Induction of Cytochrome P-450 1A2 by Oxidized Tryptophan in Hepa lclc7 Cells

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ABSTRACT

Recent studies from this laboratory have demonstrated that l-tryptophan, after oxidation either by UV-irradiation or ozone, induces ary1 hydrocarbon receptor (AhR) activation and binding of the liganded AhR complex to its specific DNA recognition site, thereby initiating transcription of the cytochrome P-450 1A1 (Cyp1a1) gene with concomitant increase of CYP1A1 protein and 7-ethoxyresorufin O-deethylase activity in wild-type mouse hepatoma cells, Hepa lclc7 (Hepa-1), in culture. Temporary inhibition of protein synthesis by cycloheximide resulted in superinduction of oxidized tryptophan-inducible CYP1A1 mRNA, protein, and 7-ethoxyresorufin O-deethylase activity in Hepa-1 cells. In the present communication, the results obtained by immunoblot analyses with monoclonal CYP1A1/1A2 antibody (NIH 1-7-1) demonstrate that both UV- or ozone-oxidized tryptophan also induce CYP1A2 protein in Hepa-1 cells. CYP1A2 mRNA, detected by reverse transcription-polymerase chain reaction, was markedly induced in the UV- or ozone-oxidized tryptophan-treated cells. Temporary inhibition of protein synthesis by cycloheximide further induced oxidized tryptophan-inducible CYP1A2 mRNA as well as the protein in Hepa-1 cells. This is the first report demonstrating the induction of CYP1A2 mRNA and protein in Hepa-1 cells.

Cytochrome P-450 (CYP) is a superfamily of hemoproteins that are able to metabolize a large number of endogenous and exogenous compounds through oxidative, reductive, and peroxidative mechanisms. According to the current estimates, the P-450 gene family comprises 74 families, 14 of which exist in all mammals examined to date (Nelson et al., 1996). In mammals, the CYP family consists of three isozymes, CYP1A1, 1A2, and 1B1 (Nelson et al., 1996). Although no endogenous substrate for CYP1A1 has been found to date, this isozyme actively metabolizes benzo[a]pyrene and several other polycyclic aromatic hydrocarbons (PAHs) (Guengerich and Shimada, 1991). In contrast, CYP1A2 exhibits a high level of catalytic activity toward 2-acetylaminofluorene, acetalidile, aflatoxin B1, 4-aminobiphenyl, and other arylamines (McMurray et al., 1984; Kailubur and Hammons, 1987; Paletto et al., 1988). Many of these PAHs and arylamine substrates are metabolized by CYP1A1 and 1A2 to carcinogenic and toxic intermediates. Therefore, the differences in individual risk of carcinogenesis or toxicity might be correlated with the level of expression of either of these genes in a particular tissue.

In general, CYP1A1 is not expressed in normal adult animal tissues but can be induced severalfold in response to several stimuli such as PAHs or halogenated aromatic hydrocarbons (for review, see Gonzalez, 1989), hyperoxia (Okamoto et al., 1993; Khatsenko et al., 1997), or oxidized tryptophan (Sindhu et al., 1996b, Sindhu and Kikkawa, 1999). CYP1A2, in contrast, is known to be constitutively expressed in animals and is inducible in the liver after treatment by a variety of exogenous substances, including PAHs, ingestion of charbroiled meat, certain unknown components of cruciferous vegetables, dietary heterocyclic amines, and certain drugs (for review, see Guengerich and Shimada, 1991; Wrighton and Stevens, 1992 and references therein). In the aryl hydrocarbon (Ah)-responsive mouse liver, the levels of constitutively expressed CYP1A2 mRNA levels appear to be at least as high as maximally induced CYP1A1 mRNA levels (Gonzalez et al., 1984). However, many liver-derived cell lines are known to rapidly lose the expression of CYP1A2. Therefore, in contrast to CYP1A1, the detailed molecular mechanism(s) underlying the expression of CYP1A2 are not very well defined.

