Regular article

Establishment of Reporter Yeasts for Guinea Pig and Syrian Hamster Aryl Hydrocarbon Receptor Ligand Activity

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Polymorphism of the aryl hydrocarbon receptor (AhR) presumably induces genetic difference in the susceptibility of animals to aryl hydrocarbons. The activation of intracellular signaling following AhR binding to aryl hydrocarbons is highly correlated with the toxicity and carcinogenicity of these chemicals. Here we developed two reporter yeasts coexpressing AhR and AhR nuclear translocator (Arnt) proteins of a guinea pig and a Syrian hamster, known as the most sensitive and most resistant laboratory rodents to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), respectively. We previously constructed reporter yeasts expressing human and mouse AhR/Arnt. We conducted reporter assays to measure ligand activities of TCDD, 3-methylcholanthrene, β-naphthoflavone and indirubin in these yeasts. Ligand treatment induced a dose-dependent increase in β-galactosidase activity from a reporter plasmid in all 4 yeast strains. The assays showed that yeast expressing guinea pig AhR/Arnt is most sensitive and yeast expressing Syrian hamster AhR/Arnt is most insensitive to these ligands. The yeasts expressing human and mouse AhR/Arnt were in-between. These different ligand activities reflect the species specificity of AhR/Arnt, and may be related to the susceptibility of rodents to aryl hydrocarbons.

Key words: aryl hydrocarbon receptor, reporter yeast, species specificity, guinea pig, syrian hamster

Introduction

Aryl hydrocarbons bind to the cellular aryl hydrocarbon receptor (AhR) in the initial step of their metabolism. AhR, also known as the dioxin receptor, is a ligand-activated transcription factor that exists in most cell and tissue types of the body (1). AhR-mediated signaling is necessary for potent xenobiotic ligands, e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls and polyaromatic hydrocarbons to produce toxicity (2,3). Following ligand activation, AhR migrates into the nucleus from the cytoplasm and interacts with the AhR nuclear translocator (Arnt) (4–8). The resultant AhR/Arnt heterodimer with the ligand binds to xenobiotic responsive element (XRE) sequences located upstream of dioxin-responsive genes like CYP1A1 to up-regulate their transcription (8–13).

Sensitivity to TCDD varies greatly among animal species. Acute oral LD50 differs over a 5,000-fold range (14). The most sensitive species is guinea pigs with an LD50 of about 1 µg/kg. The LD50 of rats (male) and mice are about 22 µg/kg and 114 µg/kg, respectively. Syrian hamsters are the most resistant laboratory rodents with an LD50 of about 5,000 µg/kg (14). Polymorphic forms of AhR are reported among animal strains and are related to genetic differences in the responsiveness and susceptibility of the animals to aryl hydrocarbons (15–17). In mammalian species, there is a distinct correlation between the number of glutamine residues in the C-terminal Q-rich subdomain and sensitivity to the acute lethality of TCDD (17).

Since the status of AhR activation and the following signal transduction are expected to be an indicator of the toxicity of aryl hydrocarbons, AhR-responsive reporter assays have been constructed in mammalian cells and mice (18,19). Several years ago, C. A. Miller III et al. established a human AhR signaling assay system in yeast (Saccharomyces cerevisiae) (20–22). In accordance with Miller’s method, we previously developed a yeast strain coexpressing mouse AhR and Arnt (23). In these strains, the level of β-galactosidase activity indicates the level of AhR/Arnt signaling activated by ligand binding. Since the yeast assay system is relatively simple, easy to handle and inexpensive, the reporter yeast system has been applied for various studies on environmental contaminants as well as on the AhR signaling pathway (24). Here we established reporter yeasts coexpressing AhR and Arnt cDNA of guinea pigs or Syrian hamsters. Using these yeasts along with the previously constructed mouse and human AhR/Arnt-expressing yeasts, we compared the level of ligand-dependent AhR/Arnt signal activation in order to know the species difference of AhR/Arnt.
Materials and Methods

