

# Point Mutation in Intron Sequence Causes Altered Carboxyl-Terminal Structure in the Aryl Hydrocarbon Receptor of the Most 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Resistant Rat Strain

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## ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent dioxin. There are exceptionally wide inter- and intraspecies differences in sensitivity to TCDD toxicity with Han/Wistar (H/W) (Kuopio) rats being the most resistant mammals tested. A peculiar feature of H/W rats is that despite their unresponsiveness to the acute lethality of TCDD, their sensitivity to other biological impacts of TCDD (e.g., CYP1A1 induction) is preserved. The biological effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR). We recently found that the AhR of H/W rats (about 98 kDa) is smaller than the receptor in other rat strains (106 kDa). In the present study, molecular cloning and sequencing of the H/W rat AhR revealed that the reason for its smaller size is a deletion/insertion-type change at the 3' end of

exon 10 in the receptor cDNA. This change emanates from a single point mutation at the first nucleotide of intron 10, resulting in altered mRNA splicing. At the protein level, the mutation leads to a total loss of either 43 or 38 amino acids (with altered sequence for the last seven amino acids in the latter case) toward the carboxyl-terminal end in the *trans*-activation domain of the AhR. H/W rats also harbor a point mutation in exon 10 that will cause a Val-to-Ala substitution in codon 497, but this occurs in a variable region of the AhR. These findings suggest that there is a relatively small region in the AhR *trans*-activation domain that may be capable of providing selectivity to its function.

TCDD and related aromatic hydrocarbons are important environmental toxicants. Because TCDD is the most potent compound in this chemical class, it has become the prototype for all others. In laboratory animals, TCDD brings about a wide spectrum of toxic or adaptive biochemical and morphological changes, including acute lethality associated with a wasting syndrome; tissue-dependent induction or inhibition of a variety of enzyme activities (the most extensively studied being induction of hepatic CYP1A1); endocrine perturbations; immune suppression coupled with thymic atrophy; lipid peroxidation; alterations in growth factors and cytokines and in their receptors; disturbed synthesis and degrada-

tion of heme; and terato- and carcinogenicity (for a review, see Pohjanvirta and Tuomisto, 1994).

The biological effects of TCDD are mediated by an intracellular protein, the AhR (Fernandez-Salquero *et al.*, 1995, 1996; Mimura *et al.*, 1997), which is a member of the bHLH-PAS protein family (for a review, see Okey *et al.*, 1994; Schmidt and Bradfield, 1996). The molecular mechanism of CYP1A1 induction by TCDD has been elucidated in detail. TCDD has the highest affinity of all ligands for the AhR (Safe, 1986). Upon binding TCDD, the AhR sheds its chaperones, two hsp90 molecules, along with other associated proteins and translocates into the nucleus. There it dimerizes with another type of bHLH protein, the ARNT, which is a common partner for several bHLH-PAS proteins (Gradin *et al.*, 1996; Probst *et al.*, 1997). The AhR-ARNT-TCDD complex then binds to DNA at one of several specific sites designated dioxin responsive elements acting as transcriptional enhancers (Whitlock, 1990). This binding induces transcription of

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**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; hsp90, 90-kDa heat-shock protein; H/W, Han/Wistar; H/Wdv, H/W deletion variant cDNA; H/Wliv, H/W longer insertion variant cDNA; H/Wsv, H/W shorter insertion variant cDNA; kb, kilobase pair(s); L-E, Long-Evans; NT, nucleotide; PAS, PER/ARNT/SIM (periodicity/aryl hydrocarbon receptor nuclear translocator protein/simple-minded); PCR, polymerase chain reaction; RT, reverse transcription; SD, Sprague-Dawley.

the gene *CYP1A1* and probably other genes under the regulation of the system by a mechanism not yet fully clarified but which may involve interactions with basal transcription factors (Rowlands *et al.*, 1996).

The bHLH domain is located at the amino terminus of the AhR protein and is responsible for DNA binding and also contributes to protein-protein heterodimerization. The PAS domain participates in dimerization and contains most of the ligand binding domain (Dolwick *et al.*, 1993). Toward the carboxyl terminus, there is a strong *trans*-activation domain consisting of several subdomains with synergistic action (Jain *et al.*, 1994; Ma *et al.*, 1995).

Regarding acute lethality in guinea pigs, TCDD is the most potent synthetic compound known with an LD<sub>50</sub> value of about 1 µg/kg (Schwetz *et al.*, 1973). However, other species are less susceptible; the other extreme, the hamster, tolerates > 1000-fold higher doses (Henck *et al.*, 1981). There are exceptionally wide differences in susceptibility also within species. Two of these, the divergence between inbred C57BL/6 and DBA/2 mice on the one hand and that between inbred L-E (*Turku AB*) and outbred H/W (*Kuopio*) rats on the other, have been exhaustively characterized and exploited as biological tools in mechanistic studies.

