Establishment of a Rat Hepatic Cell Line, KanR2-XL8, for a Reporter Gene Assay of Aryl Hydrocarbon Receptor Ligands

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To establish a highly sensitive and convenient reporter gene assay for aryl hydrocarbon receptor (AhR) ligands, the chimera plasmid xenobiotic responsible element-luciferase (XRE-Luc) containing an XRE, minimal SV40 promoter, and luciferase reporter gene, was first constructed. The XRE-Luc and the expression vector pRC/CMV containing a neomycin-resistant gene were transfected into a rat hepatic cell line, Kan-R2, and then KanR2-XL8 was selected as a cell clone, which showed the highest response to the induction of luciferase by aryl hydrocarbons, 3-methylcholanthrene (3-MC) and benzo[a]pyrene. Furthermore, AhR-regulated genes, CYP1A1 and CYP1B1, in KanR2-XL8 cells were also activated by 3-MC. In the present study, we established a hepatic cell line, KanR2-XL8, that is useful for screening of AhR ligands with two parameters, the activations of the transfected luciferase gene and the AhR-regulated genes in a host cell.

Key words —— aryl hydrocarbon receptor ligand, luciferase assay, rat hepatocyte, CYP1A1, CYP1B1

INTRODUCTION

Hepatic cytochrome P450 (P450) isoforms play important roles in the metabolism including detoxification and metabolic activation of xenobiotics and are generally induced by their own substrates. For example, hepatic CYP1A subfamily enzymes responsible for metabolic activation of environmental carcinogens, including aryl hydrocarbons (Ah) and aromatic amines, are induced by exposure to the carcinogen in experimental animals.1,2) Furthermore, there are species and sex differences in the induction of hepatic CYP1A subfamily enzyme(s), and the differences are closely correlated with differences in the carcinogenic susceptibilities of mice and rats.3–5) Induction of CYP1A subfamily enzymes, especially CYP1A1, by Ah, such as benzo[a]pyrene (B[a]P) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been known to be induced in an Ah receptor (AhR)-dependent manner.6) Furthermore, AhR-null mice have been reported to be more resistant to B[a]P-mediated carcinogenesis and TCDD-mediated teratogenesis than wild-type mice.7–9) These previous findings revealed that AhR plays an important role in not only gene activation of CYP1 family enzymes but also AhR ligand-mediated toxicity. Therefore the development of a method for screening of environmental AhR ligands is thought to be important for the prevention of chemical-mediated disease.

Recently, we have established a rat hepatic cell line, Kan-R2, which expresses constitutively the AhR gene and the AhR-dependent genes including CYP1A1 and CYP1B1 and responds to an AhR ligand-mediated CYP1A1 induction.10) In the present study, we first constructed the plasmid xenobiotic responsible element-luciferase (XRE-Luc), which contains an AhR enhancer (XRE), minimal SV40 promoter, and Luc reporter gene, and established a KanR2-XL8 cell line useful for the screening of AhR ligands by transfection of the XRE-Luc plasmid into Kan-R2 cells.

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MATERIALS AND METHODS

Chemicals ——— 3-MC, B[a]P, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, U.S.A.). G418 was obtained from Invitrogen (Gaithersburg, CA, U.S.A.). Reporter Lysis Buffer and Luciferase Assay Reagent were purchased from Promega (Madison, WI, U.S.A.).

Construction of Reporter Plasmid ——— The core XRE sequence in the promoter region of rat CYP1A1 gene (−1024 to −1007 bp)11 was used for the construction of a reporter plasmid. The DNA fragment containing three copies of the XRE sequence was synthesized with the polymerase chain reaction (PCR) and inserted into NheI-MluI site of PicaGene Promoter Vector 2 (Toyo Ink Co. Ltd., Japan), which contains the minimal SV40 promoter and luciferase reporter gene. This plasmid was designated XRE-Luc.

Stable Transfection of Reporter Plasmid ——— The rat hepatic cell line Kan-R2 was used as the host cells in the present experiment. An aliquot (1 × 10⁶ cells) of Kan-R2 cells suspended in the Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum was seeded in a 60-mm culture dish. After 24 hr, the adherent cells were washed twice with 2 ml of OPTI-MEM Reduced Serum Medium (Invitrogen), and cotransfected with 1 µg of XRE-Luc and 0.1 µg of pRC/CMV plasmid (Invitrogen) containing a neomycin-resistant gene by using of Lipofectamine 2000 (Invitrogen). After 3-hr culture, the medium was replaced with DMEM, and the cells were cultured for 24 hr. After the culture, the cells were stripped with 0.1% trypsin, and part (1 × 10⁶ cells) of the cell suspension was inoculated into a 100-mm culture dish. After 24 hr, the resultant cell lysate was frozen at −80°C for 30 min, thawed at room temperature, and centrifuged at 20000 g for 5 min. The protein concentration in the resultant supernatant was measured with a BCA-protein Assay Kit (Pierce, Rockford, IL, U.S.A.) and adjusted to 1 mg protein per ml with PBS. A portion (10 µl) of the resultant supernatant was mixed with 50 µl of Luciferase Assay Reagent, and the amount of light product was measured immediately with a Lumat LB9507 Luminometer (EG&G Berthold, Bad Wildbad, Germany). Luciferase activity was represented as a luminescence unit per mg of protein.