Mouse hepatoma-derived Hepa lclc7 (Hepa-1) cell line has been considered as an excellent model for CYP1A1 studies and the wild-type and variant Hepa-1 cells have been used by numerous laboratories to investigate the molecular and biochemical mechanisms of CYP1A1 induction. Hepa-1 cells are known to be inducible for CYP1A1 and retain the same high

ABBREVIATIONS: CYP, cytochrome P-450; PAH, polycyclic aromatic hydrocarbon; Ah, aryl hydrocarbon; OT, ozone-oxidized tryptophan; UVT, UV-oxidized tryptophan; AhR, aryl hydrocarbon receptor; EROD, 7-ethoxyresorufin O-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair; MC, 3-methylcholanthrene.

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level of inducible CYP1A1 activity during several months in culture (for review, see Hankinson, 1994). Additionally, no detectable heterogeneity in CYP1A1 activity has been observed among subclones of this line (Hankinson, 1994). Furthermore, mutants of the Hepa-1 cell line that are defective in induction of CYP1A1 have been isolated and characterized (Hankinson, 1994). However, CYP1A2 in this cell line was either thought not to be expressed or, if expressed at all, it was thought to be below the levels of detection.

For the past several years, this laboratory has been investigating the molecular and biochemical mechanisms of CYP1A1 induction by oxidized tryptophan in wild-type mouse Hepa-1 cells, in culture. As a part of this project, we have recently demonstrated that l-tryptophan, after oxidation by either ozone (OT) or UV-irradiation (UVT), induces aryl hydrocarbon receptor (AhR) activation and binding of the liganded AhR complex to its specific DNA recognition site, thereby inducing transcription of the Cyp1a1 gene with concomitant increase of CYP1A1 protein and 7-ethoxyresorufin O-deethylase (EROD) activity in wild-type Hepa-1 cells (Sindhu et al., 1996b, 1999). The induction of CYP1A1 by UV-oxidized tryptophan was effectively inhibited by administration of a 15-mer antisense phosphorothioate oligonucleotide that was targeted to the Cyp1a1 gene (Sindhu et al., 1996a). More recently, we have found that temporary inhibition of protein synthesis by cycloheximide causes superinduction of UVT- or OT-inducible CYP1A1 mRNA, protein and EROD activity in wild-type Hepa-1 cells (Sindhu and Kikkawa, 1999). The present investigation was undertaken as a corollary to this project. The results obtained in the present study demonstrate that administration of OT or UVT to wild-type Hepa-1 cells causes induction of CYP1A2 mRNA and the protein. Temporary inhibition of protein synthesis by cycloheximide was found to result in the superinduction of CYP1A2 mRNA as well as the protein.

Experimental Procedures

Materials. Cycloheximide, actinomycin D, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). L-Tryptophan was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was of highest purity commercially available. 7-Ethoxyresorufin and resorufin were purchased from Pierce (Rockford, IL). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Midwest Research Institute, Kansas City, MO. Monoclonal CYP1A1/1A2 antibody (NIH 1-7-1) was generously provided by Dr. S. S. Park of National Cancer Institute, Frederick, MD. All other chemicals were of highest purity commercially available.

Oxidation of Tryptophan. O3 was generated by passing medical grade oxygen as detailed in Yan et al. (1999). Ozonation of tryptophan was carried out by bubbling a stream of O3 (196 mg/m3, 100 ppm) through a fine capillary into 500 ml of 2 mM aqueous solution of tryptophan for 24 h. Glass-distilled water, used routinely for the experiments, was also ozonized along with tryptophan and used as one of the controls.

UV oxidation of 2 mM tryptophan was carried out with a germicidal UV light for 6 h at a distance of ~15 cm above the solution as described (Sindhu et al., 1996b). The oxidized solutions were protected from light and stored in the refrigerator.

To confirm the oxidation of tryptophan, unoxidized tryptophan, UVT, or OT were analyzed by reversed phase HPLC (Perkin-Elmer Series 410) equipped with a Zorbax C18 analytical column (4.6 x 250 mm, 5 μm) and an LC 235 diode array detector as described in Sindhu et al. (1996b, 1999) and Yan et al. (1999).