The sensitivity difference between C57BL/6 and DBA/2 mice is about 10-fold. This is true of acute lethality, CYP1A1 induction, a number of biochemical and histological changes, and teratogenicity (Poland and Knutson, 1982; Chapman and Schiller, 1985). The effects caused by TCDD in the less sensitive DBA/2 strain are qualitatively similar, but require a dose that is an order of magnitude higher to become manifest. In genetic crosses between these strains, sensitivity to AhR ligands is inherited as the dominant trait according to simple Mendelian inheritance for autosomal genes (Gielen *et al.*, 1972). The differential TCDD sensitivity reflects dissimilar AHRs in the two mouse strains. The AhR of the DBA/2 strain has a point mutation (alanine to valine) in its ligand binding domain in codon 375 resulting in markedly reduced binding affinity for TCDD (Ema *et al.*, 1994; Poland *et al.*, 1994); the apparent number of binding sites is also lower than that in C57BL/6 mice (Okey *et al.*, 1989).

The situation is considerably different in the rat model. The LD<sub>50</sub> value for L-E rats is only about 10 µg/kg, whereas that for H/W rats is >9600 µg/kg (Pohjanvirta *et al.*, 1993; Unkila *et al.*, 1994). Despite their tremendous resistance to the acute lethality of TCDD, H/W rats are nearly equal to L-E rats in sensitivity to many other biochemical and toxic effects of TCDD, such as induction of CYP1A1, decrease in serum thyroxine and melatonin levels, thymic atrophy and fetotoxicity (Pohjanvirta and Tuomisto, 1994). Also, in contrast to the mouse model, in genetic crosses between these rat strains resistance to TCDD was inherited as a dominant trait, with two or three genes probably involved (Pohjanvirta, 1990). More recent genetic studies have further implied that the *AhR* gene is the most important of these in terms of sensitivity to the acute lethality of TCDD (Tuomisto *et al.*, 1998).

Recent studies on the AHRs of these two rat strains revealed a notable size dissimilarity. The apparent molecular mass of the AhR, as assessed from Western blots, was 106 kDa in L-E (and also SD rats) but only about 98 kDa in H/W rats (Pohjanvirta *et al.*, 1998). Although the H/W rat AhR showed binding affinity to TCDD similar to that of L-E rat AhR under standard conditions, metal oxyanions (molybdate,

tungstate) as well as a high-ionic strength buffer markedly diminished or totally abolished ligand binding of the H/W rat AhR but had little effect on the L-E receptor.

In the present study, we sought to determine the reason for the small size and the physicochemically deviant nature of the H/W rat AhR by molecular cloning and sequencing of the entire coding region of the AhR cDNA. The AhR of the SD rat has previously been cloned (Elferink and Whitlock, 1994; Carver *et al.*, 1994), but for verification of the changes found, representative segments were also cloned and sequenced from L-E and SD rat samples.

## Materials and Methods

**Samples.** Male rats (female for SD) at the age of 13–14 weeks were used. For validation of the findings, additional male H/W rats that were 5 weeks old were studied. The rats were killed by decapitation. A piece of liver weighing approximately 1 g was rapidly removed by a sterile technique, flash-frozen in liquid nitrogen, and stored at –80° until analysis.

**RT-PCR.** The liver samples were homogenized while frozen and total RNA was isolated from the homogenate by the phenol-chloroform-guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) using TRIzol reagent (Life Technologies, Eggenstein, Germany). RT-PCR was performed with avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany) and either *Taq* (Pharmacia Biotech, Uppsala, Sweden) or AmpliTaq Gold (Perkin-Elmer-Roche, Branchburg, NJ, USA) DNA polymerase. Random primers were used for RT and the reaction was allowed to proceed at 42° for 30 min. For PCR, the primers were designed on the basis of a published sequence for SD rat AhR (Elferink and Whitlock, 1994). According to this paper, rat AhR cDNA has a 30-NT leader sequence, a 2562-NT coding region (NTs 31–2592) and a total size of 6.6 kb. DNA was amplified with Uno II thermocycler (Biometra, Göttingen, Germany). Thirty-five cycles were always run, but other PCR conditions were optimized for each primer pair. The coding region of the H/W rat AhR cDNA was obtained in five fragments (from 5' to 3' end: 2 or 31 to 839; 810 to 1660; 1561 or 1884 to 2609). The corresponding primers were as follows. Clone 1, 5'-CAGGTG-GAGCGGGCA-3' (forward), 5'-AGTGGAGTAGCTATTGCAAA-CAAAG-3' (reverse); clone 2, 5'-ATGAGCAGCGCGCCAACAT-CACCTAT-3' (f), 5'-AGTGGAGTAGCTATTGCAAAACAAAG-3' (r); clone 3, 5'-GTTGGCTTTGTTTGAATAGCTACT-3' (f), 5'-TC-CCTAGGTTTCTCATGATGCTATAC-3' (r); clone 4, 5'-CACACT-CAGGACGTGAACCTTA-3' (f); 5'-TTTCATCTGGCCTCGAGC-3' (r); clone 5, 5'-GCAGCTTCAGCAGCAGCA-3' (f), 5'-TTCATCTG-GCCTCGAGC-3' (r). A few additional fragments were cloned (e.g., to resolve compression regions).