RT-PCR Analysis ——— RT-PCR was performed according to the method previously described.11 Briefly, an aliquot (5 × 10⁴ cell/dish) of KanR2-XL8 cells were seeded into a 60-mm dish and precultured for 48 hr. After the preculture, cells were further incubated in the medium containing 3-MC (10⁻⁹–10⁻⁵ M) or vehicle (0.1% DMSO) for the indicated times. After the chemical treatment, total RNA was isolated from the cells with Isogen (NipponGene, Toyama, Japan) and used for the RT-PCR analysis of the expression of CYP1A1, CYP1B1, and GAPDH.

A portion (4 µg) of total RNA was converted to cDNA using poly d(N)₆ primer (Pharmacia Biotech, Piscataway, NJ, U.S.A.) and MMLV Reverse Transcriptase (Invitrogen) in an RT reaction mixture (20 µl). PCR was performed in a total reaction mixture (25 µl) containing RT reaction mixture (0.8 µl), the corresponding primer sets (12.5 pmol), and 0.625 unit of AmpliTaq Gold (Perkin-Elmer, Norwalk, CT, U.S.A.). The primer sets used in the present study were as follows: CYP1A1, 5’-TGA CCT CTT TGG AGC AGC TGG TTA-3’ (forward) and 5’-TTG AGC CTC AGC AGA T-3’ (reverse); CYP1B1, 5’-ACC GCA ACT TCA GCA ACT TC-3’ (forward) and 5’-GTG TTG GCA GTG GTG GTG GCAG TC-3’ (reverse), and GAPDH, 5’-TTC AAC GGC ACA GTC AAG G-3’ (forward) and 5’-CAT GGA CTG TGG TCA G-3’ (reverse).

The PCR amplification protocol consisted of the preactivation of AmpliTaq Gold for 10 min at 95°C, 20–30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C for CYP1A1 or 60°C for CYP1B1 and GAPDH, and elongation for 1 min at 72°C with a GeneAmp PCR system model 9600 (Perkin-Elmer). The PCR products were separated by electrophoresis on a 2% agarose gel and moved, and cells in each well were washed twice with phosphate-buffered saline (PBS). Thereafter, 1 × Reporter Lysis Buffer (100 µl) was added to each well. After 15 min, the resultant cell lysate was frozen at −80°C for 30 min, thawed at room temperature, and centrifuged at 20000 g for 5 min. The protein concentration in the resultant supernatant was measured with a BCA-protein Assay Kit (Pierce, Rockford, IL, U.S.A.) and adjusted to 1 mg protein per ml with PBS. A portion (10 µl) of the resultant supernatant was mixed with 50 µl of Luciferase Assay Reagent, and the amount of light product was measured immediately with a Lumat LB9507 Luminometer (EG&G Berthold, Bad Wildbad, Germany). Luciferase activity was represented as a luminescence unit per mg of protein.
visualized by ethidium bromide staining under UV light. The amount of each PCR product was densitometrically quantified with a computer using Kodak 1D Image Analysis Software (Macintosh 1D version 2.02). The expression level of each gene was normalized to that of GAPDH.

RESULTS

Construction of KanR2-XL8 Cells for Screening AhR Ligands

We constructed the new reporter plasmid XRE-Luc (Fig. 1) by inserting the XRE sequence (5′-CTCTTCTCACGCAAACCTC-3′), which is located at –1024 to –1007 bp in the rat CYP1A1 gene promoter, into the PicaGene Promoter Vector 2 containing the SV40 promoter and luciferase reporter gene. The XRE-Luc and pRC/CMV vector containing the neomycin-resistant gene were cotransfected into the rat hepatic cell line Kan-R2, which constitutively expresses CYP1A1 and AhR. From 30 clones selected as G418-resistant cells, 6 clones (KanR2-XL1, -XL3, -XL7, -XL8, -XL20, and -XL21) stably expressing the luciferase gene were obtained.

Among the six clones, the sensitivities toward AhR ligands were compared in a luciferase assay (Table 1). In the all clones examined, the luciferase activity was increased by treatment with the AhR ligands, 3-MC and B[a]P (10−5 M). In particular, the KanR2-XL8 had the highest potency and therefore was used for further experiments.

