A Mutation in the Aryl Hydrocarbon Receptor (AHR) in a Cultured Mammalian Cell Line Identifies a Novel Region of AHR That Affects DNA Binding*

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Introduction of a retroviral expression vector for the aryl hydrocarbon receptor (AHR) restores CYP1A1 inducibility to a mutant derivative of the Hepa-1 cell line that is defective in induction of CYP1A1 by ligands for the receptor. An AHR protein with normal ligand binding activity is expressed in the mutant but ligand treatment of mutant cell extract fails to induce binding of the AHR-ARNT (aryl hydrocarbon receptor nuclear translocator) dimer to the xenobiotic responsive element (XRE). AHR cDNAs derived from the mutant encode a protein that is unimpaired in ligand-dependent dimerization with ARNT, but the AHR-ARNT dimer so formed is severely impaired in XRE binding activity. The mutant cDNAs contain a C to G mutation at base 648, causing a cysteine to tryptophan alteration at amino acid 216, located between the PER-ARNT-SIM homology region (PAS) A and PAS B repeats. Introduction of the same mutation in the wild-type AHR sequence by site-directed mutagenesis similarity impaired XRE binding activity. Substitution with the conservative amino acid, serine, had no effect on XRE binding. The tryptophan mutation, but not the wild-type allele, was detectable in genomic DNA of the mutant. The implication that an amino acid within the PAS region may be involved in DNA binding indicates that the DNA binding behavior of AHR may be more anomalous than previously suspected.

The AHR mediates carcinogenesis by certain environmental pollutants, including the halogenated aromatic hydrocarbon TCDD, and polycyclic aromatic hydrocarbons (PAH) (reviewed in Ref. 1). Unliganded AHR is located in the cytoplasm associated with two molecules of the 90-kDa heat shock protein (HSP90) and possibly another protein(s) (2). After binding ligand, AHR dissociates from this complex and dimerizes with the ARNT protein. In the nucleus, this dimer acts as a transcription factor. Most if not all of the pathological effects mediated by AHR appear to depend on modulation of transcription by the receptor. The mechanism of transcriptional regulation is best understood for the CYP1A1 gene. Activation of transcription occurs through interaction of the AHR-ARNT dimer with several copies of short sequences, termed xenobiectic responsive elements (XREs), located in the 5'-flanking region of the gene (reviewed in Ref. 3).

The AHR and ARNT proteins both contain basic helix-loop-helix motifs toward their amino termini. More centrally located in both proteins is an approximately 300 amino acid PAS homology region, containing two approximately 50 amino acid degenerate direct repeats, PAS A and PAS B. Analysis of deletion mutants has identified putative functional domains of both proteins. Ligand binding occurs in a region encompassing the PAS B repeat of AHR (4, 5). One molecule of HSP90 appears to bind within the PAS region of AHR. The other molecule of HSP90 appears to require interaction at two sites: one over the basic helix-loop-helix region and the other located within the PAS region (6, 7). Dimerization of AHR and ARNT requires the HLH regions of both proteins. The PAS regions of both proteins facilitate dimerization. The basic submotifs of AHR and ARNT are required for DNA binding but not for dimerization (7, 8). Site-directed mutagenesis of individual amino acids confirmed this last observation and also indicated that an additional block of basic amino acids of AHR located amino-terminal to the above basic submotif also appears to contact DNA (9, 10). The carboxyl-terminal half of AHR appears to contain several discrete transcriptional transactivation domains, while a single such domain occurs toward the extreme carboxyl-terminal region of ARNT (11–15).

The AHH activity of CYP1A1 metabolizes PAH, such as benzo(a)pyrene, to cytotoxic as well as carcinogenic products. The mouse hepatoma cell line, Hepa-1, is highly inducible for CYP1A1 by PAHs and TCDD. We isolated mutants of Hepa-1 cells that are resistant to the toxicity of benzo(a)pyrene. These arose spontaneously at the relatively low rate of $2 \times 10^{-7}$ events per cell generation, and their frequency was increased markedly by mutagenesis. All of the clones had much reduced or undetectable CYP1A1-dependent AHH activities after treatment with concentrations of PAHs and HAAs that lead to maximal induction in wild-type Hepa-1 cells (16, 17). Analysis of somatic cell hybrids between individual clones and the Hepa-1 parental line demonstrated that most of the clones are recessive to the wild-type cells, whereas a few of the clones are dominant. Somatic cell hybridization experiments performed between the recessive clones permitted their assignment to four complementation groups (18, 19). These mutants have been used extensively for analyzing the mechanism of CYP1A1 induction and the mechanism of action of AHR and ARNT (20).
One complementation group corresponds to the CYP1A1 gene (21, 22). Clones assigned to the other three complementation groups are all affected in functioning of the AHR-ARNT dimer. Clones in complementation group B express markedly reduced levels of AHR mRNA and appear to be defective in a factor required for transcription of the AHR gene (23). Clones in complementation group C are defective in activity of the ARNT protein (24). One clone was isolated in complementation group D. Analysis of the D mutant demonstrated that it has moderately reduced levels of ligand binding to AHR, but appeared to be even more severely deficient in ligand-dependent translocation of AHR to the nucleus (19). The original D mutant analyzed in these previous studies exhibited an unstable phenotype. In the studies reported here we utilize a subclone of the original mutant that possesses a stable phenotype, maintaining undetectable TCDD-inducible AHH activity over many months in culture. We demonstrate here that the stable subclone carries a point mutation in the *Ahr* gene that does not affect ligand binding or dimerization with ARNT, but markedly reduces the efficiency with which the encoded AHR protein binds the XRE. This mutation changes a cysteine residue located in the PAS region to a tryptophan residue. This region was previously not thought to be involved in DNA binding, and our results point to the possibility that this region represents a novel DNA binding domain in AHR.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—The mouse hepatoma cell line, Hepa1c1c7 (Hepa-1), and a subclone, c35-3, of the D mutant strain, c35, which was derived from Hepa-1 cells (19), were maintained in nucleoside-free α-minimal essential medium (Irvine Scientific) supplemented with 10% fetal calf serum in a 5% CO₂ incubator at 37°C.

