Biochemical and Biophysical Research Communications **273,** 272–281 (2000)
doi:10.1006/bbrc.2000.2931, available online at http://www.idealibrary.com on IDEAL®

# Restructured Transactivation Domain in Hamster AH Receptor

Merja Korkalainen,* Jouko Tuomisto,* and Raimo Pohjanvirta*,†,‡

**Laboratory of Toxicology, National Public Health Institute, P.O. Box 95, FIN-70701 Kuopio, Finland; †National Veterinary and Food Research Institute, Regional Laboratory of Kuopio, Kuopio, Finland; and ‡Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland*

Received May 19, 2000

**Hamsters and Han/Wistar (*Kuopio;* H/W) rats show peculiarly selective responsiveness to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). They are extremely resistant to its acute lethality but sensitive to, e.g., enzyme induction. The biological effects of TCDD are mediated by the AH receptor (AHR). Recent studies on H/W rat AHR discovered a remodelled transactivation domain which appears to be critical for the TCDD resistance of these animals. Here, molecular cloning and sequencing of hamster AHR reveals another type of restructured transactivation domain. In hamsters, the functionally pivotal Q-rich region is substantially expanded and enriched in glutamine compared with all other AHRs cloned to date. By contrast, the amino-terminal end is highly conserved, which is in agreement with the H/W rat AHR. Because of the additional material in the transactivation domain, hamster AHR protein is larger than that in rats or mice, but the pattern of AHR mRNA expression in tissues is similar.** 

***Key Words:*** **2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD; aryl hydrocarbon receptor; hamster; cloning; transactivation; species differences.**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototype for a class of halogenated aromatic hydrocarbons that are widespread and persistent environmental contaminants. Most of the biological effects of these compounds are mediated by the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, which structurally belongs to a newly discovered family of regulatory proteins: basic helix-loop-helix (bHLH)/PAS transcription factors (1).

The molecular mechanism of AHR action has so far been only elucidated for CYP1A1 induction, but this is believed to be a general mode of gene regulation by the AHR. In an inactive state, the AHR is located in the cytoplasm in a protein conglomerate along with two molecules of the chaperone hsp90, certain immunophilins and c-src. Binding of ligand such as TCDD results in translocation of the AHR into the nucleus and disruption of the complex. Inside the nucleus, the AHR dimerizes with a related bHLH/PAS protein, ARNT, and then binds to DNA at specific sites containing a consensus hexanucleotide core. These dioxin response elements act as enhancers for genes regulated by dioxins. Since the enhancer sites are usually situated relatively far upstream of the gene promoter, gene activation by dioxins probably involves nucleosomal disruption and interaction with transcriptional coactivators and/or corepressors (2).

The AHR protein consists of distinct functional modules. The bHLH domain located in the N terminus is responsible for DNA binding and heterodimerization. The PAS motif flanking the bHLH structure affords specificity to dimerization and also contains most of the ligand-binding domain. The C terminus comprises a potent transactivation domain composed of several interacting subdomains, one of which is a Q-rich subunit. This has been shown to be essential for the transactivation function of the AHR *in vitro* (3, 4).

Hamsters are highly resistant to the acute toxicity of TCDD. Compared with guinea pigs, they tolerate an over 1000-fold higher dose (5). The difference of the same magnitude exists between a TCDD-sensitive (Long-Evans, *Turku AB; L-E*) and resistant (Han/Wistar *Kuopio;* H/W) rat strain (6). However, despite their resistance hamsters and H/W rats are susceptible to certain biochemical and even toxic effects of TCDD including enzyme induction, thymus atrophy and fetotoxicity (7). Recent cloning of H/W rat AHR revealed changes in the architecture of the transactivation domain, which appear to be the principal reason for TCDD resistance in H/W rats (8, 9). Therefore, the primary structure of hamster AHR was determined to find out if TCDD resistance has a similar basis in hamsters and to gain better understanding of the role

0006-291X/00 $35.00

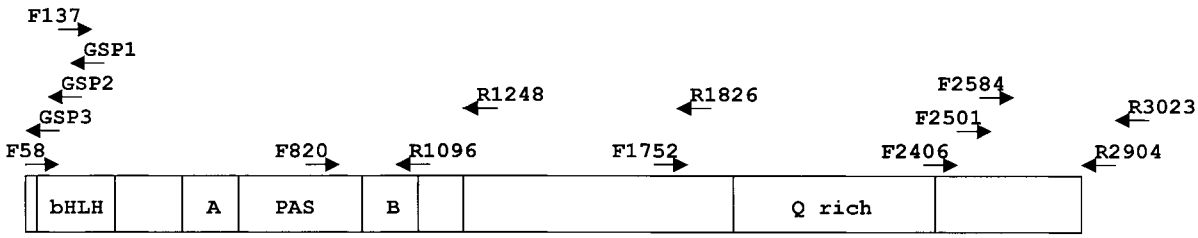


**FIG. 1.** Location of primers used in PCR for cloning the hamster AHR (F, forward, and R, reverse). The positions of the basic helix-loop-helix region (bHLH), PAS A and B repeat segments and the glutamine-rich subdomain (Q) are indicated.

of the AHR structure as a determinant of dioxin sensitivity.

## MATERIALS AND METHODS

*Animals.* Two golden Syrian hamsters, a male and a female, were purchased from Harlan Nederland (Horst, The Netherlands). The hamsters were killed by decapitation at the age of 4 weeks. The following tissues were rapidly removed, flash-frozen in liquid nitrogen, and stored at −80°C until analysis: liver, lung, heart, kidney, spleen, thymus, hypothalamus, testis and ovary.

*Primers.* The first primers (F137 and R1096) were selected from highly conserved regions of the AHR. Thereafter, one of the primers in subsequent PCR reactions was always chosen from the segment of AHR cDNA already resolved. The primers are shown in relation to the full-length AHR cDNA in Fig. 1. Their primary structures (5′-3′) were as follows: **Anchor-RT,** CCA-CCT-ACC-CAC-TAC-CCC-TCT-CAT-T(22)-V; **Anchor-R,** CCA-CCT-ACC-CAC-TAC-CCC-TCT-CA; **GSP1,** GCT-TGT-TAA-TAA-CAT-CTT-GTG; **GSP2,** TGT-CTC-TAT-GTC-GCT-TAG-AAG; **GSP3,** GGA-CTG-GCT-TCA-CTG-TTT-TCT-GCA; **Biotseq,** CTT-GCT-TAG-GAA-CGC-CTG-GGA; **Biotcomp,** CCC-AGG-CGT-TCC-TAA-GCA-A; **F2406,** CCC-GGC-GTT-CCA-AGT-GTG-CAC-A; **F2501,** TGT-TTA-CAA-GTT-CCT-GAA-AAC-C; **F2584,** CAT-GTC-CAT-GTA-YCA-GTG-CCA; **F137,** GCT-GAA-GGA-ATY-AAG-TCA-AA; **F820,** GAA-CAA-GAA-GGG-GAG-GAT-GGA; **R1096,** CAT-GCC-ACT-TTC-TCC-AGT-CTT-AAT-CAT; **R1248,** CAG-CGT-CAT-ACT-CCG-CTT-CTG; **F1752,** CAA-ACG-CAT-GCA-GAG-TGA-GGA; **R1826,** CAC-GTA-AGT-GAG-GAT-TTC-ATC-TG; **R2904,** TGG-ACA-GAC-AGT-CCC-GGT-ATC; **R3023,** CTC-TCC-CTA-TGC-TCC-TGC-AGA.