To compare the cDNA versus genomic DNA structures of the AhR in H/W and L-E rats, we used antisense strand for the shorter insertion stretch isolated from H/W rats (see below) as the reverse primer in PCR. Poly(A)<sup>+</sup>-enriched RNA was purified from total RNA with oligo dT-coated latex beads (Oligotex midi; Qiagen, Hilden, Germany). RT-PCR was then carried out from 250 ng of poly(A)<sup>+</sup> mRNA by the Titan (Boehringer-Mannheim) system. The cycling conditions were: RT 50° for 30 min, PCR 94.8° for 2 min 20 sec; 94.8° for 50 sec, 57.5° for 50 sec, 68° for 50 sec × 10; 94.8° for 50 sec, 57.5° for 50 sec, 68° for 50 sec + 5 sec/cycle × 21.

**Genomic DNA.** To determine the primary structure of intron 10, liver pieces of about 25 mg were lysed with proteinase K and genomic DNA isolated as 30–50 kDa fragments by spin chromatography (QIAamp Tissue Kit; Qiagen). Intron 10 was then amplified by PCR with the primers 5'-GGTCAGTCCTCAGGCGTACTA-3' (f; 2325–2345) and 5'-GTCCAACCTCAGATTCT-3' (r; 2655–2637). The cycling conditions were: 94.8° for 2 min 20 sec; 94.8° for 1 min, 58.7° for 1 min, 72° for 2 min 40 sec × 35; 72° for 12 min.



**Cloning and sequencing.** The PCR products were cloned by blunt-end cloning into pCR-Script SK(+)-Amp plasmids with which XLB-1 supercompetent cells were transformed (Stratagene, La Jolla, CA). The plasmids were purified by Wizard Plus (Promega, Madison, WI) or Quantum Prep (Bio-Rad, Hercules, CA) miniprep systems. The inserts were sequenced with an A:L:F-automated DNA sequencer (Pharmacia Biotech) using either AutoRead sequencing kit (Pharmacia Biotech) or Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham, Cleveland, OH).

**Cloning strategy.** Each clone was first sequenced from an H/W rat. If any differences from the published SD rat receptor base order were detected (or if there were compression areas), the reciprocal strand of the DNA double helix was analyzed on the same site or, if the clone was too large for this, another fragment over the same region was amplified with slightly different primers. If the alteration was confirmed, this region was examined in another H/W rat. If an identical change was found, its strain specificity was assessed by cloning and sequencing this region from L-E and SD rats (intron 10 was only cloned from L-E and H/W rats). The entire 3' end (NTs 1561 to 2609) of the AhR protein coding region was cloned and sequenced from all three strains. As an extra measure, a large portion of the 5' end (NTs 127–839) was also cloned from L-E and SD rats. Thus, the sections analyzed for each strain were as follows: H/W, NTs 2–2609 and intron 10; L-E, NTs 127–2609 and intron 10; SD, NTs 127–839, 1561–2609.

**Southern blotting.** After separation in a 1% agarose gel, PCR-amplified DNA stretches were denatured by submerging the gel in 0.5N NaOH, 1.5 M NaCl solution for 30 min followed by neutralization with 0.5 M Tris-HCl, 3 M NaCl, pH 7.5, for another 30 min. The PCR products were then transferred from the gel onto a positively charged nylon membrane (Hybond+; Amersham) by capillary diffusion overnight. DNA was immobilized by baking the membrane at 120° for 30 min. Prehybridization and hybridization were accomplished in bottles in a rotisserie-equipped hybridization oven (Hybaid, Teddington, UK) at 68°. The probe used was the antisense strand for the shorter insertion of H/W rat AhR (see Results) labeled with digoxigenin at its 5' end. Detection was performed with the colorimetric detection reagents 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]ditetrazolium chloride/bromo-4-chloro-3-inodolyl phosphate (NBT/BCIP) according to manufacturer's instructions (Boehringer-Mannheim).