**Retroviral Expression Vector for Mouse AHR**—pSRα(NotI)AHR and the preparation of retroviruses from this vector, have been described (23). In brief, the mouse AHR cDNA was ligated into pSRα(NotI) (a generous gift of Dr. O. N. Witte, Department of Microbiology and Molecular Genetics, UCLA, Los Angeles, CA), using the XbaI and HindIII sites, to generate pSRα(NotI)AHR. Infectious retroviruses derived from pSRα(NotI)AHR was prepared by the rapid procedure of Muller et al. (25). Hepa-1 and D cells in 100-mm culture dishes were treated with the viral suspension in growth medium supplemented with 8 μg/ml Polybrene. Two or three days later, the infected cells were trypsinized and plated in growth medium supplemented with G418 (8). The benz[a]anthracene plus near-UV reverse selection was carried out as described without prior treatment of cells with TCDD (26).

**Protein Assays**—Cell extracts were prepared as described (24). Protein concentrations were determined with the Bradford assay. Western blot analysis of cytosolic extracts of either Hepa-1 or D cells was performed as described previously using affinity-purified polyclonal antibodies to AHR (24). Immune complexes were detected using the enhanced chemiluminescence (ECL) detection system (Pierce) with a secondary antibody coupled to horseradish peroxidase. The AHH assay and the *in vivo* AHR ligand binding assay (using the photoaffinity ligand, 2-azido-3-[[125]I]ido-7,8-dibromodibenzofuran-dioxin) were performed as described (7).

**RNA Analysis**—Total RNA was generated from Hepa-1 and D cells using the method of Chomczynski and Sacchi (27). Messenger RNA was prepared directly from the cells using a FastTract mRNA isolation kit (Invitrogen). For quantification of steady-state messenger RNA levels by competitive RT-PCR, internal standards for mouse Arh receptor and ribosomal large protein 7 (ML7) (28) were generated as follows: modified (down- and 5'-end) cDNA fragments for AHR and ML7 were derived from RT-PCR reactions with the primers designed to generate an 80-bp internal deletion located toward the 5'-end (see Table I) and then cloned into a TA cloning vector, pCRII, and transcribed into cRNA from the TCDD polymerase promoter in the vector.

| Table I | Primers for PCR and reverse transcription |
|---|---|
| **I. AHR cloning and sequence analysis** |
| Reverse transcription (22b): | AAATT TCATC CTGGG ATGGG AG |
| Full-length, forward (24b): | GGAGA CGGAC CATCT TCCAG AGGGG AG |
| Full-length, reverse (18b): | TCTTC GCTCG GTGGC TGGCA TAG |
| Upstream, forward (20b): | AGCTT GGCCC TGAGG GTTTC TCCTC |
| Upstream, reverse (18b): | TCAGG TGGGA CTAAA CC |
| Genomic fragment, forward (20b): | CGCCA TGAAT TACCC AGCAG |
| Genomic fragment, reverse (20b): | TTGTT TTTTT TTTTT TTT TG CGGAG GTGGG |
| **II. Competitive RT-PCR** |
| A. AHR | TAAAT CGACT CACTA TAGGA GCTTC ATCCT CTTCA |
| Generating internal standard | GAGAA CGGAC CATCT TCCAG GAGGG AG |
| Forward (57b): | ATTCG GTGAG AGGAT GCAAT GCTTG |
| Generating internal standard | GCTCA TCTAT GAGAA GGAAA |
| Reverse (54b): | AGCTT CCTCC AGAGA ACGCC |
| PCR, forward (19b): | TGGCC ATGTT GGATT CTG |
| RT and PCR, reverse (18b): | GCTCA TCTAT GAGAA GGAAA |
| B. Ribosomal L7 (ML7) | ATTCG GTGAG AGGAT GCAAT GCTTG |
| Generating internal standard | GCTCA TCTAT GAGAA GGAAA |
| Forward (42b): | GCTCA TCTAT GAGAA GGAAA |
| PCR, forward (22b): | GCTCA TCTAT GAGAA GGAAA |
| RT and PCR, reverse (18b): | GCCAC AATTCC GCGAC ATG |

* Internal standard (I.S.) cDNA for AHR was generated by PCR with indicated forward and reverse primers from an oligo(dT)-primed reverse transcriptase and transcribed into cRNA from a T7 polymerase promoter included in the forward PCR primer.

