151	130

[a] Alternatively spliced short insertion variant
[b] Alternatively spliced longer insertion variant

TABLE 2

Predicted secondary AHR protein structures of transactivation domains in different species and strains.

Protein	Dioxin LD_{50} µg/kg (male)	Alpha helix (%)	Random Coil (%)	Ambiguous states (%)	Extended strand (%)
Rat					
L-E WT	18	19.1	74.0	1.59	5.4
H/W	> 9,600				
IV		19.9	72.2	0.36	7.6
DV		18.4	74.3	1.47	5.9
Mouse					
C57BL/6	128	19.4	73.3	0.64	6.7
DBA/2J	2,600	19.4	73.3	0.64	6.7
Guinea Pig	1-2	15.2	76.1	2.27	6.5
Hamster	3,000-5,000	33.2	62.6	0.52	4.7
Human	Unknown (resistant)	19.9	72.6	0.33	7.2

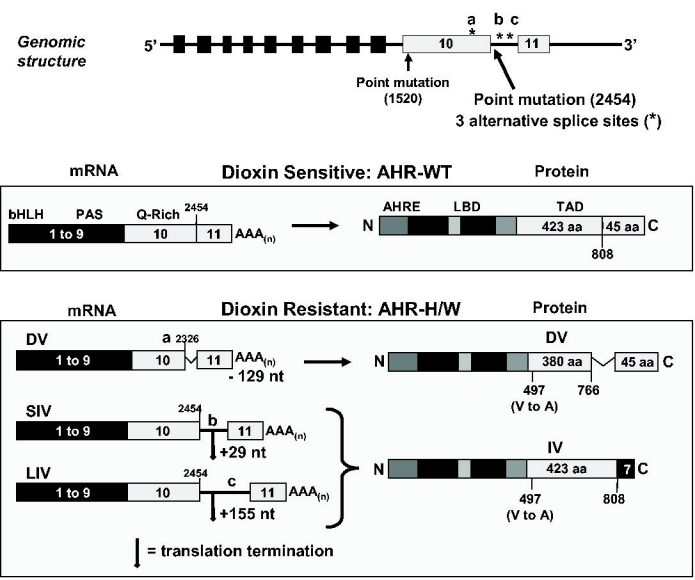

Genomic structure
5'
a
b c
10
11
3'
Point mutation (1520)
Point mutation (2454)
3 alternative splice sites (*)
mRNA
Dioxin Sensitive: AHR-WT
Protein
bHLH
PAS
Q-Rich
2454
1 to 9
10
11
AAA(n)
AHRE
LBD
TAD
N
423 aa
45 aa
C
808
mRNA
Dioxin Resistant: AHR-H/W
Protein
DV
a 2326
- 129 nt
380 aa
497
766
(V to A)
SIV
b
+29 nt
LIV
c
+155 nt
IV
7
= translation termination

Figure 1

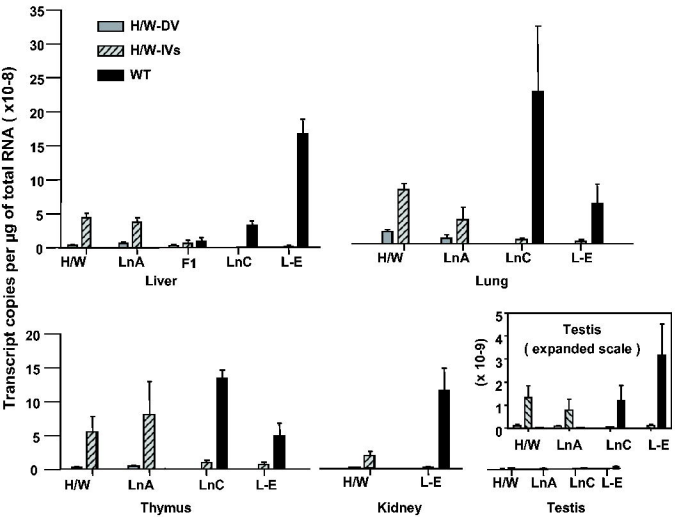

H/W-DV
H/W-IVs
WT
Transcript copies per µg of total RNA (x10-8)
Liver
Lung
Thymus
Kidney
Testis
Testis
(expanded scale)
(x 10-9)
H/W
LnA
F1
LnC
L-E