*RT-PCR for cloning.* The liver samples were homogenized and total RNA was isolated using Trizol reagent (Life Technologies, Eggenstein, Germany). Protein was isolated simultaneously (10). cDNA was synthesized with enhanced avian myeloblastosis virus reverse transcriptase (AMV-RT) (Sigma, St. Louis, MO) or with Omniscript reverse transcriptase (Qiagen, Hilden, Germany). For cDNA synthesis, either oligo-dT or random hexamers were used as primers in all cases except for cloning of the ends (see below). PCR was performed with DyNAzyme EXT DNA polymerase blend (Finnzymes, Espoo, Finland) and with either Uno II or TGradient thermocycler (Biometra, Göttingen, Germany). The "touchdown" method was applied throughout all reactions: the annealing temperature was set at about 5°C above the melting temperature of primers for the first cycle and then decreased by 1°C/cycle down to the desired final temperature (determined with the aid of the gradient feature of the TGradient thermocycler). A total of 35 cycles were usually run.

To obtain the 3′ end of the cDNA, an anchored primer (Anchor-RT) was employed in the RT phase followed by a total of 3 seminested PCR reactions (Anchor-R as reverse primer with 3 different forward primers: F2406, F2501 and F2584). The 5′ end was first pursued by a modified ligation-based method (11). In brief, RT was conducted with a gene specific primer (GSP1). cDNA was purified of the primer with the Qiaquick PCR Purification Kit (Qiagen). The RNA template was degraded by alkaline treatment (0.3N NaOH for 30 min at 50°C followed by neutralization with 0.3 M acetic acid for 30 min at 50°C). The single-stranded cDNA was concentrated with Pellet Paint co-precipitant (Novagen, Madison, WI). A special 29-base oligonucleotide (Biotseq) with a phosphate group at its 5′ end and the 3′ end OH-group blocked with biotin was ligated to the 3′ end of the cDNA with T4 RNA ligase (MBI Fermentas, Vilnius, Lithuania) (22°C for 16 h, 25°C for 6 h). Two seminested PCR reactions were then run with a constant forward primer (Biotcomp; complementary to Biotseq) and two nested gene specific reverse primers (GSP1 and GSP2). Since this method yielded a product suggestive of alternative splicing of exon 1 (see below), the result was verified with a different approach. A homopolymeric dA tail was added to the cDNA generated with the GSP1 primer using terminal deoxynucleotidyl transferase (MBI Fermentas). The first PCR reaction was performed with the Anchor-RT and GSP1 primers. This was followed by two seminested PCR reactions with Anchor-R as the forward primer and GSP2 or GSP3 as the reverse primer.

*Cloning and sequencing.* PCR products were cloned by blunt-end cloning into pCR-Script SK(+) Amp plasmid (Stratagene, La Jolla, CA) or pT7Blue-2 plasmid (Novagen). XLB-1 supercompetent cells were used in transformations (Stratagene). The plasmids were purified by Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The inserts were sequenced with an A.L.F. or A.L.Fexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) using either Thermo Sequenase fluorescent labelled primer cycle sequencing kit or Thermo Sequenase CY5 Dye Terminator Kit (Amersham Pharmacia Biotech). All ambiguities were resolved by auxiliary clonings. The entire coding region was cloned and sequenced from both the female and male hamster with identical results.

*Northern blotting.* Poly(A)+ -RNA was purified from total RNA with oligo dT-coated latex beads (Oligotex midi, Qiagen) and concentrated using Pellet Paint Co-Precipitant (Novagen). 2 $\mu$l 10 × MOPS, 10 $\mu$l formaldehyde and 3.3 $\mu$l formamide were added to 4.7 $\mu$l mRNA sample, denatured for 10 min at 70°C and chilled on ice. After electrophoresis in a 1.2% formaldehyde gel, the samples were transferred onto a positively charged nylon membrane (Hybond+, Amersham Pharmacia Biotech) by capillary diffusion overnight. Prehybridization and hybridization were accomplished in bottles in a rotisserie-equipped hybridization oven (Hybaid, Teddington, UK) at 42°C in ULTRAhyb hybridization solution (Ambion, Austin, TX). A rat oligo showing 100% homology to hamster AHR was 3′ end-labelled using digoxigenin-ddUTP and terminal transferase (MBI Fermentas). Detection was performed with the colorimetric detection reagents NBT/BCIP according to manufacture's instructions (Roche, Mannheim, Germany).