## Results

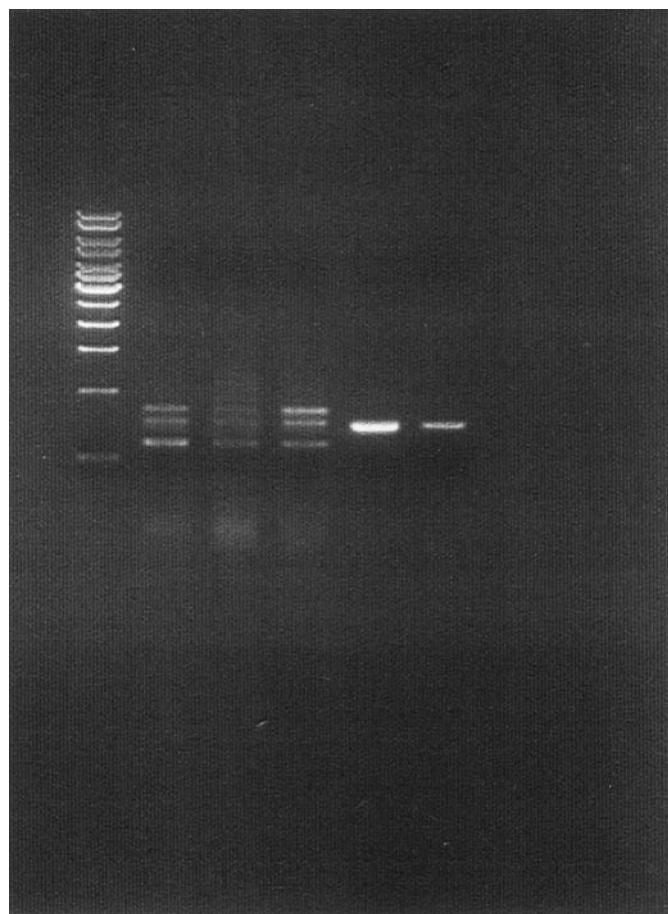
The 5' end of the H/W rat AhR, comprising the bHLH and PAS domains (NTs 109–276 and 358–1185, respectively), proved to be identical to that of the previously published sequence for SD rats (Elferink and Whitlock, 1994; Carver *et al.*, 1994). This was also true of the fragment (NTs 127–839) cloned from L-E and SD rats.

A transition mutation was recorded in H/W rats in the variable region flanking downstream the PAS domain. The second base of codon 497 (NT 1520) was altered from T to C, resulting in an amino acid change from valine to alanine at the protein level. This point mutation was strain specific, because it did not occur in L-E rats that had the same sequence published previously for SD rats.

A more conspicuous change was found toward the 3' end of the coding region of the cDNA. Although the reverse-transcribed RNA from L-E and SD rat livers always provided the expected single band in the subsequent PCR amplification, three distinct bands were constantly detected in the case of H/W rats (Fig. 1). One of the bands was about 100 bases smaller than the expected size. The other two were some 30 and 150 bases larger than expected. These cDNA stretches

were isolated, purified, and reamplified by PCR. They were then individually cloned and sequenced.

The bottom band turned out to contain a deletion of 129 bases (NTs 2326–2454). The ends of this deletion matched codon boundaries; therefore, it would not lead to a frame-shift in the translated protein, but only a loss of 43 amino acids (AAs 766–808). The middle band was the expected sequence with an insertion of 29 bases between NTs 2454 and 2455. It contained a termination codon (UAA) in its sequence and would give rise to an AhR protein with the last 45 carboxyl-terminal amino acids missing (AAs 809–853) and seven new ones added to its carboxyl terminus, for a total loss of 38 amino acids. The top band represented the expected DNA stretch with an insertion of 134 bases between the same NTs 2454 and 2455. At its 5' end, it encompassed the entire sequence of the smaller insertion. Because of the stop codon included, this mRNA would translate into the same protein as the 29-base insertion. We will call the three corresponding full-length cDNA structures in H/W rats H/Wdv, H/Wsiv, and H/Wliv for deletion variant, shorter insertion variant and longer insertion variant, respectively. The departures of the H/W rat AhR primary structure from that of SD and L-E rats are summarized in Fig. 2.

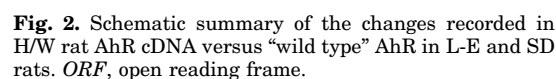


**Fig. 1.** PCR amplification products from the 3' end of the rat AH receptor coding region. The forward and reverse primers recognized bases 1884–1901 and 2609–2592, respectively, and thus the expected size of the PCR product was 726 nucleotides. Lane 1, 1 kb DNA molecular mass marker (MBI Fermentas, Vilnius, Lithuania); lanes 2–4, H/W rats; lane 5, SD rat; lane 6, Product 5 diluted 1:5 (to show the exact size). The rat samples used in PCR were RT products obtained with random primers from 1  $\mu$ g total RNA (2.5  $\mu$ l/50  $\mu$ l PCR reaction volume).