* I.S. cDNA for ML7 was generated by PCR with indicated forward and reverse primers from an oligo(dT)-primed reverse transcriptase from Hepa-1 cells. The I.S. cDNA was then cloned into a TA cloning vector, pCR II, and transcribed into cRNA from the T7 polymerase promoter in the vector.

**Primers for PCR and reverse transcription**

- **AHR cloning and sequence analysis**
  - Reverse transcription (22b):
    - AAATT TCATC CTGGG ATGGG AG
  - Full-length, forward (24b):
    - GGAGA CGGAC CATCT TCCAG AGGGG AG
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    - TCTTC GCTCG GTGGC TGGCA TAG
  - Upstream, forward (20b):
    - AGCTT GGCCC TGAGG GTTTC TCCTC
  - Upstream, reverse (18b):
    - TCAGG TGGGA CTAAA CC
  - Genomic fragment, forward (20b):
    - CGCCA TGAAT TACCC AGCAG
  - Genomic fragment, reverse (20b):
    - TTGTT TTTTT TTTTT TTT TG CGGAG GTGGG

- **II. Competitive RT-PCR**
  - A. AHR
    - Generating internal standard
    - Forward (57b):
      - TAAAT CGACT CACTA TAGGA GCTTC ATCCT CTTCA
    - Generating internal standard
    - Reverse (54b):
      - ATTCG GTGAG AGGAT GCAAT GCTTG
    - PCR, forward (19b):
      - GCTCA TCTAT GAGAA GGAAA
    - RT and PCR, reverse (18b):
      - GCTCA TCTAT GAGAA GGAAA
  - B. Ribosomal L7 (ML7)
    - Generating internal standard
    - Forward (42b):
      - GCTCA TCTAT GAGAA GGAAA
    - PCR, forward (22b):
      - GCTCA TCTAT GAGAA GGAAA
    - RT and PCR, reverse (18b):
      - GCCAC AATTCC GCGAC ATG

2 O. N. Witte, unpublished data.

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Mutation in the Ahr Gene in Hepa-1 Cells
spanning the coding region. (The “full-length forward primer” contained the first 20 nucleotides of the coding sequence, while the “full-length reverse primer” (see Table I) corresponded to a sequence in the 3'-untranslated region (see Table I).) Superscript II reverse transcriptase (Life Technologies, Inc.) was used in the RT reactions. A mixture of the primers (Ser-5, 5'-AGC GGCTGAGGTGC-3'; Ser-9, 5'-GCACCTCAGGGGGCTCCTGAGAAGCCTCTC-3'; and Trp-3, 5'-GCACCTCAGGGCCACCTGAGAAGCCTCTC-3'). The external 5′-primer corresponds to AHR bases 190–211 and the external 3′-primer corresponds to AHR bases 878–894. The secondary PCR products were digested with Bpu110I and Eco47III and then ligated to similarly digested pDNA3-AHR. The generated clones were sequenced to confirm their mutations.

In Vitro Transcription and Translation—Constructions were expressed in the TNT T7 coupled reticulocyte lysate system (Promega Biotech) in the presence or absence of [35S]methionine (Amerham Corp.). Expression of each construct was assayed by SDS-polyacrylamide gel electrophoresis of an aliquot of the reaction mixture performed in the presence of [35S]methionine. Quantification of each construct's level of expression was then performed by β-scanning.

**Dimerization of AHR Proteins with ARNT Proteins—**Dimerization of AHR proteins with ARNT proteins was performed as described (10). Briefly, ARNT protein was synthesized as described above in the presence of [35S]methionine, whereas all the AHR clones were transcribed and translated into protein in the absence of the isotope. AHR proteins were mixed with an equimolar amount of ARNT in the presence or absence of 10 nM TCDD. The protein mixture was then incubated with affinity purified anti-AHR polyclonal antibody. The resultant immune complexes were then precipitated with protein A-Sepharose CL-4B beads, washed, and analyzed by SDS-polyacrylamide gel electrophoresis. β-Scanning of the gel was used to estimate the relative ARNT heterodimerization activity of the AHR clone in question (expressed as a percentage of the XRE binding activity of the wild-type AHR cDNA). Mean values were calculated from three independent experiments.

**XRE Binding Assay—**This assay was performed either with nuclear extracts prepared from cells that had been incubated with 10 nM TCDD for 90 min or with in vitro generated AHR and ARNT proteins. In the former case, nuclear extracts were prepared as described previously (31). The XRE binding assay was performed as described (8). Briefly, each in vitro synthesized, unlabeled AHR protein was mixed with unlabeled ARNT in an equimolar ratio and the mixture incubated in the presence of 10 nM TCDD for 1.5 h. A poly(dI-dC)-containing binding buffer was then added. A 32P-labeled double-stranded synthetic oligonucleotide containing mouse XRE1 was added to the mixture for an additional 20 min at room temperature. Resulting samples were analyzed by nondenaturing polyacrylamide gel electrophoresis in 200 mM HEPES, 100 mM Tris, 5 mM EDTA, pH 8.0. Autoradiography was used as well as β-scanning of the gel provided a quantitation of the relative XRE binding activity of each AHR clone in question. The results are expressed as a percentage of the XRE binding activity of the wild-type AHR cDNA kindly provided by Dr. C. Bradfield (Mc Ardle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, WI) and designated cXRE1. This construct is referred to as A. Mean values were calculated from four or more independent experiments.