*Tissue specific expression.* RT-PCR was carried out from 60 ng or 200 ng of total RNA by RobusT RT-PCR Kit (Finnzymes). Sequence specific primers from exon 10 (F1752) and exon 11 (R2904) of the coding region of hamster AHR were used (spanning of intron 10

```
     GAGGCTCACCTGGGCGCCGCCTCCGGGCCGCAGGTGGAGCGGGCAGGGCGCGGGCACCATGAGCAGCGGC   70
1                                                                M  S  S  G

     GCCAACATCACCTACGCCAGCCGCAAGCGGCGCAAGCCGGTGCAGAAAACAGTGAAGCCAGTCCCCGCT   139
5     A  N  I  T  Y  A  S  R  K  R  R  K  P  V  Q  K  T  V  K  P  V  P  A

     GAAGGAATCAAGTCAAATCCTTCTAAGCGACATAGAGACAGGCTAAACACAGAGTTGGACCGCCTGGCT   208
28    E  G  I  K  S  N  P  S  K  R  H  R  D  R  L  N  T  E  L  D  R  L  A

     AGTCTGCTGCCCTTCCCACAAGATGTTATTAACAAGCTGGACAAACTCTCAGTTCTTAGGCTCAGTGTC   277
51    S  L  L  P  F  P  Q  D  V  I  N  K  L  D  K  L  S  V  L  R  L  S  V

     AGCTACCTGAGGGCCAAGAGCTTCTTCGATGTTGCGTTAAAATCCTCCCCCGCTGACAGGAATGGAGGC   346
74    S  Y  L  R  A  K  S  F  F  D  V  A  L  K  S  S  P  A  D  R  N  G  G

     CAGGAGCAGTGTAGAGCGTTCAGAGATGGACTGGACCTGCAAGAAGGAGAGTTCTTATTGCAGGCACTG   415
97    Q  E  Q  C  R  A  F  R  D  G  L  D  L  Q  E  G  E  F  L  L  Q  A  L

     AACGGCTTCGTACTGGTTGTCACAGCAGATGCCTTGGTCTTCTATGCCTCCTCTACTATCCAAGATTAC   484
120   N  G  F  V  L  V  V  T  A  D  A  L  V  F  Y  A  S  S  T  I  Q  D  Y

     CTGGGCTTTCAGCAATCTGATGTCATCCATCAGAGTGTCTATGAGCTCATCCATACCGAAGACCGAGCT   553
143   L  G  F  Q  Q  S  D  V  I  H  Q  S  V  Y  E  L  I  H  T  E  D  R  A

     GAGTTCCAGCGCCAGCTTCACTGGGCTCTAAACCCCGCACAGTGTACAGACTCTGCACAAGGAGGGGAT   622
166   E  F  Q  R  Q  L  H  W  A  L  N  P  A  Q  C  T  D  S  A  Q  G  G  D

     GAATCTCATGGCCTCTCACAGCCTCCGGCAGTCTACTTCAACCCAGACCAGCTTCCTCCGGAGAGCGCC   691
189   E  S  H  G  L  S  Q  P  P  A  V  Y  F  N  P  D  Q  L  P  P  E  S  A

     TCCTTCCTGGAGAGGTGCTTCATCTGCCGGCTCCGGTGTCTGCTGGATAATTCCTCTGGTTTTCTGGCA   760
212   S  F  L  E  R  C  F  I  C  R  L  R  C  L  L  D  N  S  S  G  F  L  A

     ATGAATTTCCAAGGGCGGCTAAAGTATCTCCATGGACAGAACAAGAAGGGGAAGGATGGAACACTACTG   829
235   M  N  F  Q  G  R  L  K  Y  L  H  G  Q  N  K  K  G  K  D  G  T  L  L

     CCCCCGCAGCTGGCTCTGTTTGCAATAGCCACCCCACTTCAGCCCCCGTCCATACTGGAAATCCGAACC   898
258   P  P  Q  L  A  L  F  A  I  A  T  P  L  Q  P  P  S  I  L  E  I  R  T

     AAAAACTTCATCTTCAGGACCAAACACAAACTAGACTTCACACCTATTGGTTGCGATGCCAAAGGGCAG   967
281   K  N  F  I  F  R  T  K  H  K  L  D  F  T  P  I  G  C  D  A  K  G  Q

     CTTGTCCTGGGCTACACGGAAGCGGAGCTGTGCACCCGAGGCTCGGGGTACCAGTTCATCCACGCTGCC  1036
304   L  V  L  G  Y  T  E  A  E  L  C  T  R  G  S  G  Y  Q  F  I  H  A  A

     GACATGCTCTACTGCGCGGAGTTCCACGTCCGCATGATTAAGACTGGGGAAAGCGGCATGACAGTTTTC  1105
327   D  M  L  Y  C  A  E  F  H  V  R  M  I  K  T  G  E  S  G  M  T  V  F

     CGGCTTCTGGCAAAGCACAGTCGATGGAGGTGGGTCCAGTCCAACGCTCGCTTGATCTATAGAAACGGG  1174
350   R  L  L  A  K  H  S  R  W  R  W  V  Q  S  N  A  R  L  I  Y  R  N  G

     AGGCCAGATTACATCATCGCAACTCAGAGGCCACTAACGGATGAAGAAGGAAGAGAGCACTTACAGAAG  1243
373   R  P  D  Y  I  I  A  T  Q  R  P  L  T  D  E  E  G  R  E  H  L  Q  K

     CGGAGTATGACGCTGCCCTTCATGTTCGCTACAGGAGAGGCTGTATTGTACGAGATCTCCAGCCCTTTC  1312
396   R  S  M  T  L  P  F  M  F  A  T  G  E  A  V  L  Y  E  I  S  S  P  F

     CCTCCCATAATGGATCCCTTGCCAATCCGTACCAAAAGCGGCACCGGTGCAAAGGACTGGGTTCCGCAG  1381
419   P  P  I  M  D  P  L  P  I  R  T  K  S  G  T  G  A  K  D  W  V  P  Q
```

**FIG. 2.** Nucleotide and deduced amino acid sequence of the hamster AHR cDNA. Amino acids are numbered on the left and nucleotides on the right. The termination codon is indicated with an asterisk (*). Repetitive DNA sequences and the possible alternative start site (see text) are underlined.

prevented amplification of genomic DNA). The 12.5 $\mu$l RT-PCR reaction contained 4 $\mu$l template, 2.5 pmoles of primers, 200 $\mu$M each dNTP, 1.25 units AMV reverse transcriptase, 0.5 units DyNAzyme EXT DNA polymerase, 1.5 mM $MgCl_2$ in RobusT reaction buffer. The cycling conditions were: 46°C for 60 min, 94.5°C for 2 min 25 sec; 94.5°C for 35 sec, 66°C (1°C decrement per cycle) for 40 sec; 72°C for 2 min $\times$ 7; 94.5°C for 35 sec, 59°C for 40 sec, 72°C for 2 min $\times$ 25; 72°C for 10 min.

*In vitro protein synthesis.* The full-length hamster AHR constructed by PCR was subcloned into a mammalian expression vector containing the expression cassette from pCI plasmid (Promega) in the polylinker of pCR-Script SK(+) AMP plasmid (Stratagene) with a kozak sequence incorporated immediately upstream the first ATG of the insert. *In vitro* transcription and translation were carried out using Single Tube Protein System3 (Novagen). 0.5 $\mu$g or 1 $\mu$g plasmid DNA was added to T7 transcription mix and incubated at 30°C for 30 min. Methionine and translation mix were added directly to transcription reaction and incubation was continued for 2 hours.