The two insertion fragments in H/W rat AhR cDNA proved to emanate from intron 10 (Fig. 5). Moreover, the intron 10 sequence was found to be highly homologous between L-E and H/W rats. In the 5' end, there was only one difference within the first 150 NTs: the first base, the invariant G, was substituted by A in H/W rats. At the 3' end, the first divergence was at NT 69. The total number of nonmatching bases was 6/485 and 6/439 for the 5' and 3' ends, respectively. Both

The two previous publications on the amino acid sequence of rat AhR differ from one another at one position, 794, which was reported to be histidine by Elferink and Whitlock (1994) for SD rats but glutamine by Carver *et al.* (1994) for Fisher 344 rats. At the corresponding cDNA NT position, 2412, we obtained G for all three strains of rat studied, including SD, instead of T reported by Elferink and Whitlock (1994). This G would convert the codon to that of glutamine. Therefore, the difference is probably attributable to an error in amplification or sequencing in the Elferink and Whitlock (1994) paper.

The H/W strain stands out from all other rat strains for its exceptional TCDD resistance. Previous studies have shown

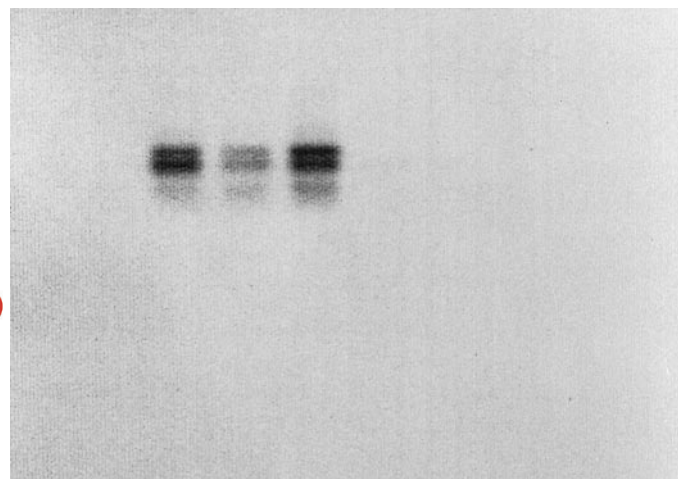




that this resistance is not due to altered overall kinetics of TCDD (Pohjanvirta *et al.*, 1990), which may contribute to the 5-fold sensitivity variation among substrains of Long-Evans rats (Viluksela *et al.*, 1996). Involvement of the AhR in the resistance was suggested earlier by two lines of evidence. Firstly, both strains were equally susceptible to the acute lethality of perfluorodecanoic acid (Unkila *et al.*, 1992), which causes a wasting syndrome similar to that of TCDD but does not act through the AhR. Secondly, the fold-difference in LD<sub>50</sub> values between the strains narrows in a step-by-step manner, with an increase in the number of chlorine atoms in the dioxin molecule, as follows: >1000, 320, 20, 10 for the most potent congeners of tetra-, penta-, hexa- and heptachlorodibenzo-*p*-dioxins, respectively (Pohjanvirta *et al.*, 1995).

In support of this line of reasoning, the AHRs of the two strains were found to have dissimilar molecular masses by immunoblotting (Pohjanvirta *et al.*, 1998). Very recent genetic crossing and molecular biological studies imply that the smaller size (~98 kDa) of H/W rat AhR [compared with that of L-E rat AhR (~106 kDa)] stems from the altered H/W rat AhR cDNA structure described here and that rats with the deviant AhR are about 50 times more resistant to TCDD lethality than those with the wild-type receptor (Tuomisto *et al.*, 1998; Pohjanvirta R, unpublished observations). However, the unresponsiveness of H/W rats to TCDD is peculiarly endpoint-dependent, and they fully exhibit many classic AhR-mediated effects, such as CYP1A1 induction (Pohjanvirta *et al.*, 1988). Recent studies further demonstrate that there is no discernible difference in the affinity of ligand binding between the AHRs of L-E and H/W rats, although receptor density is somewhat (2–3 times) higher in L-E rats; the ARNT proteins of the two strains are also similar in size (Pohjanvirta *et al.*, 1998).