**In Vivo Functionality—**PCRIII derivatives containing AHR cDNA inserts or the parental plasmid vector PCRIII (0.2–0.5 µg) were co-transfected along with the CAT reporter plasmid, pMC6.3k (1–2 µg) (32) into 2 × 105 D cells/35-mm tissue culture well, using the lipofectAMINE method according to the provider (Life Technologies, Inc.).

RESULTS

**AHR Functionality in the D Mutant Cells—**Both the D mutant strain and the Hepa-1 cells were analyzed for the steady-state expression level of AHR mRNA by RT-PCR. The expression of AHR mRNA was substantial (8 × 106 molecules/µg mRNA) in the D cells (Fig. 1A, panel i), although significantly reduced compared with that in the Hepa-1 cells (3.2 × 107 molecules/µg of mRNA). The amount of the mRNA for the constitutively expressed ribosomal protein L7 (LM7) was measured in parallel from the same RNA samples (Fig. 1A, panel ii) to provide a control for possible differences in sample preparation.

Expression of AHR protein was then analyzed in cytosolic extracts prepared from the D mutant and the Hepa-1 cells. The AHR protein level in the D mutant cells varied between one-third and two-thirds of that in Hepa-1 cells in different experiments and extracts. Results of one experiment are presented in Fig. 1B. The capacity of the AHR in the cytosol of the D mutant strain to bind the photoaffinity ligand 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin was also evaluated. In comparison to the Hepa-1 cells, the D mutant cells exhibited approximately one-half as much ligand binding capacity (Fig. 1C). Thus, the levels of expression of AHR mRNA, AHR protein, and ligand binding activity are all reduced by about three-quarters to one-half in the D strain. The AHR protein expressed by the D mutant cells, therefore, appears to have normal ligand binding activity.

To evaluate the overall in vivo functionality of AHR expressed by the D mutant cells, nuclear extracts were prepared from either vehicle (Me6SO) or 10 nM TCDD-treated Hepa-1 and D cells. The binding capacity of the nuclear extracts to a 32P-labeled double-stranded synthetic oligonucleotide containing mouse XRE1 was analyzed by electrophoretic mobility shift assay. As shown in Fig. 1D, formation of the AHR/ARNT/XRE complex was observed in the Hepa-1 cells after TCDD treatment, whereas no similar XRE complex was detectable in D cells, with or without ligand activation. (The band indicated as the AHR-ARNT/XRE complex in Fig. 1D has been unambiguously demonstrated to correspond to this complex in previous publications from this laboratory (36).) Thus, the D cells have...
lost ligand-inducible XRE binding activity.

**Rescue of the D Mutant Phenotype by Infection with a Retroviral Vector Expressing AHR**—Construction of an ecotropic recombinant retroviral vector containing both the neo gene and the AHR cDNA has been described previously (23). Hepa-1 and D cell cultures were infected with either pSR

\[\text{AHR cDNA on its own (data not shown), probably due to its low template possessiveness.} \]

infected D cells survived the reverse selection at a frequency less than 0.01% (Table II and Fig. 2B). Restoration of CYP1A1 inducibility was further demonstrated when both pSRot(Not1)- and pSRot(Not1)AHR-infected D cells were analyzed for expression of CYP1A1-dependent AHH activity with or without TCDD induction (Table II). While TCDD-inducible AHH activity in the D cells infected with the parental retroviral vector pSRot(Not1) was 1% of that in Hepa-1 cells, TCDD-inducible AHH activity was restored to 46% of wild-type levels in D cells infected with pSRot(Not1)AHR. These data provide direct evidence that the CYP1A1-noninducible phenotype of the D strain is principally, if not completely, ascribable to loss of AHR activity.

**RT-PCR Cloning of the Full-length Coding Region of AHR cDNA**—To test whether the AHR protein encoded by the AHR gene in the D cells is defective, RT-PCR reactions were performed to generate the AHR cDNA covering the entire protein coding region (the forward primer contains the first 20 nucleotides of the coding sequence) from the total RNA of D cells. A mixture containing two different thermostable DNA polymerases was used in the PCR reactions to maximize fidelity. The proofreading polymerase *Pfu* failed to amplify the AHR cDNA on its own (data not shown), probably due to its low template possessiveness.) AHR cDNA products from different RT-PCR reactions were cloned separately into the eukaryotic expression vector, PCRIII. Thus, any sequence artifacts intro-
Cells—of AHR cDNA from the Hepa-1 cells. The cDNA fragments of AHR independently derived RT-PCR products. The same procedure produced by the RT-PCR reactions could be detected by comparing ing restoration of AHR mRNA expression to D cells infected with pSRα(NotI)-AHR. Cells were subjected to the reverse selection two weeks after infection. All infectants were assayed 8 weeks after infection. Upper arrow, AHR mRNA; lower arrow, CHOβ mRNA; left, size markers (kilobase pairs). mRNA (5 μg) was probed with a mixture of mouse AHR cDNA and CHOβ cDNA. B, resistance of pSRα(NotI)-AHR-infected D cells to the reverse selection. D cells infected with pSRα(NotI) (left) or pSRα(NotI)AHR (right) were selected with G418. Two weeks after infection, surviving cells were subjected to the reverse selection at 5 × 10⁵ cells/dish. The dishes were stained after a further 9 days of incubation. Infection with the AHR-expressing vector allowed the D cells to survive this selection procedure, which selects for cells expressing CYP1A1 inducibility.