*Western blotting.* The *in vitro*-generated proteins as well as the hamster lung and liver protein fraction were analyzed by immuno-

```
     TCAACACCAAGCAATGATTCCCTCCACCCCAGCTCGCTTATGAATTGCATGATCCAACAGGATGAGTCC  1450
442   S  T  P  S  N  D  S  L  H  P  S  S  L  M  N  C  M  I  Q  Q  D  E  S

     ATCTATCTCTGTCCTCCTTCGAGTGCTGCGCCGCTAGACAGCCATTTTCTCACCCACGGGAGTGAAGGC  1519
465   I  Y  L  C  P  P  S  S  A  A  P  L  D  S  H  F  L  T  H  G  S  E  G

     GATGGTTGGCAGGACAGTATTGCATCAATAGGAAGTGAAGCTGAGTTGAAACATGAACAAATCGGACAT  1588
488   D  G  W  Q  D  S  I  A  S  I  G  S  E  A  E  L  K  H  E  Q  I  G  H

     GGTCAGGACATGAACCCTGCAGTCTCTGGAGGCCCCCCGGGGCTCTTTCCAGATAATAGAAATAGTGAC  1657
511   G  Q  D  M  N  P  A  V  S  G  G  P  P  G  L  F  P  D  N  R  N  S  D

     TTGTACAGCATCATGAAAAACCTAGGGATCGATTTCGATGACATCAAACGCATGCAGAGTGAGGAGTTC  1726
534   L  Y  S  I  M  K  N  L  G  I  D  F  D  D  I  K  R  M  Q  S  E  E  F

     TTCAGGACTGAGCTGGCTGGCGAGGTTGACTTCAGAGACATCGACATAACAGATGAAATCCTCACTTAC  1795
557   F  R  T  E  L  A  G  E  V  D  F  R  D  I  D  I  T  D  E  I  L  T  Y

     GTGCAAGACTCTCTAAACAGGTCGACCTTGCTGAGTTCTGCCAGCCAGCAGCAGCCTGTGACTCAGCAC  1864
580   V  Q  D  S  L  N  R  S  T  L  L  S  S  A  S  Q  Q  Q  P  V  T  Q  H

     CTGAGCTGCATGCTGCAGGAACGCCTGCATCTGGGGCAGCGGCAGCTGCAGCAGCACGAAACTCAGGCA  1933
603   L  S  C  M  L  Q  E  R  L  H  L  G  Q  R  Q  L  Q  Q  H  E  T  Q  A

     GCGGAGCCCCAGCAGCAGCTGGGTCATCAGACGGCGCCCCAGCAAGAGCTGTGTCACCAGACGGCGCCC  2002
626   A  E  P  Q  Q  Q  L  G  H  Q  T  A  P  Q  Q  E  L  C  H  Q  T  A  P

     CAGCAACAGATGTGTCTTCAGATGGCGCCCCAGCAAGAGCTGTGTCATCAGATGGAACCCCAACAGCAG  2071
649   Q  Q  Q  M  C  L  Q  M  A  P  Q  Q  E  L  C  H  Q  M  E  P  Q  Q  Q

     CTGTGTCTTCAGATGGCGCCCCAGCAGCAGCTGTGTCACCAGACGGCACCCCAGCAACAGCTGTGTCTT  2140
672   L  C  L  Q  M  A  P  Q  Q  Q  L  C  H  Q  T  A  P  Q  Q  Q  L  C  L

     CAGATGGCGCCCCAGCAAGAGCTGTGTCACCAGACGGCGCCCCAGCCGGAGCTGGGTCAGAAAATGAAT  2209
695   Q  M  A  P  Q  Q  E  L  C  H  Q  T  A  P  Q  P  E  L  G  Q  K  M  N

     CATGCGCAAGTCAATGGCATGTTTGCAAGTTGGAACCCCACCCCTCTCGTGCCTTTCAGCTGTCCTCAG  2278
718   H  A  Q  V  N  G  M  F  A  S  W  N  P  T  P  L  V  P  F  S  C  P  Q

     CAGGAACTGAAGCATTATGACGTCTTTTCAGACTTGCAGGGGGCGATTGAGGAGTTCCCCTACAAATCT  2347
741   Q  E  L  K  H  Y  D  V  F  S  D  L  Q  G  A  I  E  E  F  P  Y  K  S

     GAGATGGACAGTATGCCTTACACACAGAGCTTTGCTCCCTGTAATCAGTCTGTGCTACCCCAGCGTTCC  2416
764   E  M  D  S  M  P  Y  T  Q  S  F  A  P  C  N  Q  S  V  L  P  Q  R  S

     AAGTGTGCACAGCTGGACTTGCCTGGAAAGGGTTTCGAACCATCCCTGCACCCCAACACTTCTAATGTA  2485
787   K  C  A  Q  L  D  L  P  G  K  G  F  E  P  S  L  H  P  N  T  S  N  V

     GGAGATTTTGTCACTTGTTTACAAGTTCCCGAAAACCAAAGACACGAGGTGCATCCACAGTCGGCCATG  2554
810   G  D  F  V  T  C  L  Q  V  P  E  N  Q  R  H  E  V  H  P  Q  S  A  M

     GTCGCTCCTCAGACATACTATGCTGGGGCCATGTCCATGTACCAGTGCCAGCCAGGACCTCAGCACGTC  2623
833   V  A  P  Q  T  Y  Y  A  G  A  M  S  M  Y  Q  C  Q  P  G  P  Q  H  V

     CCTGTAGAGCAGATGCAGTACAGCCCTGCAGTCCCAGACTCCCAGGCATTTCTAAACAAGTTTCAGAAT  2692
856   P  V  E  Q  M  Q  Y  S  P  A  V  P  D  S  Q  A  F  L  N  K  F  Q  N

     CAGGGAGTGTTAAATGAAACCTATTCGTCTGAATTAAACAGCGTTGGTCACAGGCAGACCACTGCTCAT  2761
879   Q  G  V  L  N  E  T  Y  S  S  E  L  N  S  V  G  H  R  Q  T  T  A  H

     CTTCATCACCCTGCAGAAGGGAGGCCGTTTCCTGATATCACACCCAGCGGATTCCTGTAGCTTTGGGAA  2830
902   L  H  H  P  A  E  G  R  P  F  P  D  I  T  P  S  G  F  L  *

     CAAGATACCGGGACTGTCTGTCCATGTTGGCCATCAGCACTCTTTTTCCAAGCCAGATTTTAATCTTTG  2899
     TGTTGGGAAGAGGAGTTTAAAAACTGTTATCGGTTACCTATAAAATGGGAAGCTCTGCAGGAGCATAGG  2968
     GAGAGACCACAAGCCTTTAAGTAGCTTTAGCACCTCAGCGTGCACATGCCCACAGCGGAGAGCTTTGGG  3037
     GTTCTGGAGAGCTGAGCGTCTCCACTCAGTGACCAGCCAGAAAGACAACTTCAGGATTCCCGATGGTGT  3106
     ACCAAATACAGTTAGAAGACTAATTGATTTTAACCTCTCTGTGTCTCAGATGTTAGAATAAATGGTTTG  3175
     GTGCTTTTATCAAAAAAAAAAAA...
```

**FIG. 2**—*Continued*

blotting after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A 6% polyacrylamide minigel was run (Bio-Rad, Hercules, CA). The proteins were transfered onto a nitrocellulose membrane and then subjected to Western blot analysis using anti-AHR, rabbit polyclonal antibody (1:50,000) as a primary antibody (BioMol Research Laboratories, Plymouth Meeting, PA)