Figure 2

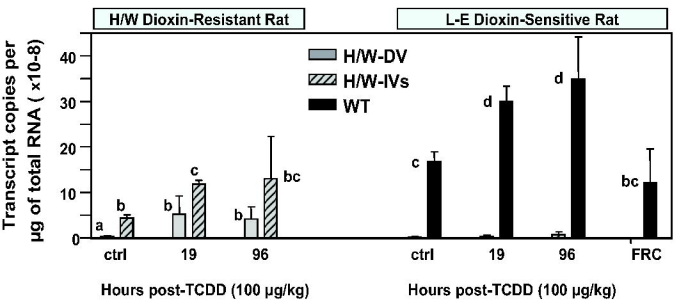

H/W Dioxin-Resistant Rat
L-E Dioxin-Sensitive Rat
H/W-DV
H/W-IVs
WT
Transcript copies per µg of total RNA (x10-8)
0
10
20
30
40
a
b
b
c
b
bc
c
d
d
bc
ctrl
19
96
ctrl
19
96
FRC
Hours post-TCDD (100 µg/kg)
Hours post-TCDD (100 µg/kg)

Figure 3

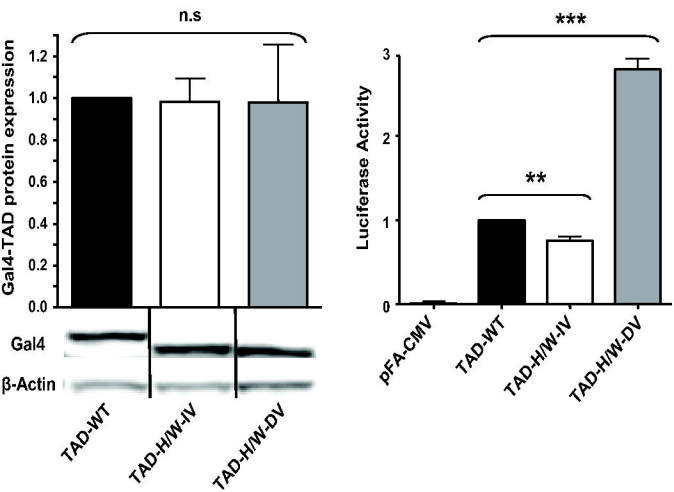
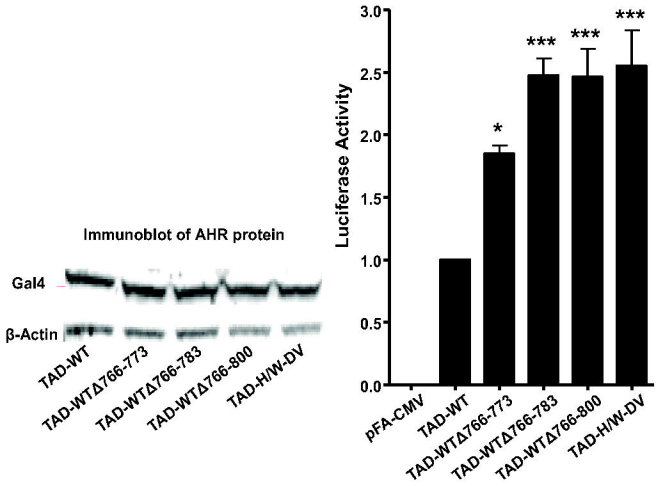

n.s
Gal4-TAD protein expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Gal4
β-Actin
TAD-WT
TAD-H/W-IV
TAD-H/W-DV
***
**
Luciferase Activity
3
2
1
0
pFA-CMV
TAD-WT
TAD-H/W-IV
TAD-H/W-DV