**FIG. 2** Rescue of the D mutant phenotype by infection with a retroviral vector expressing AHR. A, Northern blot analysis showing restoration of AHR mRNA expression to D cells infected with pSRα(NotI)-AHR. Cells were subjected to the reverse selection two weeks after infection. All infectants were assayed 8 weeks after infection. Upper arrow, AHR mRNA; lower arrow, CHOβ mRNA; left, size markers (kilobase pairs). mRNA (5 μg) was probed with a mixture of mouse AHR cDNA and CHOβ cDNA. B, resistance of pSRα(NotI)-AHR-infected D cells to the reverse selection. D cells infected with pSRα(NotI) (left) or pSRα(NotI)AHR (right) were selected with G418. Two weeks after infection, surviving cells were subjected to the reverse selection at 5 × 10⁵ cells/dish. The dishes were stained after a further 9 days of incubation. Infection with the AHR-expressing vector allowed the D cells to survive this selection procedure, which selects for cells expressing CYP1A1 inducibility.

**TABLE II** Survival in the reverse selection and AHH activities of cells infected with the AHR retroviral expression vector

| Cell strain | Retroviral vector | Selection | Survival in reverse selection | AHH specific activity | −TCDD | +TCDD |
|-------------|------------------|-----------|-------------------------------|-----------------------|-------|-------|
| Hepa-1      | None             | None      | 100                           | 2.4                    | 100   |       |
|             | pSRα(NotI)      | G418      | 100                           | 2.1                    | 56    |       |
| D (c35–3)   | pSRα(NotI)AHR   | G418      | 100                           | 2.5                    | 150   |       |
|             | pSRα(NotI)AHR   | G418      | 0.01                          | 1.0                    | 0.6   | 46    |

The greatly attenuated XRE binding activity of AHR proteins encoded by the cDNAs derived from the D cells was determined to be in all cases less than 10% of the activity expressed by the cDNAs derived from the Hepa-1 cells. The low XRE binding activity of AHR derived from the D cells remained ligand-inducible and could be competed by excess unlabeled XRE.

To analyze the overall biological functionality of the AHR protein derived from the D cells, AHR cDNAs derived from the D cells (D1-7 and D1-14) were cotransfected into the D cells together with a plasmid containing a CAT reporter gene driven by the upstream regulatory region of the rat CYP1A1 gene. A wild-type AHR cDNA as well as the empty parental vector pCRII were also included as positive and negative controls respectively (Fig. 3C). There was no detectable constitutive or TCDD-inducible CAT activity in the D cells cotransfected with the CAT reporter construct plus the parental vector lacking the AHR insert. In D cells transfected with all the AHR cDNAs, TCDD-inducible CAT activity was detected. However, the levels of CAT activity were markedly lower in cells transfected with the AHR cDNAs derived from the D cells than that in cells transfected with a cDNA derived from wild-type Hepa-1 cells. The CAT activity was influenced by the amount of AHR cDNA transfected as well as its ratio to the reporter construct. Nevertheless, AHR cDNAs derived from the D cells generated markedly lower CAT activities than the cDNA derived from Hepa-1 under all conditions tested (data not shown).
Sequence Analysis—Cycle sequencing analysis was performed on two AHR cDNA clones derived from the D cells (D1-7 and D2-8) encompassing the entire AHR coding region (although the forward PCR primer used to generate D1-7 and D2-8 contained the first 20 nucleotides of the coding sequence). AHR cDNA clones derived either from the wild-type Hepa-1 cells (U1-6) or from the normal AHR clone (L5) were also analyzed in parallel. Sequencing results obtained were compared with the normal AHR cDNA sequence in GenBank™ (34). All the noted sequence variations were further confirmed or eliminated by analyzing the complementary strand or sister clones.

A sequence variation was located at bases 221 and 222, which read GC rather than CG. This would change the amino acid coding from threonine to serine at position 74. Since the above GC sequence was observed in all the AHR cDNA analyzed (derived from both Hepa-1 and the D mutant), we concluded that the sequence in GenBank™ is inaccurate at that location. Our observation confirms other published AHR sequences (37, 38) at this location. A sequence variation existed in the AHR cDNA clones derived from the D cells but not in any of the normal clones (Fig. 4). This single C to G mutation at base 648 would cause an amino acid alteration from cysteine to tryptophan at position 216. To rule out the possibility of the alteration being a cloning artifact, 10 other D mutant AHR cDNA clones derived from three different RT-PCR reactions were analyzed. All contained the above C to G point mutation.