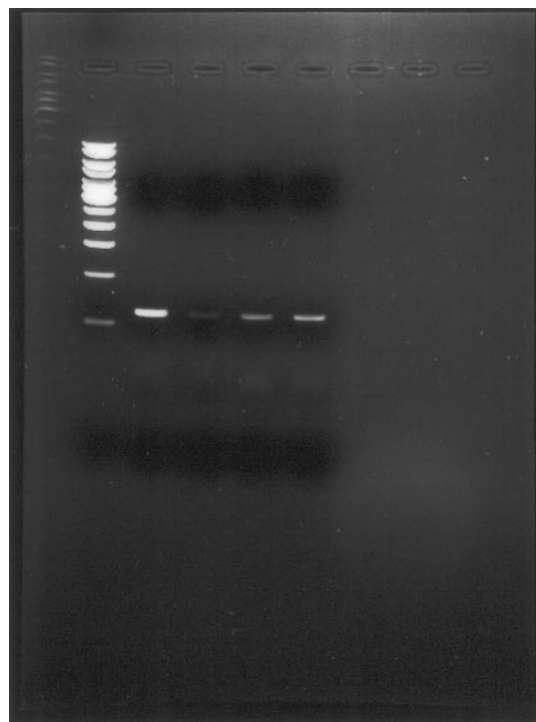
Collectively, the data gathered on H/W rats imply that their AhR is somehow deviant, but the domains responsible for ligand and DNA binding as well as heterodimerization are probably intact. In full accordance with this hypothesis, we now found that the entire 5' end of the H/W rat AhR cDNA encompassing these conserved domains is identical to that in other strains. This clearly distinguishes the rat model from its mouse counterpart at the molecular level, because the higher resistance to TCDD of DBA/2 mice compared with



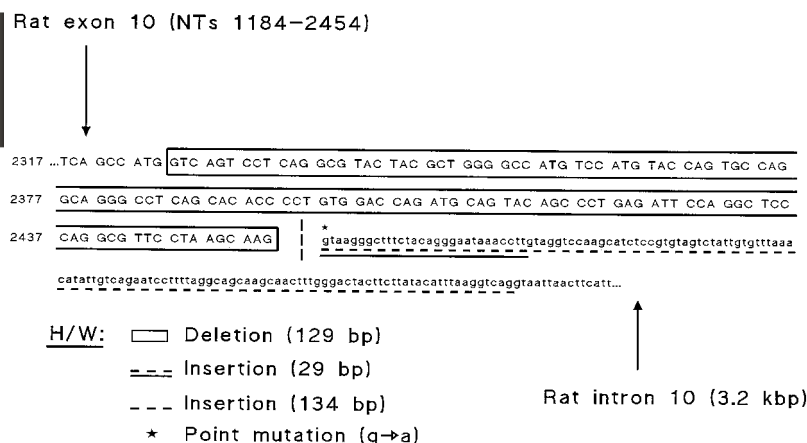
**Fig. 3.** Southern blot of the gel shown in Fig. 1. The probe used was digoxigenin-labeled antisense strand for the shorter insertion. The probe recognizes only H/W samples.

C57BL/6 mice results from reduced binding of TCDD to DBA/2 AHRs due to a single amino acid change (alanine to valine) within the ligand binding domain (Ema *et al.*, 1994; Poland *et al.*, 1994).

The segment that showed a striking departure from the normal rat AhR structure in H/W rats was the 3' end of the cDNA coding region. A transition mutation (G to A) in the first invariant NT of intron 10 abolishes the default exon/intron junction leading to the usage of three nearest cryptic splice sites. This in turn gives rise to a deletion and two insertions, the smaller insertion being the 5' end of the larger. At the protein level, the two insertions would yield the same product, an AhR variant that lacks the last 38 amino acids from its carboxyl terminus and has an altered sequence for seven amino acids in this end. The deletion would translate into a protein with 43 amino acids missing at a site 45 amino acids away from the carboxyl terminus. The calculated molecular masses of these deficiencies are 5445 (H/Wdv) and 4572 Da (H/Wsiv and H/Wliv). They are slightly smaller than the figure obtained for the size difference from immunoblots (~8000 Da), but the match is seldom perfect. For example, the calculated molecular mass of the SD rat AhR is 96,231 Da, but immunoblots consistently give a value of 106 kDa (Elferink and Whitlock, 1994; Pohjanvirta *et al.*, 1998), possibly reflecting aberrant mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The two predicted protein products in H/W rats are so close to one another in size that at the moment it is unknown whether both of them



**Fig. 4.** PCR amplification products from reverse-transcribed poly A<sup>+</sup> mRNA (lanes 2–3) or from genomic DNA (lanes 4–5). The reverse primer was digoxigenin-labeled antisense strand for the shorter insertion fragment from H/W rat AhR cDNA (the first 29 NTs of rat AhR intron 10 with a G-to-A mutation at the 5' end) and the forward primer was the 18-mer 1884–1901 from the rat AhR cDNA. Lane 1, 1 kb marker (MBI Fermentas); lanes 2 and 4, samples from the same H/W rat; lanes 3 and 5, samples from the same L-E rat. Note that the amplified segment contains the possible supernumerary exon/intron junction at 2325–2326.