Induction of Luciferase Activity by AhR Ligands in KanR2-XL8 Cells

KanR2-XL8 cells were treated with an AhR ligand, either 3-MC or B[a]P (each 10−5 M) for 1, 3, 6, 12, and 24 hr, and the time course of change in luciferase activity was examined (Fig. 2). Significant increases in luciferase activity were observed at 1 and 3 hr after treatment with 3-MC and B[a]P, respectively. The increased levels reached the maximum at 3 hr and were retained up to 24 hr.

Therefore the dose dependency of the induction of luciferase activity by 3-MC and B[a]P was examined 3 hr after AhR ligand treatment (Fig. 3). Luciferase activity in KanR2-XL8 cells was significantly increased at a concentration of 10−9 M either 3-MC or B[a]P, and the increased activity reached the maximum at concentrations of 10−7 M and

<table>
<thead>
<tr>
<th>(Clone)</th>
<th>Vehicle alone</th>
<th>3-MC (10−6 M)</th>
<th>B[a]P (10−6 M)</th>
<th>Induction ratio (vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KanR2-XL1</td>
<td>2.88 × 10^6</td>
<td>5.64 × 10^6</td>
<td>4.78 × 10^6</td>
<td>1.96</td>
</tr>
<tr>
<td>KanR2-XL3</td>
<td>5.69 × 10^5</td>
<td>13.13 × 10^5</td>
<td>12.70 × 10^5</td>
<td>2.31</td>
</tr>
<tr>
<td>KanR2-XL7</td>
<td>4.09 × 10^4</td>
<td>8.48 × 10^4</td>
<td>10.38 × 10^4</td>
<td>2.07</td>
</tr>
<tr>
<td>KanR2-XL8</td>
<td>4.11 × 10^4</td>
<td>13.33 × 10^4</td>
<td>11.50 × 10^4</td>
<td>3.23</td>
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<tr>
<td>KanR2-XL20</td>
<td>1.85 × 10^5</td>
<td>2.86 × 10^5</td>
<td>2.18 × 10^5</td>
<td>1.55</td>
</tr>
<tr>
<td>KanR2-XL21</td>
<td>4.70 × 10^4</td>
<td>7.89 × 10^4</td>
<td>6.26 × 10^4</td>
<td>1.68</td>
</tr>
</tbody>
</table>

Each cell clone was treated with vehicle alone, 3-MC (10−6 M), or B[a]P (10−6 M) for 24 hr, and luciferase assay was performed as described in the MATERIALS AND METHODS section. The luciferase activity and induction ratio represented are the mean of four individual samples.
The EC₅₀ values of 3-MC and B[a]P for the induction of luciferase were 2.92 × 10⁻⁹ M and 1.13 × 10⁻⁹ M, respectively.

**Activation of AhR-Regulated Genes by 3-MC**

Changes in expression levels of AhR-regulated genes, such as CYP1A1 and CYP1B1, 3 hr after the treatment of KanR2-XL8 cells with 3-MC (10⁻⁵ M) were first examined using RT-PCR. Significant increases in expression levels of CYP1A1 and CYP1B1 after 3-MC treatment were observed (Fig. 4).

Subsequently, the dose effects of 3-MC on the expression of CYP1A1 and CYP1B1 were examined. Expression levels of CYP1A1 and CYP1B1 increased in a dose dependent manner over the concentration range from 10⁻⁹ to 10⁻⁷ M (Fig. 5). In addition, no significant change in the expression level of GAPDH was observed at any concentration examined.

**DISCUSSION**

In the present study, we established a rat hepatic cell line, KanR2-XL8, by transfecting the XRE-Luc reporter plasmid into Kan-R2, which was found useful as a luciferase reporter gene assay for AhR ligands. The reporter gene assay with KanR2-XL8 had higher sensitivity, as judging from 3-MC- or B[a]P-induced luciferase activity, than those reported previously (Table 2). Although several luciferase reporter plasmids, such as pGudLuc1.1,12,13) pL1A1N,14) and pGreen1.1,15) have been constructed for screening for AhR ligands (Table 2), these plasmids contain not only the XRE core sequence but also other transcription factor binding sequences of the CYP1A1 promoter, and assays with these plasmids might not be sufficiently selective in screening for AhR ligands.