To verify the existence of the altered sequence in the genome of the D cells, we also cloned a short 1.2-kilobase pair fragment of genomic DNA corresponding to the segment between bases 516

FIG. 3. Functionality of AHR protein derived from the Hepa-1 and the D mutant cells. A, ARNT heterodimerization activity of AHR cDNA clones. Equal amounts of AHR proteins synthesized from cDNA clones derived from the D mutant (D1-7 and D3-3) and Hepa-1 cells (U1-5 and U2-6), as well as L5 (the wild-type AHR cDNA) were used in the analysis described. p = preimmune IgG; * = affinity-purified AHR antibodies. B, XRE binding activity of AHR cDNA clones. Equal amounts of AHR protein synthesized from cDNA clones derived from D mutant (from three separate PCR reactions: D1, D2, and D3) and Hepa-1 cells (two separate RT-PCR reactions: U1 and U2), as well as L5 (the wild-type AHR cDNA) were analyzed for their XRE binding activity. C, CAT activity of D cells cotransfected with pMC6.3K and AHR cDNA. 2 × 10^5/dish D cells were cotransfected with 2 µg of pMC6.3k plus 0.5 µg of empty vector (pCRIII) or pCRIII-AHR derived from either the D mutant (D1-7 and D1-14) or Hepa-1 cells (U1-5). CAT activity was assayed from cell extracts prepared from the transfectants and is expressed as a percentage of the specific activity of TCDD-treated cells cotransfected with pMC6.3k plus U1-5.
and 680 of the cDNA, which spans intron E and encompasses the site of the mutation. All 17 D mutant genomic clones so derived from three different PCR reactions contained the C to G point mutation when analyzed by either restriction digestion (the C to G mutation leads to the loss of a *Nae*I restriction site) or sequence analysis. None of the 4 corresponding cloned genomic *Ahr* fragments from Hepa-1 cells contained the mutated sequence. There were two other possible sequence alterations observed in certain D derived AHR clones, but since they were each found in only 1 out of seven sister clones analyzed (Fig. 4), they were considered to be cloning artifacts.

Since the forward primer which we used for generating the AHR cDNAs corresponded to the first 20 nucleotides of the coding region, potential mutations in this region would be overlooked. Therefore, 210-bp fragments extending from -170 bp through the first 40 bp of the coding sequence of the AHR cDNA were generated by PCR from the genomic DNA of both the D and the Hepa-1 cells, and these were cloned and sequenced. No mutations were observed in the first 20 bp of the coding sequence of the D-derived AHR gene.

**Substitution at Position 216 of AHR by Site-directed Mutagenesis—**To confirm the affect of the above tryptophan mutation on AHR, *in vitro* mutagenesis was performed to substitute Cys with Trp at position 216 in the wild-type AHR (pcDNA3-AHR). In addition we substituted the same position with serine. The two mutant forms of AHR cDNA were transcribed and translated into proteins *in vitro* as described above. As expected, Trp<sup>216</sup>-AHR had about 5–10% of DNA binding activity compared with the wild-type AHR (Cys<sup>216</sup>-AHR) when analyzed in the XRE gel shift experiment (Fig. 5). This result confirmed our observation that the same single mutation in the AHR derived from the D cells was responsible for its loss of DNA binding ability. Interestingly, serine substitution at position 216 did not affect the DNA binding activity of AHR.

**Determination of Chromosome Aneuploidity by FISH—**To determine if the observed decreased expression of AHR in the D mutant is due to a reduced copy number of the *Ahr* gene, FISH analysis was conducted. Metaphase chromosomes were prepared from both Hepa-1 and D cells, then hybridized with a probe specific for the centromeric region of mouse chromosome 12, where the *Ahr* gene is located (39). Analysis of 80 metaphase cells from each cell strain indicates that Hepa-1 cells contain three chromosomal sites that hybridize with the probe (Fig. 6A) while the D cells contain only two (Fig. 6B).

**DISCUSSION**

Our previous studies demonstrated that the non-inducibility phenotype of the mutant is expressed in a recessive fashion in somatic cell hybrids formed between D cells and Hepa-1 cells, indicating that the mutant does not express a novel activity that eliminates inducibility. These studies also found that binding to AHR is moderately reduced in the D mutant, but that nuclear translocation of AHR is more severely affected. We proposed that the strain is either mutated in AHR or in a protein required for functionality of AHR (19). Our previous results were obtained with the original D mutant, c35, and several subclones of the mutant that exhibit an unstable phenotype in that they slowly reacquired TCDD-inducible AHH activity as they were maintained in culture. The current results were obtained with a subclone (c35-3) of the original D mutant, that possesses a stable phenotype, maintaining low TCDD-inducible AHH activity (less than 1% of that of wild-type Hepa-1 cells) over many months in culture (Ref. 19 and data not shown). In the current studies we thereby avoided potential confounding effects of an unstable phenotype. In this paper we
Generation of the Trp mutation by site-directed mutagenesis of
the mutant. Furthermore, we show that the D mutant possesses levels
of AHR mRNA and AHR protein equivalent to its ligand binding
activity (in each case about one third to one half of the
magnitude in Hepa-1 cells). Thus, the AHR protein that is
expressed in the D mutant appears to have normal ligand
binding activity, indicating that the defect in the mutant does
not reside in a protein required for ligand binding (such as
HSFP90) or in a segment of AHR required for this function.
Reversal of the mutant phenotype by a retroviral expression
vector for AHR indicated that loss of CYP1A1 inducibility in
the mutant is principally, if not totally, ascribable to its loss of
AHR function.