**Fig. 5.** Primary structure of the 3' end of exon 10 and 5' end of intron 10 in the rat AhR gene. The deviations occurring in H/W rats are depicted.

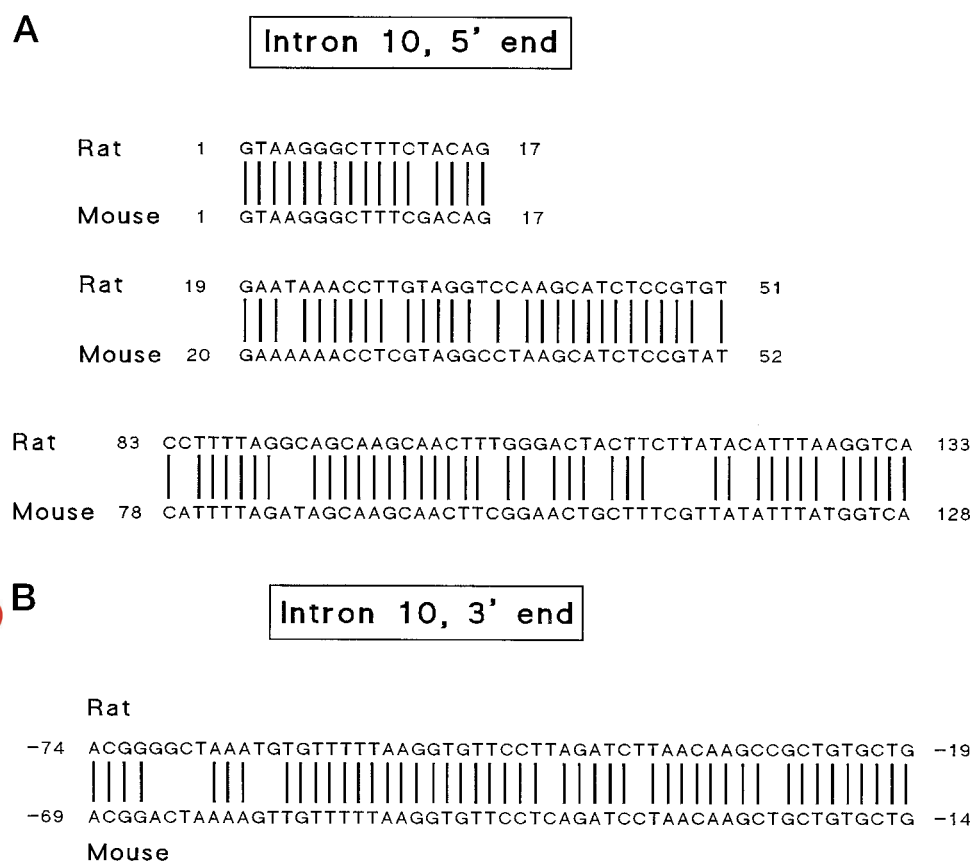
are really synthesized *in vivo*. Verifying this will require raising specific antibodies against the missing parts.

The insertion-based missing segment is rich in leucine (15.6%), alanine (13.3%) serine (13.3%) and proline (11.1%). The skipped part stemming from deletion is rich in glutamine (16.3%), alanine, proline and serine (all three 11.6%). They both belong to the *trans*-activation domain of the AhR (Jain *et al.*, 1994; Whitelaw *et al.*, 1994; Ma *et al.*, 1995), which may be reflected in the high glutamine content in the latter defect. Studies on the mouse AhR have revealed that the carboxyl-terminal end of the AhR actually consists of several subdomains with synergistic coefficients (Ma *et al.*, 1995). It is conceivable that the contribution of each subdomain to regulation of gene activity varies from gene to gene,

which might account for the selective responsiveness of H/W rats to TCDD.

Another mechanism for the unique pattern of responsiveness in H/W rats could be that the gene(s) responsible for acute lethality of TCDD are predominantly controlled by the AhR partner of the AhR/ARNT complex, whereas for some other dioxin-regulated genes (e.g. *CYP1A1*) the converse is true. In support of this hypothesis, by using AhR and ARNT chimeras, Whitelaw *et al.* (1994) showed that relative *trans*-activational potencies of the two partners varied with cell type and promoter architecture.