Figure 4



←MVSPQAYYAGAMSMYQCQAGPQHTPVDQMHYSPEIPGSQAFLSKF→ TAD-WT

←M GAMSMYQCQAGPQHTPVDQMHYSPEIPGSQAFLSKF→ TAD-WTΔaa766-773
766 773
VSPQAYYA

←M GPQHTPVDQMHYSPEIPGSQAFLSKF→ TAD-WTΔaa766-783
766 783
VSPQAYYAGAMSMYQCQA

←M GSQAFLSKF→ TAD-WTΔaa766-800
766 800
VSPQAYYAGAMSMYQCQAGPQHTPVDQMHYSPEIP

←M F→ TAD-H/W-DV
766 808
VSPQAYYAGAMSMYQCQAGPQHTPVDQMHYSPEIPGSQAFLSK

Figure 5

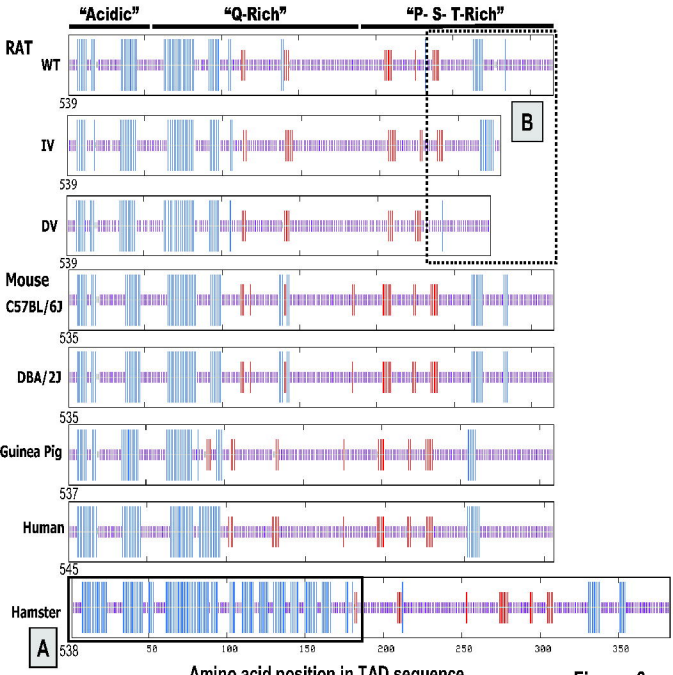

"Acidic"
"Q-Rich"
"P- S- T-Rich"
RAT
WT
539
B
IV
539
DV
539
Mouse
C57BL/6J
535
DBA/2J
535
Guinea Pig
537
Human
545
Hamster
A
538
50
100
150
200
250
300
350
Amino acid position in TAD sequence

Figure 6

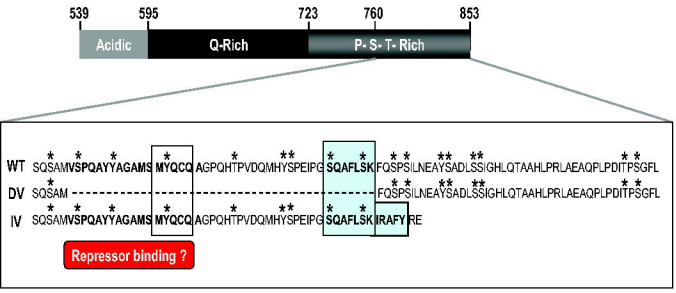

539
595
723
760
853
Acidic
Q-Rich
P- S- T- Rich
WT SQSAMVSPQAYYAGAMS MYQCQ AGPQHTPVDQMHYSPEIPG SQAFLSK FQSPSILNEAYSADLSSIGHLQTAAHLPRLAEAQPLPDITPSGFL
DV SQSAM FQSPSILNEAYSADLSSIGHLQTAAHLPRLAEAQPLPDITPSGFL
IV SQSAMVSPQAYYAGAMS MYQCQ AGPQHTPVDQMHYSPEIPG SQAFLSK IRAFY RE
Repressor binding ?

Figure 7