XRE binding in nuclear extracts obtained from D mutant
cells was found to be severely reduced. Three possible explana-
tions for the reduced XRE binding in the D mutant were en-
visioned: viz. that the D strain is mutated in (i) a portion of AHR
that affects DNA binding or (ii) in a protein required for a
necessary step preceding DNA binding or (iii) in a protein
directly required for AHR to bind DNA. (This last protein could
be a protein kinase, for example, since AHR appears to require
phosphorylation to bind the XRE (but not to dimerize with
ARNT) (40), or it could be a protein required to maintain AHR
in the reduced state required for DNA binding (41).) To distin-
guish between these possibilities, we analyzed AHR cDNAs
derived from the D mutant. The AHR proteins expressed from
the D mutant-derived cDNAs exhibited normal dimerization
activity toward ARNT. However, seven different cDNAs, de-

erived from three independent RT-PCR reactions performed on
the D mutant, exhibited markedly reduced XRE binding activ-
ities, demonstrating that the D mutant carries a mutation in
the AHR gene, and in particular, one that affects XRE binding.
Subsequent sequencing of the cDNAs identified this mutation
as being a C to G transversion, leading to a Trp from Cys
mutation located between the PAS A and PAS B domains.

Generation of the Trp mutation by site-directed mutagenesis of
the wild-type AHR confirmed that this mutation leads to a
dramatic decrease in XRE binding activity. Interestingly, con-
servative substitution of cysteine with serine had no effect on
XRE binding.

The D mutant cDNAs were also markedly deficient in their
ability to transactivate transcription from an AHR-dependent
reporter gene. This deficiency is probably secondary to the
defect in DNA binding, since no evidence indicates that the
PAS region possesses transcriptional transactivation activity
(12, 13). The fact that the activity of the D mutant cDNAs in the
transactivation assay are less markedly reduced than their
activities in the XRE binding assay may reflect differences in
the ratios or absolute amounts of AHR, ARNT, and/or DNA in
these two assays. We have observed a similar discrepancy with
regard to the results obtained with these two assays for in vitro
generated mutants of AHR that are affected in XRE binding
(9). The nearly complete absence of TCDD-inducible AHH ac-
tivity in D mutant cells (1% of that in Hepa-1 cells) is probably
ascribable to a combination of both reduced AHR expression
(one-quarter to one-half of wild-type) as well as reduced XRE
binding activity of the expressed protein (less than 10% of
wild-type).

Fluorescence in situ hybridization analysis demonstrated
that the D mutant cells contain two chromosomal segments
that hybridize to a probe that maps to the centromeric region of
mouse chromosome 12, very close to where the Ahr gene is
located (39), while Hepa-1 cells contain three copies. It is likely
therefore that D and Hepa-1 cells contain two and three copies
of the Ahr gene, respectively. However, only the mutant AHR
sequence (Trp<sup>216</sup>) was detected in genomic DNA from the
D mutant, while only the wild-type sequence was detected in the
genomic DNA of Hepa-1 cells. Furthermore, all 12 cDNAs
derived from the D mutant contained the mutation, while neither
of those derived from the Hepa-1 strain contained it. Thus the
mutation must have arisen de novo in the D mutant. A possible
scenario for the origin of the c35-3 mutant strain is that it arose
from Hepa-1 cells by loss of two copies of the Ahr gene and
mutation of one copy (not necessarily in that order), followed by
duplication of the mutant allele. This suggestion is compatible
with the observation that the strain expresses reduced levels of
AHR compared with Hepa-1 cells. The proposed mode of origin
of the D strain is consistent with the known mechanism of
action of gamma irradiation, which was used to induce the D
mutant (19). Gamma irradiation is known to cause G to C
transversions as well as gross chromosomal deletions, includ-
ing loss of whole chromosomes, in mammalian cells (42–44).

We previously observed that the originally isolated D mutant
strain, c35, and certain subclones derived from it exhibited an
unstable phenotype, is that they slowly increased in TCDD-
ducible CYP1A1 activity as they were maintained in culture
(19). It is possible that each of these strains consists of a
heterogenous population of cells, some possessing and some
lacking the wild-type Ahr allele, and that instability is due to
an increase in the proportion of cells containing the wild-type
allele. The clone used in the present paper, c35–3, exhibited a
stable phenotype.