Materials: General chemicals, essentially analytical grade, were purchased from Sigma-Aldrich Japan (Tokyo, Japan). TCDD was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-methylcholanthrene (3-MC) and β-naphthoflavone (β-NF) were purchased from Sigma-Aldrich Japan. Induribin (25) was kindly supplied by Dr. Tomonari Matusuda, Kyoto University, Japan. Syrian hamster (Slc: syrian, 4 weeks old) and guinea pig (Std: Hartley, 5 weeks old) lungs were purchased from Japan SLC Inc. (Shizuoka, Japan). The yeast strain W303a, the reporter plasmid pTXRE5-Z and the yeast bidirectional expression vector YEpplac181 were kindly supplied by Dr. Charles A. Miller III, Tulane University, USA (22). The yeast integration vector pAUR101 and its selection marker antibiotic, aureobasidin, were purchased from Takara Bio Inc. (Shiga, Japan). The DH5α strain of Escherichia coli was used for the subcloning of plasmids. Generally, polymerase chain reactions (PCR) were carried out with high fidelity PCR polymerase KOD plus (Toyobo Co. Ltd., Osaka, Japan). Oligonucleotides for PCR primers and DNA sequencing primers were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). Restriction enzymes MfeI and Acc65I were purchased from New England Biolab Inc. (Beverly, MA, USA). All other restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase, were obtained from Toyobo Co. Ltd. DNA sequences were determined with the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Co., Foster City, CA, USA) using the ABI377 automatic DNA sequencer (Perkin-Elmer Co.).

Molecular cloning of Arnt cDNAs of guinea pigs and Syrian hamsters: RNA was extracted from the frozen lungs of a guinea pig and a Syrian hamster using the Qiashredder and RNasey Mini kit (QIAGEN GmbH, Hilden, Germany). A set of primers (5’-CGRC-CATGGCGGCGACTACG- and 5’TCTATTTCDGAAAA-GGGGGAACAA) was synthesized. As the sequences of guinea pig and Syrian hamster Arnt have not been reported, these primers were designed from conserved sequences of 5’- and 3’-ends of coding regions of human, mouse and rat Arnt. Using the set of primers and the extracted RNA, Arnt cDNAs of guinea pigs and Syrian hamsters were amplified with the Takara RNA PCR kit AMV Ver. 2.1 (Takara Bio Inc.). Amplification mixtures were separated by agarose gel electrophoresis, and the amplified products (bands of around 2.4 kb length) were recovered with the Ultraclean Gel Spin DNA Purification kit (MO Bio Laboratories Inc., Carlsbad, CA). DNA sequences of the cDNAs were determined using an ABI377 automatic DNA sequencer. The resultant nucleotide sequence data were registered in DDBJ/EMBL/GenBank databases under accession number AB263100 for guinea pig Arnt and AB263099 for Syrian hamster Arnt.

Plasmid constructions: Using the ReverTra Dash kit (Toyobo Co., Ltd.), the sequence of guinea pig AhR cDNA (17) was amplified from lung RNA as described in the previous section with a set of gene-specific primers having adapter sequences, 5’-ATCACGTACGATGAA-CAGCGGCGAGCGCAAA and 5’-TAGACAAATTGTCGACAGAATCCACTGGGTGC (adapter sequences are underlined). The sequence information of cDNA was obtained from the GenBank database. Amplified guinea pig AhR cDNA was digested using restriction enzymes BsiWI and MfeI, and subsequently inserted into the Acc65I/EcoRI region of the bidirectional expression vector YEpplac181. The resultant plasmid was designated as YEpplac181gpAhR. Guinea pig Arnt cDNA was subjected to PCR with a set of primers: 5’-ATGCTAGATGGCGGCGACTACGCTAACCC and 5’-ATCACGTACGAGGCCGAATTCTGAAAAGGGG-GGAAC (adapter sequences are underlined), and the amplified product was digested by restriction enzymes XbaI and SfI. The amplified product was subsequently inserted into the XbaI/SfI region of YEpplac181gpAhR. The resultant plasmid was designated as YEpplac181gpAhR/Arnt.