Cells and Growth Conditions. Wild-type Hepa-1 cells, generous gifts from Dr. Oliver Hankinson of UCLA, were propagated in ribonucleoside-free-minimum essential medium (Life Technologies, Paisley, Scotland) containing 5% heat inactivated fetal bovine serum (Gemini Biological Products, Calabasas, CA) and 1% antibiotic-antimycotic (Life Technologies) with 25-cm2 canted neck tissue culture flasks (Becton Dickinson, Mountain View, CA) as described previously (Sindhu et al., 1996b, 1999; Yan et al., 1999). When the cells were ~95% confluent, these were incubated with OT or UVT for a total of 4 h at 37°C as described in the text. The cells were treated with cycloheximide as indicated during the final minutes of oxidized tryptophan incubation period. At the end of treatment period, the medium containing oxidized tryptophan and cycloheximide was taken out and the cells were washed twice with 10 ml each of sterile PBS. After adding 10 ml of fresh medium, actinomycin D (1 μg/ml) was added and the cells were incubated at 37°C for 3 more hours. The cells were then washed twice with ice-cold PBS and scraped. The harvested cell suspension was centrifuged at 1500g for 10 min and the cell pellet was resuspended in 250 μl of sonication buffer (150 mM Tris-HCl, pH 8.0, containing 7.5% sucrose). The cell suspensions were sonicated on ice with a cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) at 50% power and an output setting of three with two periods of 10 s, interrupted by one interval of the same duration on ice to avoid overheating.

EROD activity was determined as described by Prough et al. (1978). Total protein content of the sonicated cells was measured with the Bio-Rad protein assay kit with bovine plasma γ-globulin as the standard.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. The disrupted cell extracts (10 μg of protein) were boiled with an equal volume of SDS-sample solubilization buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 125 mM Tris-HCl, pH 6.8) for 5 min. The samples were allowed to cool to room temperature and proteins were resolved by denaturing SDS-PAGE on discontinuous polyacrylamide (10%) slab gels. The proteins were then electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). The filters were blocked with SuperBlock blocking buffer (Pierce) containing 5% nonfat milk, washed thoroughly with Tris-buffered saline containing 0.1% Tween 20 and probed with monoclonal anti-CYP 1A1/1A2 (1-7-1). The antigen-antibody complexes were detected with a horseradish peroxidase-conjugated antibody and bovine IgG (Amersham, Arlington Heights, IL) with the enhanced chemiluminescence detection system (Amersham).

Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Hepa-1 cells, grown as described earlier, were incubated with OT or UVT (100 μM) for a total of 2 h in the presence or absence of cycloheximide. Cycloheximide (5 μg/ml medium) was included during the final 1 h of oxidized tryptophan treatment period. At the end of the incubation period, total RNA was isolated with ultrapure total RNA isolation reagent (Biotex Laboratories, Inc., Houston, TX) according to the manufacturer’s instructions except that the aqueous solution containing RNA was treated three times with phenol/chloroform/isoamyl alcohol (25:24:1) and then precipitated with isopropanol. The RNA pellet was washed twice with 75% ethanol and gently dried under house vacuum in a desiccator. Finally, the RNA was dissolved in 100 μl of nuclease-free H2O and quantified.

The isolated RNA solution (5 μg each) was diluted with 10.15 μl of nuclease-free H2O to a total volume of 11.15 μl and 1 μl (5 pmol) of poly (dT)15 (Promega, Madison, WI) was then added. The mixture was kept in boiling water for 5 min and then cooled on ice for 5 min. After adding 4 μl of reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, Life Technologies), 1 μl of 10 mM deoxynucleoside-5′-triphosphate mixture (Promega), 2 μl of 0.1 M dithiothreitol (Life Technologies), 0.25 μl (10 U) of RNAsin (Promega), and 0.6 μl of Moloney murine leukemia virus reverse transcriptase (120 U), these were incubated at 37°C for 1 h. After the cDNA synthesis, reverse transcriptase was inactivated by incubation.
at 75°C for 10 min. Ribonuclease H (1 µl, 1.5 U; Promega) was then added and incubated for 20 min at 37°C. The single-stranded cDNA thus obtained was used for PCR amplification.