The DNA binding behavior of AHR is very different from that
of other basic helix-loop-helix PAS proteins, including ARNT.
DNA contacts within the basic domain of AHR are restricted to
its carboxyl-terminal half. An amino-terminal basic segment of
AHR separated from the above basic domain may also contact
the XRE (although this latter point is controversial) (9, 10, 45,
46). The mutated cysteine residue, Cys<sup>216</sup> is embedded in an
arginine and cysteine-rich region of AHR, R<sup>218</sup>RCRCRLR<sup>220</sup>,
that is conserved in rat and human AHR (except for Arg<sup>215</sup>,
which is Ile in human AHR). This region is highly basic like the

![Image](https://example.com/image.png)
above two regions of AHR believed to contact DNA. It is thus possible that DNA binding by AHR is even more anomalous than hitherto suspected, with DNA contacts also occurring within the PAS region where Cys216 is located. Several large deletions in the PAS region of AHR have previously been characterized by several investigators. Large deletions encompassing Cys216 eliminate XRE binding (6, 7, 47), consistent with the notion that Cys216 contacts DNA, although these deletion mutations may compromise other activities preceeding DNA binding (such as dimerization), rather than DNA binding per se.

Another explanation for the phenotype of the D mutant is that Cys216 does not contact DNA, but that mutation of Cys216 to Trp216 alters the tertiary structure of AHR in such a way as to eliminate DNA binding. If indeed the loss of DNA binding by the D mutant protein is due to an alteration in tertiary structure rather than a specific effect on DNA binding, then this alteration must be relatively minor, since the above mutation does not affect either ligand binding or dimerization with ARNT.

Dougherty and co-workers (41) demonstrated that binding of the AHR-ARNT heterodimer to the XRE in vitro could be reversibly activated or inhibited by agents that reduce or oxidize cysteine residues, respectively, and that dimerization of AHR with ARNT was not affected by these agents. A number of considerations suggested to us that the (or a) target for redox regulation of AHR-ARNT DNA binding might be Cys216 of AHR: (i) no cysteine residues exist in the first 100 residues of AHR, that encompasses all previously identified domains involved in DNA binding, and no cysteine residues occur in the basic region of AHR, which is its only known DNA binding region (8, 10). (ii) Basic residues are located either side of a cysteine residue dramatically enhances its reactivity (48), potentially making it particularly susceptible to oxidation. (iii) Redox regulation of DNA binding by Jun and Fos is mediated by single cysteine residues in the DNA binding domains of these proteins. Like Cys216 of AHR, these cysteine residues are each flanked on both sides by basic residues (49). To investigate this possibility we tested the effect of oxidation/reduction on the XRE binding activities of in vitro transcription/translation-generated, dialyzed, and TCDD-treated AHR-ARNT dimers. Diamide treatment reduced XRE binding of the wild-type AHR-ARNT dimer by 2-fold, and this effect was reversed by dithiothreitol.3 Thus Cys216 of AHR does not appear to mediate redox regulation of XRE binding.

We previously reported that the D mutant is severely affected in ligand-dependent nuclear translocation of AHR, as assessed by conventional subcellular fractionation analysis. One possible explanation for these results is that amino acids 212–220 represent part or all of a nuclear translocation signal (this region is rich in basic amino acids like other known nuclear translocation signals) and that the Trp216 mutation negates activity of this signal. An alternative explanation is that, because of its defect in DNA binding, the AHR mutant binds with reduced avidity to DNA and is therefore more readily extracted from nuclei during the subcellular functionality procedure. Immunocytochemical analysis will be required to determine whether nuclear translocation of AHR is genuinely defective in the mutant.

We now possess mutants affected in the major proteins involved in induction of CYP1A1. Certain of our mutants (originally called A mutants) are mutated in the Cyp1a1 gene, others (originally called C mutants) are defective in ARNT function and are probably mutated in the Arnt gene, while we identify here a mutation (in the D mutant) in the Ahr gene. These mutants, which are all derived from the same cell line, together provide a powerful experimental system for investigating the role of this induction mechanism in any cellular process that is expressed in these cells. AHR knockout mice have previously been reported by two research groups (50, 51). AHR null mutant cell lines that are potentially obtainable from these mice strains would constitute a complementary system for studying the role of AHR in cellular processes.

It should be noted that another class of mutants of Hepa-1 cells, the B mutants, have previously been used to investigate the role of AHR in particular cellular functions (52, 53). However, we recently demonstrated that the B mutants are deficient in a factor required for expression of the Ahr gene, rather than being mutated in the Ahr gene itself, and may potentially be defective in expression of many other genes besides Ahr (23). Our demonstration that the D mutant is specifically mutated in the Ahr gene makes this mutant much preferable to the B mutants for investigating the role of AHR in cellular physiological processes. Our observation that the D strain is mutated in the Ahr gene also sheds light on certain previous observations. For example, we previously studied differentiated and dedifferentiated derivatives of the rat hepatoma line, H4IE-C3, and found that dedifferentiated variants express CYP1A1 inducibility while differentiated variants generally do not. Treatment of the differentiated variants with 5-azacytidine or sodium butyrate, which are known to be able to reactivate expression of silenced genes, restored inducibility and AHR activity to these cells. Furthermore, the non-inducibility phenotype of the differentiated variants was complemented in somatic cell hybrids between these cells and the A, B, and C mutant classes of Hepa-1 cells, but was not complemented in somatic cells hybrids formed between these cells and the D mutant (54). We can now conclude that lack of CYP1A1 inducibility in the differentiated variants is due to silencing of the AHR gene in these cells.

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