The sequence of Syrian hamster AhR cDNA (16) was amplified by reverse transcriptase PCR (RT-PCR) with a set of gene-specific primers with adapter sequences: 5’-ATCACGTACGATGAA-CAGCGGCGAGCGCAAA and 5’-TAGACAAATTGTCGACAGAATCCACTGGGTGC (adapter sequences are underlined). The sequence information of cDNA was obtained from the GenBank database. Amplified Syrian hamster AhR cDNA was digested by restriction enzymes BsiWI and MfeI, and subsequently inserted into the Acc65I/EcoRI region of YEpplac181. The resultant plasmid was designated as YEpplac181shAhR. Syrian hamster Arnt cDNA was subjected to PCR with a set of primers: 5’-ATGCTAGATGGCGGCGACTACGCTAACCC and 5’-ATCACGTACGAGGCCGAATTCTGAAAAGGGG-GGAAC (adapter sequences are underlined), and the amplified product was digested by restriction enzymes XbaI and SfI. The amplified product was subsequently inserted into the XbaI/SfI region of YEpplac181shAhR. The resultant plasmid was designated as YEpplac181shAhR/Arnt.

The region between guinea pig Arnt and AhR in YEpplac181gpAhR/Arnt was amplified by PCR with primers 5’-ATGACAAATTGTCGACAGAATCCACTGGGTGC, and then digested by SfI and phosphorylated by T4 polynucleotide kinase. The resulting fragment that contains guinea pig AhR cDNA, a bidirectional promoter sequence and guinea pig Arnt cDNA was inserted into the SfI/SmaI region of the integration vector pAUR101. The resultant plasmid was designated as YEpplac181gpAhR/Arnt.
designated as pAUR101gpAhR/Arnt.

The region between Syrian hamster Arnt and AhR in YEPlac181shAhR/Arnt was amplified by PCR with primers 5’-TAGATCTAGACTACAGGGAATCCGGCTG-GGTTGTG and 5’-TAGACCTGCAGCTATCCWGA-AAAGGGGGAAC, and then phosphorylated by T4 polynucleotide kinase. The resulting fragment containing Syrian hamster AhR cDNA, a bidirectional promoter sequence and Syrian hamster Arnt cDNA was inserted into the Smal site of integration vector pAUR101. The resultant plasmid was designated as pAUR101shAhR/Arnt. Base sequence analysis was carried out to confirm no mutations caused by PCR during plasmid construction.

**Establishment of assay yeasts:** After linearization of pAUR101gpAhR/Arnt and pAUR101shAhR/Arnt by BsiWI, these plasmids were introduced into W303a (MATa, ade2-1, can1-100, his3-11,15, leu2-3,11, trp1-1, ura3-1), and transformants were selected by aureobasidin. The aur1 gene on chromosome XI is the target of plasmid integration by homologous recombination. Integration of single pAUR101gpAhR/Arnt or pAUR101shAhR/Arnt into the yeast chromosome was confirmed by PCR (data not shown). The TRP1-marked reporter plasmid pTXRE5-Z, which has five XREs upstream of the lacZ gene (22), was then introduced into the transformants. Several clones were randomly chosen, and one of them was used for further study. The resultant reporter yeast strains expressing guinea pig AhR/Arnt and Syrian hamster AhR/Arnt were designated as OPU-GP and OPU-SH, respectively. Since cDNAs of AhR and Arnt are inserted downstream of the reporter plasmid, pTXRE5-Z, which has five XREs upstream of the lacZ gene (22) was then introduced into the transformants. Several clones were randomly chosen, and one of them was used for further study. The resultant reporter yeast strains expressing guinea pig AhR/Arnt and Syrian hamster AhR/Arnt were designated as OPU-GP and OPU-SH, respectively. Since cDNAs of AhR and Arnt are inserted downstream of the lacZ gene (22), these yeasts have the same genetic background except for the species of AhR/Arnt. In these yeasts, AhR and Arnt cDNAs are integrated into chromosome XI. AhR and Arnt are expressed from galactose-regulated GAL1 and 10 promoters. Transcriptional activation mediated by the AhR/Arnt heterodimer is assessed by β-galactosidase activity. Expression of the lacZ reporter plasmid, pTXRE5-Z, is directed by AhR/Arnt complex binding to five artificially synthesized xenobiotic responsive element (5×XRE) sequences in the promoter region (a minimal basal promoter of the yeast cytochrome C gene (CYC1 promoter)).