*CYP1A1* and *CYP1B1* are well-known AhR-
Fig. 4. Time-Course Changes in the Expression of CYP1A1 and CYP1B1 after Treatment of KanR2-XL8 Cells with 3-MC

KanR2-XL8 cells were treated with 3-MC (10⁻⁹ M) or vehicle alone (0.1% DMSO) for the indicated times. Total RNA was prepared from each chemical-treated cells and used for RT-PCR analysis of the expression of CYP1A1 and CYP1B1. RT-PCR products from four individual samples in each experimental group were mixed, and the mixture was subjected to agarose gel electrophoresis (A). The values on the right side of each graph represent the number of PCR cycles performed. The expression levels of CYP1A1 and CYP1B1 were measured using individual samples in each experimental group, calculated on the basis of that of GAPDH, and compared with the corresponding controls (B). Closed circles show the gene expression level in individual samples, and open circles represent the mean in each experimental group (n = 4). Significant differences from the corresponding controls assayed with ANOVA and Dunnett’s test; *p < 0.05, **p < 0.01.

regulated genes. Therefore the altered expression of CYP1A1 and CYP1B1 by 3-MC was further examined in KanR2-XL8 cells. Expression levels of these AhR-regulated genes were significantly increased by treatment with 3-MC at a concentration of greater than 10⁻⁹ M. Thus KanR2-XL8 was demonstrated to respond to the AhR ligand-induced activation of both the transfected luciferase gene and the AhR-regulated genes in host cells.

In conclusion, we establish a rat hepatic cell line, KanR2-XL8, transfected with the plasmid XRE-Luc and demonstrated that the cell line is a useful tool for the assay of AhR ligands with two parameters, the activation of the transfected luciferase gene and the AhR-regulated genes in the host cells.

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Fig. 5. Dose-Dependent Changes in the Expression of CYP1A1 and CYP1B1 after Treatment of KanR2-XL8 Cells with 3-MC
KanR2-XL8 cells were treated with various concentrations of 3-MC for 3 hr. Total RNA was prepared and used for RT-PCR analysis. RT-PCR products from four individual samples in each experimental group were mixed, and the mixture was subjected to agarose gel electrophoresis (A). The values on the right side of each graph represent the number of PCR cycles performed. The expression levels of CYP1A1 and CYP1B1 were independently measured using individual samples in each experimental group, calculated on the basis of that of GAPDH, and compared with the corresponding controls (B). Closed circles show the gene expression level in individual samples, and open circles represent the mean in each experimental group (n = 4). Significant differences from the corresponding controls assayed with ANOVA and Dunnett’s test; *p < 0.05, **p < 0.01.

Table 2. Comparison of the Current Hepatocyte-Based Reporter Gene Assays for Detection of AhR Ligands

<table>
<thead>
<tr>
<th>Cell line (Origins)</th>
<th>Species</th>
<th>Vector name</th>
<th>Enhancer</th>
<th>Promoter</th>
<th>Reporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>KanR2-XL8 (Kan-R2)</td>
<td>Rat</td>
<td>XRE-Luc</td>
<td>Three copies of rat CYP1A1 XRE sequence (−1024 to −1007)</td>
<td>SV40</td>
<td>Luciferase</td>
</tr>
<tr>
<td>H4L1.1c4 (H4IIE)</td>
<td>Rat</td>
<td>pGudLuc1.1</td>
<td>rat CYP1A1 Promoter (−1301 to −819)</td>
<td>MMTV</td>
<td>Luciferase</td>
</tr>
<tr>
<td>101L (HepG2)</td>
<td>Human</td>
<td>pL1A1N</td>
<td>human CYP1A1 Promoter (−1612 to +292)</td>
<td>SV40</td>
<td>Luciferase</td>
</tr>
<tr>
<td>H1G1.1c3 (Hepa-1)</td>
<td>Mouse</td>
<td>pGreen1.1</td>
<td>rat CYP1A1 Promoter (−1301 to −819)</td>
<td>MMTV</td>
<td>GFP</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line (Origins)</th>
<th>EC₅₀ (3-MC)</th>
<th>EC₅₀ (B[a]P)</th>
<th>Reaction time (hr)</th>
<th>Reference</th>
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<tr>
<td>KanR2-XL8 (Kan-R2)</td>
<td>2.9 × 10⁻⁹ M</td>
<td>1.1 × 10⁻⁹ M</td>
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<td>This paper</td>
</tr>
<tr>
<td>H4L1.1c4 (H4IIE)</td>
<td>2.9 × 10⁻⁹ M</td>
<td>1.1 × 10⁻⁹ M</td>
<td>3</td>
<td>Machala, M., 2001¹²</td>
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<tr>
<td>101L (HepG2)</td>
<td>4.2 × 10⁻⁷ M</td>
<td>8.1 × 10⁻⁶ M</td>
<td>12</td>
<td>Postlind, H., 1993¹⁴</td>
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<tr>
<td>H1G1.1c3 (Hepa-1)</td>
<td>2.9 × 10⁻⁹ M</td>
<td>1.1 × 10⁻⁹ M</td>
<td>24</td>
<td>Nagy, S. R., 2002¹⁵</td>
</tr>
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</table>

a) Not determined. b) The EC₅₀ values were calculated from the figures of individual reports.
REFERENCES