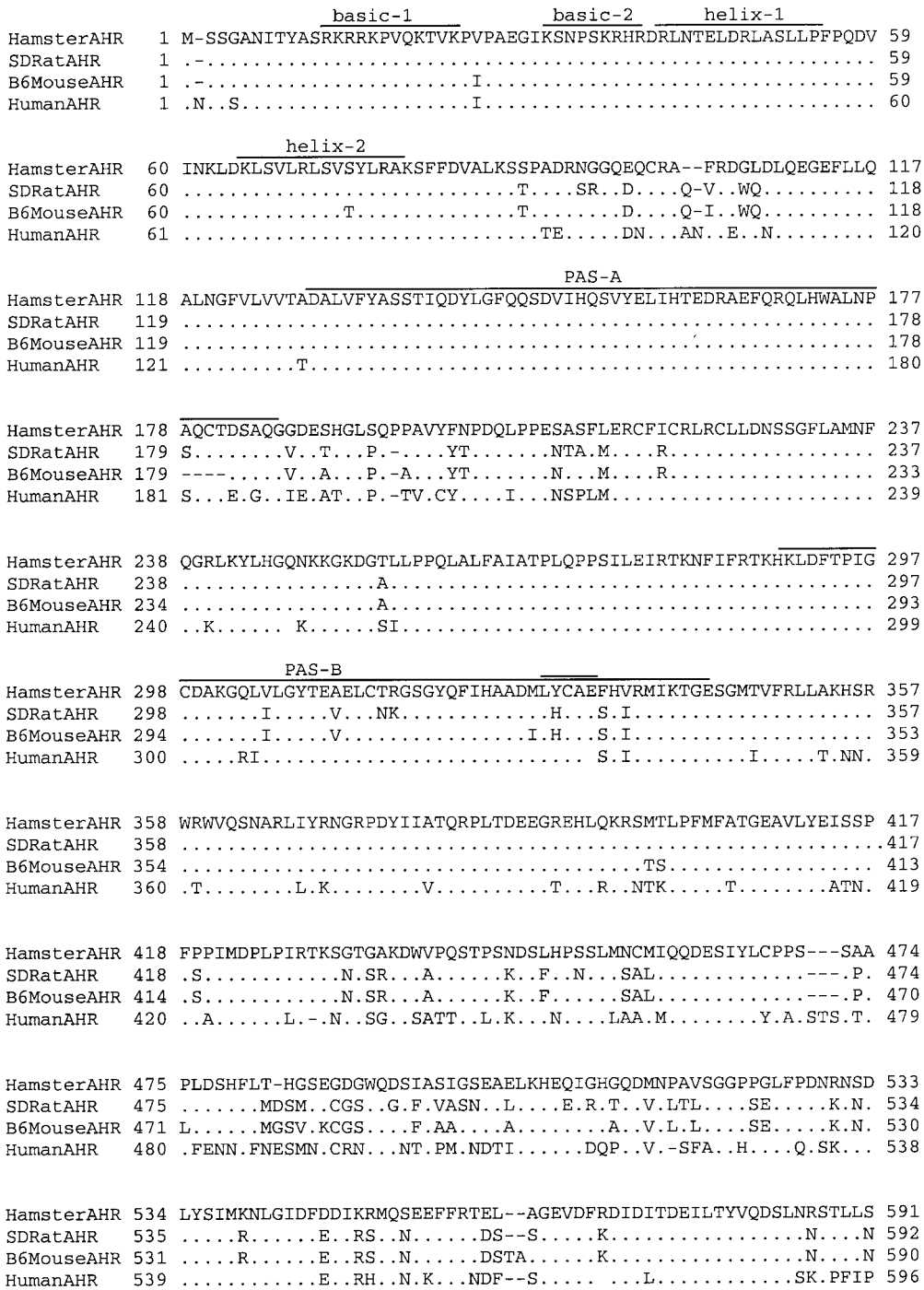


**FIG. 3.** Comparison of hamster, rat, mouse, and human AHR sequences. Dots indicate identities while dashed lines indicate gaps inserted to improve alignment. The basic helix-loop-helix region, PAS domain and glutamine (Q) rich domain are indicated by lines over the domain and LXCXE motif by a double line (20). The sequence alignment was performed using Clustal W (1.7). The GenBank Accession Numbers of the AHR sequences used are: Sprague-Dawley rat (U09000), C57BL/6 mouse (M94623), and human (L19872).

and an alkaline phosphatase-labelled anti-rabbit IgG as a second antibody (Roche). The bands were visualized with NBT/BCIP (Roche).

## RESULTS

The coding region of hamster AHR cDNA proved to contain 2763 nucleotides encoding a protein of 921 amino acids (Fig. 2). The calculated molecular weight was 7.1 kDa larger than the wild-type rat receptor, which was due to auxiliary material in the C-terminal transactivation domain. The cDNA contained 6 repetitive sequences of satellite-like DNA incorporated towards the 3′ end of the receptor (underlined in Fig. 2).

```
                                                    Q-rich
HamsterAHR 592 SASQQQP-VTQHLSCMLQERLHLGQRQLQQHETQAAEPQQQLGHQTAPQQELCHQTAPQQ 650
SDRatAHR   593 ..C....-.S..........Q.E.-------------....Q-.QH.T---------- 626
B6MouseAHR 591 ..C....-............Q.E.-------------....Q-.PPP---------- 623
HumanAHR   597 .DY...QSLALNS...V..H...E.-------------...HHQKQVV---------- 631

                                                    Q-rich
HamsterAHR 651 QMCLQMAPQQELCHQMEPQQQLCLQMAPQQQLCHQTAPQQQLCLQMAPQQELCHQTAPQP 710
SDRatAHR   627 -------------QTL...R...QVEV..H------------------------------ 643
B6MouseAHR 624 -------------QAL.......QMVC...------------------------------ 640
HumanAHR   632 ---------------V.......QK.K-------------------------------- 643

                                                    Q-rich
HamsterAHR 711 ELGQKMNHAQVNGMFASWNPTPLVPFSCPQQELKHYDVFSDLQGAIEEFPYKSEMDSMPY 770
SDRatAHR   644 .....TK.M...........A.P.S.......R...SL..G...TAQ.......V..... 703
B6MouseAHR 641 D..--PK.T.I..T........P.S.N.........QL..S...TAQ.......V..V.. 698
HumanAHR   644 --------.M......EN..SNQF...N....DPQQ.N..T..H.ISQ.............. 696

HamsterAHR 772 TQSFAPCNQSVLPQRSKCAQLDLPGKGFEPSLHPNTSNVGDFVTCLQVPENQRHEVHPQS 830
SDRatAHR   704 ..N.......L..EH..GT...F..RD..R.....A..LE...S..........GINS.. 763
B6MouseAHR 699 ..N......PL..EH..SV...F..RD.......T...LD-FVS........S.GINS.. 757
HumanAHR   697 ..N.IS...P....H...TE..Y.MGS....PY.T..SLE.......L....K.GLN... 756

HamsterAHR 832 AMVAPQTYYAGAMSMYQCQPGPQHVPVEQMQYSPAVPDSQAFLNKFQNQGVLNETYSSEL 890
SDRatAHR   764 ...S..A.............A....T..D..H...EI.G.....S...SPSI...A..AD. 823
B6MouseAHR 758 ...S..A................RT..D.T...SEI.G.....S.V.S---------- 805
HumanAHR   757 .IIT...C....V.......E...TH.G....N.VL.GQ.........-.......PA.. 815

HamsterAHR 892 NSVGHRQTTAHLH---HPAEGRPFPDITPSGFL 920
SDRatAHR   824 S.I..L..A...P---RL..AQ.L......... 853
B6MouseAHR 805 -------------------------------- 805
HumanAHR   816 .NINNT...T..QPLH..S.A.....L.S.... 848
```