A third conceivable mechanism is based on the recent discoveries that the unliganded AhR is associated with the protein kinase c-Src, which becomes activated upon ligand



**Fig. 6.** Alignment for homology between rat and mouse intron 10 sequences at the 5' (a) and the 3' (b) ends. Lines, identical bases.

binding (Enan and Matsumura, 1996) and that c-Src deficient mice are resistant to many endpoints of TCDD toxicity including acute lethality (Matsumura *et al.*, 1997). c-Src is usually bound to hsp90 (Pratt, 1997), which makes contact with the AhR at two distinct sites within the bHLH and PAS domains (Perdew and Bradfield, 1996). The metal oxyanions molybdate and tungstate stabilize AhR ligand binding (Manchester *et al.*, 1987; Okey *et al.*, 1989), probably by the same mechanism as in the case of steroid receptors, strengthening the association between hsp90 and AhR (Pratt, 1997). We recently detected, however, that in H/W rats, these metal oxyanions surprisingly labilized the AhR (Pohjanvirta *et al.*, 1998), which suggests that the association between hsp90 and AhR may be altered in these rats. If this holds true, the activation of c-Src may also not take place in a normal manner in H/W rats. In this context, it is intriguing to note that c-src knockout mice, in spite of their resistance to the acute lethality of TCDD, are fully responsive to CYP1A1 induction (Matsumura *et al.*, 1997), as are H/W rats.

The biological significance of the exon 10 mutation is uncertain. The transition mutation at the second base of the codon causes an amino acid shift from valine to alanine at position 497. Because both of them are aliphatic, nonpolar, hydrophobic amino acids and the mutation occurs in an hypervariable region, it is not likely that this change would play a major role in the resistance of H/W rats to TCDD. In fact, the mouse AhR has alanine at the corresponding position.

The high degree of conservation of intron 10 sequences between rat and mouse was a surprising finding. Usually, the

only sites in introns showing evolutionary conservation are the splicing junctions (especially the dinucleotides GT...AG) and the branch site located near the 3' end (Lewin, 1994). For example, Hughes and Yeager (1997) studied 42 pairs of orthologous genes from rats and mice and found that the evolutionary rate in introns reflected the mutation rate. In light of this evidence, the 5' end homology of intron 10 is quite striking. It would be interesting to know whether this apparent conservation applies only to intron 10 or whether it is a general phenomenon in the AhR.

The example provided here by H/W rats demonstrates that intron sequences may have functional significance and should not be overlooked in genetic analyses. Krawczak *et al.* (1992) found that point mutations within splice sites represent some 15% of known point mutations causing human genetic disease. Mutations in the 5' splice site were much more frequent than in the 3' junction. Overall, 81% of all point mutations within the 5' splice site involved substitution of the intron first G residue, and in 64% of these cases, the substituting base was A, as in H/W rats.

In conclusion, the most TCDD-resistant rat strain, H/W, harbors two point mutations in the *AhR* gene that manifest as alterations in the AhR protein structure. One of them is in exon 10 and results in a change of a single amino acid within a variable region of the AhR. The other mutation is in the first invariant NT at the 5' end of intron 10 and leads to usage of cryptic splice sites to form 3 mRNA species but resulting in only two possible proteins. The variant H/Wdv uses a cryptic splice site between NTs 2325 and 2326 and results in an in-frame deletion at NT 2326 giving rise to a 43-amino acid deletion in exon 10 of the AhR protein. The variant H/Wsiv mRNA results from the use of a cryptic splice site at +29 NTs in intron 10 causing a 29-bp insertion that includes a termination codon (UAA), giving rise to an AhR protein with a total deletion of 38 amino acids. The variant H/Wliv mRNA results from the use of a cryptic splice site at +134 NTs in intron 10; since this includes the termination codon found in H/Wsiv, this mRNA translates into the same protein as for H/Wsiv mRNA. Thus there are two possible H/W AhR proteins deleted of either 43 or 38 amino acids (with altered sequence for the last seven amino acids in the latter case) near the end of the carboxyl terminus of the *trans*-activation domain. The functional consequences of these changes remain to be characterized.

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#### Exon/intron junctions

##### Consensus (5' end of intron)

-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
A/C	A/C	A	G	<b>G</b>	<b>T</b>	A	A	G	T
63%	73%	62%	77%	100%	100%	60%	74%	84%	50%
Exon				Intron					

##### "Wild type" (SD, L-E) junction

-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
C	A	A	G	<b>G</b>	<b>T</b>	A	A	G	g

##### Cryptic junction -129 (deletion)

-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
C	A	t	G	<b>G</b>	<b>T</b>	c	A	G	T

##### Cryptic junction +29 (smaller insertion)

-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
C	C	t	t	<b>G</b>	<b>T</b>	A	g	G	T

##### Cryptic junction +134 (larger insertion)

-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
t	C	A	G	<b>G</b>	<b>T</b>	A	A	t	T

**Fig. 7.** Splice site structures at the exon/intron 10 junction in comparison with the vertebrate consensus sequence (Padgett *et al.*, 1986). The relative frequencies of occurrence for NTs at each position in the consensus sequence are shown. **Bold**, invariant GT dinucleotide; *lower-case letters*, NTs deviating from the consensus sequence.



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