The primers for PCR amplification of CYP1A2 were determined with the computer program HYBsimulator (Advanced Gene Computing Technologies, Irvine, CA) as described (Mitsuhashi, 1996). The sequence of the sense and the antisense primers for CYP1A2 were as follows: 5'-CTACCGATACATCCCTTTTTGCCC-3' and 5'-GATGGCTCGACATCTTTCACTTGGA-3'. β-Actin was amplified as a control. The sequence of sense and the antisense primers for β-actin has been described (Sindhu et al., 1996b). cDNA (6 µl) was mixed with 3 µl of 10× PCR buffer (Promega), 2.4 µl of 25 mM MgCl₂ (Promega), 1.5 µl of each sense and antisense primers (0.1 mg/ml), 2.4 µl of 10 mM deoxynucleoside-5'-triphosphate mixture (Promega), and 0.2 µl of Taq polymerase (Fisher, Pittsburgh, PA) in a final volume of 30 µl and was amplified in a programmable thermal controller (MJ Research, Cambridge, MA) with 30 cycles by denaturation at 94°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 4 min.

**Statistical Analysis.** Statistical analysis of the data was performed with StatView II software. Results are expressed as means ± S.D. Statistical significance of the difference between the control and experimental group was determined by one-way ANOVA and Dunnett's test.

**Results**

Induction of EROD Activity by OT and UVT and Its Superinduction by Cycloheximide. Wild-type Hepa-1 cells were incubated with OT or UVT (100 µM) and TCDD (2 nM) for 4 h in the presence or absence of cycloheximide as described in the text. Cycloheximide (5-µg/ml medium) was added during the final 2 h of oxidized tryptophan treatment. The cells were washed twice and incubated for an additional 3 h in fresh medium containing actinomycin D (1-µg/ml medium). The cells were then washed twice, harvested, and EROD activity was determined as described above. Both OT and UVT caused a significant induction of EROD activity compared with the untreated controls (Table 1). Treatment with cycloheximide caused a superinduction of both OT- and UVT-inducible EROD activity. Similar results were obtained with TCDD (Table 1).

**TABLE 1**

Superinduction of OT-, UVT-, and TCDD-inducible EROD activity of Hepa-1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EROD Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>OT</td>
<td>3.1 ± 0.3b</td>
</tr>
<tr>
<td>OT + cycloheximide</td>
<td>25 ± 2**</td>
</tr>
<tr>
<td>UVT</td>
<td>6.1 ± 0.5b</td>
</tr>
<tr>
<td>UVT + cycloheximide</td>
<td>24 ± 1**</td>
</tr>
<tr>
<td>TCDD</td>
<td>16 ± 1**</td>
</tr>
<tr>
<td>TCDD + cycloheximide</td>
<td>42 ± 7**</td>
</tr>
</tbody>
</table>

a EROD activity is expressed as picomoles of resorufin formed/milligram protein/min ± S.D.
b Significantly different from the untreated control group (P < .01).
c Significantly different from the respective group where no cycloheximide was added (P < .01).

Induction of CYP1A2 protein by TCDD in the absence and presence of cycloheximide is shown in Fig. 2. No CYP1A2 protein (lower band) could be detected in the untreated control cells (lanes 1 and 2). However, TCDD caused an induction of CYP1A2 protein (lanes 3–5) that was further induced after treatment with cycloheximide (lanes 6–8). CYP1A1 protein (upper band) was detected as a faint band in the controls (lanes 1 and 2) that was markedly induced by TCDD (lanes 3–5), and further induced by cycloheximide treatment (lanes 6–8).