**FIG. 3**—*Continued*

Thus the C-terminus of the translated protein would have some 60 extra amino acids compared with the corresponding region in the rat AHR (Fig. 3). More specifically, this change affected the Q-rich subdomain so that this region was much larger in hamster than in other AHRs cloned to date. The Q-rich region contains 49 glutamine residues in hamster, while the corresponding figure for rat, mouse, and human receptors is 28, 27, and 25, respectively. The amino acid sequences of rat, mouse and human AHRs were compared with the corresponding hamster sequence (Fig. 3). This analysis revealed a high degree of conservation in the amino terminal half of the hamster receptor including the basic helix-loop-helix and PAS domains. The amino acid identity was lowest in the C-terminal transactivation domain (Table 1). The overall amino acid identity to the rat, mouse and human receptors was 70%, 82% and 61%, respectively.

The mRNA size of hamster AHR was determined by Northern blot analysis (Fig. 4). It turned out to be larger than in rat, approximately slightly over 7 kb as compared with 6.6 kb in rat (12). Based on Northern blot data, the UTR we resolved by 3′ race-PCR was only partial (expected size ca. 4.2 kb), probably due to a short stretch of successive adenines in the UTR resulting in unspecific annealing of the anchor primer.

The tissue distribution of the hamster AHR was studied by the RT-PCR method (Fig. 5). Liver, lung and thymus expressed the highest levels of AHR, with lower levels observed in hypothalamus, heart, kidney and spleen. To demonstrate the ability of hamster AHR cDNA to encode protein, we used *in vitro* transcription/translation system to make translation products (Fig. 6). Although the yield was low, the apparent molecular

**TABLE 1**

Identities of AHR Functional Domains

| Domain | Hamster vs SD rat % | Hamster vs C57 mouse % | Hamster vs human % |
|---|---|---|---|
| DNA binding (9-20, 27-39, 220) | 100 | 100 | 100 |
| NLS (13-39) | 100 | 96.3 | 96.3 |
| NES (55-75) | 100 | 95.2 | 100 |
| HSP90 binding (27-79, 185-378) | 92.3 | 92.7 | 87.4 |
| Dimerization (40-79, 120-293) | 93.9 | 92.5 | 88.8 |
| pRB binding (~330; *LXCXE*) | 100 | 100 | 100 |
| Transactivation (493-920) | 60.0 | 52.1 | 51.2 |

*Note.* Numbering is based on the hamster sequence. NLS, nuclear localization signal; NES, nuclear export signal (35); pRb binding, binding site for the retinoblastoma tumor suppressor protein (20).

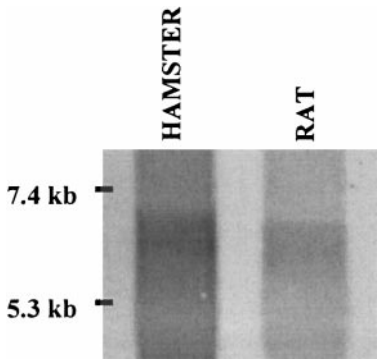


**FIG. 4.** Northern blot analysis of hamster and rat AHR. Poly-A$^+$ RNA was isolated from 250 $\mu$g of total RNA from liver.

mass of the translated protein (the highest band) was similar to the protein recognized by the anti-AHR antibody in hamster liver and lung samples being about 120 kDa. The AHR in H/W and L-E rats was notably smaller (98 kDa and 106 kDa, respectively).

The 5′ end of the hamster AHR mRNA exhibited surprising variation. The expected 5′ structure was elicited only in the case that the reverse primer in the final PCR reaction was selected 5′ to the junction of exon/intron 1 (nucleotides 120/121 in hamster cDNA), as inferred from the organization of the mouse AHR gene (13). If the reverse primer site was located downstream this junction, typical intron structure complete with the invariant 3′ AG dinucleotide was obtained 5′ to the junction. Both independent methods used yielded the same result, although the number of the probably intron-derived nucleotides preceding the junction differed slightly (28 by ligation, 18 by tailing).

## DISCUSSION

There are large inter- and intraspecies differences in acute TCDD toxicity. The classical strain difference exists between two mouse strains, the sensitive C57BL/6 and the resistant DBA/2 strains, where the sensitivity difference is about tenfold. DBA/2 strain has a point mutation in the ligand binding domain of AHR, which results in markedly reduced binding affinity for TCDD (14, 15). The sensitive L-E and resistant H/W rats show the largest intraspecies variation (*ca.* 1000-fold) in TCDD lethality. Recent cloning of H/W rat AHR cDNA revealed a critical point mutation in the first invariant nucleotide of intron 10 leading to altered splicing of the mRNA. This gives rise to two different proteins, both of which bear unique transactivation domains (8). Genetic studies implied that the altered AHR is the major determinant of TCDD resistance in H/W rats (9). The largest interspecies difference in acute TCDD toxicity exists between guinea pig and hamster. Guinea pig is the most sensitive mammal with an LD50 value of *ca.* 1 $\mu$g/kg, while hamster is extremely resistant with an LD50 value of about 3000 $\mu$g/kg (16, 5).