**Results**

The features of the recombinant yeast strain. Species-specific AhR and Arnt cDNAs are integrated into chromosome XI. AhR and Arnt are expressed from galactose-regulated GAL1 and 10 promoters. Transcriptional activation mediated by the AhR/Arnt heterodimer is assessed by β-galactosidase activity. Expression of the LacZ reporter plasmid, pTXRE5-Z, is directed by AhR/Arnt complex binding to five artificially synthesized xenobiotic responsive element (5×XRE) sequences in the promoter region (a minimal basal promoter of the yeast cytochrome C gene (CYC1 promoter)).

The features of the recombinant yeast strain. Species-specific AhR and Arnt cDNAs are integrated into chromosome XI. AhR and Arnt are expressed from galactose-regulated GAL1 and 10 promoters. Transcriptional activation mediated by the AhR/Arnt heterodimer is assessed by β-galactosidase activity. Expression of the LacZ reporter plasmid, pTXRE5-Z, is directed by AhR/Arnt complex binding to five artificially synthesized xenobiotic responsive element (5×XRE) sequences in the promoter region (a minimal basal promoter of the yeast cytochrome C gene (CYC1 promoter)).

Calculated by the following formula: (absorbance at 405 nm × 1000) ÷ (absorbance at 595 nm) × (mL of cell suspension added) × (minute of reaction time). The increase in lacZ units was calculated by subtracting the lacZ units for the negative control (i.e. yeast exposed to (CH3)2SO).

**Establishment of AhR Reporter Yeasts**

![Image](https://via.placeholder.com/150)

**Fig. 1.** The features of the recombinant yeast strain. Species-specific AhR and Arnt cDNAs are integrated into chromosome XI. AhR and Arnt are expressed from galactose-regulated GAL1 and 10 promoters. Transcriptional activation mediated by the AhR/Arnt heterodimer is assessed by β-galactosidase activity. Expression of the LacZ reporter plasmid, pTXRE5-Z, is directed by AhR/Arnt complex binding to five artificially synthesized xenobiotic responsive element (5×XRE) sequences in the promoter region (a minimal basal promoter of the yeast cytochrome C gene (CYC1 promoter)).
targeted only chromosome XI in this study.

Using the above five strains, we examined ligand activities of TCDD, 3-methylcholanthrene, β-naphthoflavone and indirubin. Animal species specificity of the toxicity of these chemicals except for TCDD has not been reported, but these compounds are known as potent ligands to human AhR (22,25). The yeast without AhR/Arnt cDNA (OPU-C) did not respond to the ligands at any concentration (data not shown). In contrast, the strains with AhR/Arnt cDNAs responded dose-dependently to all test chemicals (Fig. 2).

The EC₅₀ values of the tested chemicals in the four yeast strains with AhR/Arnt cDNAs are summarized in Table 1. The EC₅₀ values of indirubin were 0.07-40 nM in the four yeast strains, and indirubin was the most intense ligand among the four chemicals in all strains. The EC₅₀ values of TCDD and β-NF were 0.3–400 nM, and the ligand activities of 3-MC were weakest (EC₅₀ = 15–1000 nM) in all strains. Against these ligands, OPU-GP was most sensitive, and OPU-SH was most insensitive. OPU-H and OPU-M were in-between, and OPU-H was slightly more sensitive than OPU-M.
Table 1. EC₅₀ values [nM] of the ligands in the yeast strains expressing various AhR/Arnt

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Yeast strain* (Origin of AhR/Arnt)</th>
<th>OPU-GP (Guinea pig)</th>
<th>OPU-H** (Human)</th>
<th>OPU-M** (Mouse)</th>
<th>OPU-SH (Syrian hamster)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirubin</td>
<td></td>
<td>0.07</td>
<td>0.4</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>TCDD</td>
<td></td>
<td>2.5</td>
<td>10</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>β-NF</td>
<td></td>
<td>0.3</td>
<td>7</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>3-MC</td>
<td></td>
<td>15</td>
<td>70</td>
<td>90</td>
<td>1000</td>
</tr>
</tbody>
</table>

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; β-NF: β-naphthoflavone; 3-MC: 3-methylcholanthrene;

*In addition to these strains, OPU-C containing no AhR/Arnt cDNA was established as a negative control. OPU-C did not respond to these ligands.