The results presented in Fig. 3 show the presence of only CYP1A2 protein band (lanes 1 and 2) in uninduced mouse liver microsomes (10 µg of protein), whereas OT-administered samples (10 µg of protein) that also had been treated with cycloheximide show the presence of both CYP1A1 (upper band) as well as CYP1A2 (lower band).

Superinduction of Oxidized Tryptophan-Inducible CYP1A2 mRNA Expression by Cycloheximide. Results obtained with RT-PCR amplification of CYP1A2-specific

![Fig. 1](https://via.placeholder.com/150)

Fig. 1. Induction of oxidized tryptophan-inducible CYP1A2 protein. Immunoblot analysis of the cell extracts (10 µg of protein in each lane) was carried out with monoclonal anti-CYP1A1/1A2 (NIH 1-7-1). Lanes 1 and 2 had extracts from untreated and cycloheximide-treated cells. Lanes 3 and 4 were from OT-treated extracts, whereas lanes 5 and 6 had extracts of UVT-treated cells. Cycloheximide was included during the final 2 h of a total of 4 h of OT (lanes 7 and 8) or UVT (lanes 9 and 10) treatment.
cDNA (Fig. 4, upper panel) show that no CYP1A2 cDNA could be detected in the unoxidized tryptophan-treated controls (lane 1) or cycloheximide-treated samples (lane 2). However, with the cells that were incubated with UVT for a total of 2 h at the final concentration of 100 μM, an expected 440-base pair (bp)-long fragment of CYP1A2 cDNA was readily detected (lane 3). Furthermore, to the cells to which cycloheximide was added during the final 1 h of UVT treatment, CYP1A2 mRNA was further induced (lane 4). Similarly, CYP1A2 cDNA could be detected in cells that were incubated with OT alone (lane 5) and was further induced when cycloheximide was added after treatment of the cells with OT (lane 6). β-Actin-specific cDNA of 338 bp was detected in all the groups (Fig. 4, bottom), namely, control (lane 1), cycloheximide (lane 2), and UVT (lane 3), UVT plus cycloheximide (lane 4), OT (lane 5), and OT plus cycloheximide (lane 6) RT-PCR amplifications were carried out at least three times with similar results. Similar results were obtained with TCDD (data not shown).

Restriction Digestion of CYP1A2 cDNA with AvaII.

An analysis of the restriction enzyme site map of CYP1A2 cDNA revealed an AvaII restriction site at 1485 bp resulting in two fragments of 306 and 134 bp, respectively. PCR-amplified CYP1A2 cDNA was incubated with and without AvaII (20 U) overnight at 37°C in a total volume of 30 μl and 15 μl of the reaction mixtures was separated by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining. The results reported in Fig. 5 show that incubation of the CYP1A2 cDNA with AvaII resulted in its cleavage confirming its identity.

Sequencing of CYP1A2 cDNA. The PCR amplified CYP1A2 cDNA was purified with QIAquick PCR purification kit (Qiagen, Chatsworth, CA) and sequenced. DNA sequencing was performed on an ABI PRISM 377 DNA Sequencer with protocols recommended by the manufacturer with dRhodamine chemistry. Standard runs were performed on 36-cm...
well-to-read gels. An analysis of the observed cDNA sequence showed 100% homology with the predicted CYP1A2 sequence, thus confirming that the PCR-amplified cDNA was indeed that of CYP1A2.

**Discussion**

In recent years, the role of AhR in the induction of CYP1A1 has been extensively studied. Induction of CYP1A1 seems to be regulated exclusively at the transcriptional level (for review, see Hankinson, 1995). The unliganded AhR, found in the cytosol by conventional subcellular fractionation, has a molecular weight of ~280 kDa and is comprised of AhR monomer, two molecules of the 90-kDa heat shock protein, and possibly other proteins (Hankinson, 1995). After ligand binding, AhR interacts with aryl hydrocarbon receptor nuclear translocator and the heterodimer of AhR and aryl hydrocarbon receptor nuclear translocator constitutes a transcription factor, referred to as the transformed AhR complex, which stimulates the synthesis of CYP1A1 protein and several other proteins involved in xenobiotic metabolism (Hankinson, 1995). Activation of transcription occurs through interaction of the transformed AhR complex with several copies of short sequences, termed xenobiotic-(dioxin) responsive elements, located within the 5′-flanking region of the Cyp1a1 gene.