Hamsters and H/W rats share a number of features, e.g., both are extremely resistant to the acute lethality of TCDD, but sensitive to CYP1A1 induction by TCDD, the mediation of which is the best-known function of AHR. The present findings disclose that similar to H/W rat AHR, hamster also has an altered C-terminal end in its AHR. In H/W rats, mutations lead to two kinds of receptor proteins, which have shortened transactivation domains. In hamsters, the Q-rich subunit of the transactivation domain is strikingly larger than in rats, mice and humans because of incorporation of short DNA repeats. This repetetive sequence contains codons for glutamine in the reading frame and thus it increases the number of glutamine residues. The Q-rich region is thought to play an important role in mediating transactivation by the AHR (4). In some *in vitro* studies, it has been shown to exhibit the strongest transcriptional activity of several activation subdo-

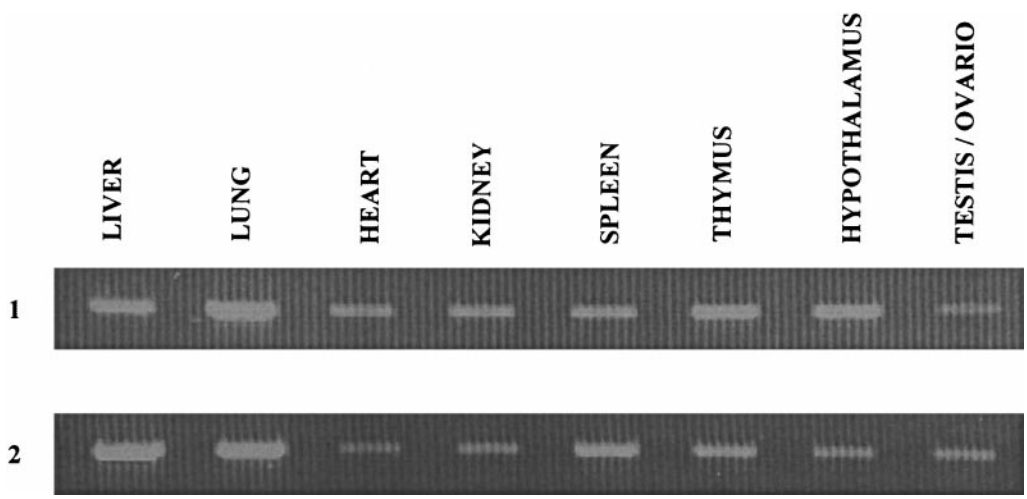


**FIG. 5.** Tissue distribution of hamster AHR gene expression. Lane 1, male hamster and lane 2, female hamster.

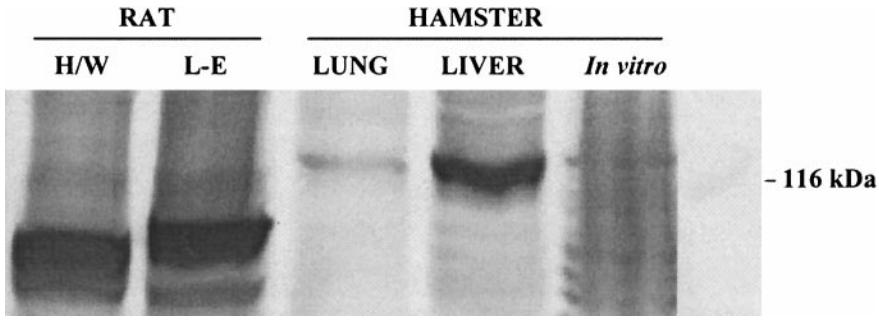


**FIG. 6.** Western blot analysis of AHR.

mains in the C-terminal half of the AHR sequence (17). In other studies, the transactivation potency of the Q-rich subdomain as such has proved to be low, but it has been able to remarkably augment functions of other subunits in this domain (18). A completely different function is exerted in the Krüppel protein of *Drosophila*, where the Q-rich region is reported to be crucial for transactivation repression (19). It is also noteworthy that an important regulator of cell cycle, the retinoblastoma tumor suppressor protein, has recently been found to be associated with the AHR, and the Q-rich subdomain is one of the two sites at which it makes contact with the AHR (20). Interestingly, the Q-rich structure is lacking in the AHR of some extremely TCDD-sensitive teleost fish such as rainbow trout (21). In contrast, a fish species, *Fundulus heteroclitus*, which is sensitive to dioxins but capable of developing dioxin resistance after multigenerational exposure, has been reported to possess a Q-rich subdomain in one of its two AHRs (22).

The evidence obtained of alternative splicing of hamster AHR mRNA at its 5′ end was totally unexpected because it is unprecedented in all other species studied hitherto. Since two independent methods relying on entirely different primer structures provided essentially the same result, it is unlikely to be due to contamination with genomic DNA. The more probable explanation is skipping of exon 1 in mRNA processing. If the alternative transcript is translated, the first ATG in frame with the wild-type start codon is at nucleotide 761. Usage of this codon as the translational start site would generate a protein 134 amino acids smaller than the full-length AHR and devoid of the critical N-terminal bHLH structure. However, the neighbor nucleotides of this codon do not favor efficient translation (23). Furthermore, although hamsters are resistant to acute lethality of TCDD, they exhibit the typical AHR-mediated phenomenon, P450 induction (24). Hamster AHR is also fully able to bind to its cognate response element in DNA (25). Therefore, alternative splicing of AHR mRNA at the 5′ end may be a peculiarity specific for hamster but of little, if any, functional consequence.

There are differences in molecular weights of AHR protein among species and also among strains in a given species. The hamster receptor is one of the largest studied (26, 27). Although the calculated molecular mass is 103.3 kDa, Western analysis yielded a size of approximately 120 kDa, which is in accordance with earlier findings (26). This difference between the calculated size and the mobility of protein has been observed previously for rat, mouse and human AHR and possibly reflects aberrant mobility during SDS–PAGE electrophoresis or post-translational modifications of proteins (8, 28, 29). The mRNA of hamster AHR was also larger than that of the rat (~7 kb vs ~6.6 kb) based on Northern analysis. Provided that we managed to clone the full leader sequence, the 3′ untranslated region constitutes the bulk of the mRNA paralleling the situation in the rat. The tissue distribution of *AHR* gene expression resembled that in rats (12, 30), mice (31) and humans (28).

The N-terminal end of the hamster AHR containing the bHLH and the PAS domains is highly conserved. This high degree of homology is not surprising since the bHLH domain has been shown to mediate DNA binding and the PAS region is important in ligand binding and AHR/ARNT dimerization (32, 33). It is obvious that the reason for dioxin resistance in hamsters cannot be found in this region. In contrast, the hamster AHR stands out from all other AHRs cloned to date for its C-terminal structure, especially for the expanded Q-rich subdomain. This could conceivably account for the peculiarly selective responsiveness of hamsters to TCDD if one assumes promoter context-dependent interplay between the transactivation domains of AHR and ARNT (34). However, further studies are needed to elucidate the functional significance of the structural alteration found.

## ACKNOWLEDGMENTS

This study was financially supported by grants from the Finnish Research Program for Environmental Health (SYTTY, the Academy of Finland, Research Council for Health, Grant 42386), the Academy of Finland, Research Council for Health (Grant 43984), and the European Commission (Contract QLK4-1999-01446).