**OPU-H and OPU-M were indicated as OPUH-11 and OPUM-11 in the previous report, respectively (23).

Discussion

The yeast strains established in this study have an identical promoter for AhR/Arnt. They should have the same cell membrane permeability and metabolism for the chemicals because of the same genetic background. Thus the difference in the ligand activity should be due to species-specific AhR/Arnt.

AhR protein consists of several functional modules. A highly conserved DNA binding domain is located in the N-terminus and a ligand binding domain is situated in the center. The C-terminus of AhR contains a potent transactivation domain composed of several interacting subdomains, one of which is a Q-rich subunit, which has been shown to be essential for the transactivating function of AhR in vitro (17,26–28). Korkalainen et al. reported a distinct correlation between the number of glutamine residues in the Q-rich subdomain and the LD₅₀ values of TCDD, i.e., the numbers of glutamine residues of the most sensitive guinea pig, human, mouse (C57BL/6) and most resistant Syrian hamster were 23, 25, 28 and 49, respectively (17). In this study, OPU-GP was most sensitive to TCDD, and OPU-SH was most insensitive. OPU-H and OPU-M were in-between. The level of ligand activity in our reporter yeasts may reflect the variation of the primary structure in the Q-rich subunit.

In this study, we cloned Arnt cDNAs of guinea pig and Syrian hamster. The base sequences showed that the open-reading frame (ORF) of guinea pig cDNA consists of 2373 bp and encodes a 790-amino acid protein, and the ORF of Syrian hamster cDNA consists of 2337 bp and encodes a 797-amino acid protein. Unlike AhR (16), the amino acid sequence of Arnt is highly conserved among these four species. The overall amino acid identity of guinea pig and Syrian hamster to human Arnt (789-amino acid protein) was 96% and 90%, respectively. Because of such high similarity, the genetic difference of Arnt would not contribute significantly to the different ligand activities of the yeasts.

Unexpectedly, OPU-GP showed the low level of β-galactosidase activity without ligand treatment (data not shown). This background activity would be ligand-independent activation of AhR. The increase in lacZ units, calculated by subtracting the lacZ units for the negative control, was shown in Fig. 2 in order to represent ligand dependency. Similarly, the reporter system of HeLa cells that express guinea pig AhR shows a high background level of AhR activation (Osako et al. unpublished observations). These differences of ligand-independent AhR activation are supposed to be dependent on the response to unknown endogenous ligands.

The AhR ligand potency alone does not explain the toxic effects of aryl hydrocarbons, but contributes to most of the effects. A set of our reporter yeasts that express guinea pig, human, mouse and Syrian hamster AhR/Arnt cDNAs would be valuable to examine the species-specific effects of TCDD and TCDD-like chemicals to estimate the human risk of these chemicals from animal data.

In the AhR study, reporter systems using mammalian cells are frequently used to investigate ligand activity. However, the mammalian cells could metabolize test chemicals by their intrinsic enzymes, and consequently convert to other chemical forms. Thus, the determined ligand activity of the test chemicals might be inaccurate in some cases. Unlike mammalian cells, yeasts do not have these intrinsic enzymes, and therefore, our yeast reporter system is suitable for toxicological research.

Instrumental analysis of environmental pollutants provides reliable data only when the analytes are known and analytical standards are available for quantitative analysis. In our reporter yeasts, the level of β-galactosidase activity indicates the level of transcriptional activation following ligand binding to AhR. Therefore this bioassay system can be a powerful tool to evaluate total AhR signaling activity in environmental samples.

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