## REFERENCES


1. Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252,** 954–958.
2. Rowlands, J., and Gustafsson, J.-Å. (1997) Aryl hydrocarbon receptor-mediated signal transduction. *CRC Crit. Rev. Toxicol.* **27,** 109–134.
3. Fukunaga, B. N., Probst, M. R., Reisz-Porszasz, S., and Hankinson, O. (1995) Identification of functional domains of the aryl hydrocarbon receptor. *J. Biol. Chem.* **270,** 29270–29278.
4. Jain, S., Dolwick, K. M., Schmidt, J. V., and Bradfield, C. A. (1994) Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxyl termini. *J. Biol. Chem.* **269,** 31518–31523.
5. Henck, J. M., New, M. A., Kociba, R. J., and Rao, K. S. (1981) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: acute oral toxicity in hamsters. *Toxicol. Appl. Pharmacol.* **59,** 405–407.
6. Pohjanvirta, R., Unkila, M., and Tuomisto, J. (1993) Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. *Pharmacol. Toxicol.* **73,** 52–56.
7. Pohjanvirta, R., and Tuomisto, J. (1994) Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in laboratory animals: Effects, mechanisms, and animal models. *Pharmacol. Rev.* **46,** 483–549.
8. Pohjanvirta, R., Wong, J. M. Y., Li, W., Harper, P. A., Tuomisto, J., and Okey, A. B. (1998) Point mutation in intron structure causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain. *Mol. Pharmacol.* **54,** 86–93.
9. Tuomisto, J. T., Viluksela, M., Pohjanvirta, R., and Tuomisto, J. (1999) The Ah receptor and a novel gene determine acute toxic responses to TCDD: Segregation of the resistant alleles to different rat lines. *Toxicol. Appl. Pharmacol.* **155,** 71–81.
10. Chomczynski, P. (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15,** 532–537.
11. Dumas, J. B., Edwards, M., Delort, J., and Mallet, J. (1991) Oligodeoxyribonucleotide ligation to single-stranded cDNAs: A new tool for cloning 5′ ends of mRNAs and for constructing cDNA libraries by *in vitro* amplification. *Nucleic Acids Res.* **19,** 5227–5232.
12. Elferink, C. J., and Whitlock, J. P., Jr. (1994) Dioxin-dependent, DNA sequence-specific binding of a multiprotein complex containing the Ah receptor. *Receptor* **4,** 157–173.
13. Schmidt, J. V., Carver, L. A., and Bradfield, C. A. (1993) Molecular characterization of the murine *Ahr* gene. Organization, promoter analysis, and chromosomal assignment. *J. Biol. Chem.* **268,** 22203–22209.
14. Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., and Fujii-Kuriyama, Y. (1994) Dioxin binding activities of polymorfic forms of mouse and human aryl hydrocarbon receptors. *J. Biol. Chem.* **269,** 27337–27343.
15. Poland, A., Palen, D., and Glover, E. (1994) Analysis of the four alleles of the murine aryl hydrocarbon receptor. *Mol. Pharmacol.* **46,** 915–921.
16. Olson, J. R., Gasiewicz, T. A., and Neal, R. A. (1980) Tissue distribution, excretion, and metabolism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the hamster. *Toxicol. Appl. Pharmacol.* **56,** 78–85.
17. Sogawa, K., Iwabuchi, K., Abe, H., and Fujii-Kuriyama, Y. (1995) Transcriptional activation domains of the Ah receptor and Ah receptor nuclear translocator. *J. Cancer Res. Clin. Oncol.* **121,** 612–620.
18. Ma, Q., Dong, L., and Whitlock, J. P., Jr. (1995) Transcriptional activation by the mouse Ah receptor. Interplay between multiple stimulatory and inhibitory functions. *J. Biol. Chem.* **270,** 12697–12703.
19. Licht, J. D., Hanna-Rose, W., Reddy, J. C., English, M. A., Ro, M., Shaknovich, R., and Hansen, U. (1994) Mapping and mutagenesis of the amino-terminal transcription repression domain of the *Drosophila* Krüppel protein. *Mol. Cell. Biol.* **14,** 4057–4066.
20. Ge, N.-L., and Elferink, C. J. (1998) A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. *J. Biol. Chem.* **273,** 22708–22713.
21. Abnet, C. C., Tanguay, R. L., Hahn, M. E., Heideman, W., and Peterson, R. E. (1999) Two forms of aryl hydrocarbon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*). *J. Biol. Chem.* **274,** 15159–15166.
22. Karchner, S. I., Powell, W. H., and Hahn, M. E. (1999) Identification and functional characterization of two divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitus*. *J. Biol. Chem.* **274,** 33814–33824.
23. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266,** 19867–19870.
24. Henry, E. C., and Gasiewicz, T. A. (1987) Changes in thyroid hormones and thyroxine glucuronidation in hamsters compared with rats following treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **89,** 165–174.
25. Bank, P. A., Yao, E. F., Phelps, C. L., Harper, P. A., and Denison, M. S. (1992) Species-specific binding of transformed Ah receptor to a dioxin responsive transcriptional enhancer. *Eur. J. Pharmacol.* **228,** 85–94.
26. Poland, A., and Glover, E. (1987) Variation in the molecular mass of the Ah receptor among vertebrate spacies and strains of rats. *Biochem. Biophys. Res. Commun.* **146,** 1439–1449.
27. Poland, A., Glover, E., and Bradfield, C. A. (1990) Characterization of polyclonal antibodies to the Ah receptor prepared by immunization with a synthetic peptide hapten. *Mol. Pharmacol.* **38,** 20–26.
28. Dolwick, K. M., Schmidt, J. V., Carver, L. A., Swanson, H. I., and Bradfield, C. A. (1993) Cloning and expression of a human Ah receptor cDNA. *Mol. Pharmacol.* **44,** 911–917.
29. Burbach, K. M., Poland, A., and Bradfield, C. A. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89,** 8185–8189.
30. Carver, L. A., Hogenesch, J. B., and Bradfield, C. A. (1994) Tissue specific expression of the rat Ah-receptor and ARNT mRNAs. *Nucleic Acids Res.* **22,** 3038–3044.
31. Li, W., Donat, S., Döhr, O., Unfried, K., and Abel, J. (1994) Ah receptor in different tissues of C57BL/6J and DBA/2J mice: Use of competitive polymerase chain reaction to measure AH-receptor mRNA expression. *Arch. Biochem. Biophys.* **315,** 279–284.
32. Dolwick, K. M., Swanson, H. I., and Bradfield, C. A. (1993) *In vitro* analysis of Ah receptor domains involved in ligand-activated DNA recognition. *Proc. Natl. Acad. Sci. USA* **90,** 8566–8570.
33. Whitelaw, M. L., Göttlicher, M., Gustafsson, J.-Å., and Poel-

linger, L. (1993) Definition of a novel ligand binding domain of a nuclear bHLH receptor: Co-localization of ligand and hsp90 binding activities within the regulable inactivation domain of the dioxin receptor. *EMBO J.* **12,** 4169–4179.

34. Whitelaw, M. L., Gustafsson, J.-Å., and Poellinger, L. (1994) Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor ARNT: Inducible versus constitutive modes of regulation. *Mol. Cell. Biol.* **14,** 8343–8355.
35. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1998) Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem.* **273,** 2895–2904.