The regulation of Cyp1a2 gene expression, in contrast, is not yet very well understood partly because of lack of stable cell line(s) that respond to the induction of this gene by PAHs or halogenated aromatic hydrocarbons. In addition, although the Hepa-1 cell line has been successfully used as a model system for delineating the mechanism(s) of CYP1A1 induction by numerous laboratories, no serious attempt seems to have been made to date to investigate the presence of CYP1A2 in these cells. The induction characteristics of CYP1A1 and 1A2 mRNAs in Ah-responsive and Ah-nonresponsive mouse strains have been shown to be similar (Tukey and Nebert, 1984), suggesting that these isozymes may share a common mechanism of induction. Therefore, although the induction of Cyp1a2 gene appears to require the AhR, there is very little similarity between the rodent CYP1A1 and CYP1A2 5′-flanking regions, where the regulatory elements are generally thought to reside (Gonzalez et al., 1985). After treatment with PAHs or TCDD, substantial increase in the rate of Cyp1a2 gene transcription was observed in the livers of the mouse (Okino et al., 1992) and rat (Pasco et al., 1993), although post-transcriptional regulation also has been reported to contribute to the induced CYP1A2 mRNA levels (Silver and Krauter, 1988). However, some laboratories also have reported either no increase (Pasco et al., 1988) or only a small increase (Silver and Krauter, 1988) in the rate of Cyp1a2 gene transcription and the reason for this discrepancy is currently unknown. Recently, Quattrochi et al. (1994) suggested that human CYP1A2 may be regulated through two mechanisms: AhR specific- and promoter-specific elements.

Although the expression of CYP1A2 was initially described only in the liver, recent studies have suggested that this isoform is more widely spread than originally thought. CYP1A2 expression has been described in the brain (Farin and Omiecinski, 1993) and in cultured umbilical vein endothelium (Farin et al., 1994). There is also evidence that CYP1A2 mRNA is present in human duodenum following treatment with omeprazole (McDonnell et al., 1992). Pineau et al. (1995) generated a transgenic mouse line that lacks expression of CYP1A2 and found that mice homozygous for a targeted mutation in the Cyp1a2 gene were nonviable. Lethality occurred shortly after birth with symptoms of severe respiratory distress, suggesting that CYP1A2 is critical for neonatal survival. However, Liang et al. (1996) developed a CYP1A2-deficient mouse line by homologous recombination in embryonic stem cells that is completely viable and fertile.

In adult mammals, the primary sites of constitutive expression of CYP1A2 have been shown to be the liver (Sakuma et al., 1998) and olfactory epithelium (Ding et al., 1992). Suggestions have been made that the hepatic enzyme may be involved in an alternate disposal pathway of bilirubin (Kapitulnik and Gonzalez, 1993). With solution hybridization technology, Raval et al. (1991) determined the steady-state level of control in rat hepatic CYP1A2 mRNA to be six molecules/cell, which was increased to >30-fold on i.p. administration of 3-methylcholanthrene (MC). CYP1A1 mRNA, in contrast, was increased from <3 to 68 molecules by MC treatment (Raval et al., 1991). However, in liver-derived cell lines such as mouse Hepa-1, rat H4IE1, and human HepG2, the levels of induced CYP1A2 mRNA were found to be either negligible or below the levels of detection as judged by Northern blot analysis (Jaiswal et al., 1985; Xu and Bresnick, 1990; Chung and Bresnick, 1994). However, with RT-PCR, HepG2 cells have recently been shown to constitutively express CYP1A2, which was induced by MC treatment (Chung and Bresnick, 1994).

In the present investigation, immunoblot analyses of Hepa-1 cell-free extracts with monoclonal antibody (NIH 1-7-1), which is known to cross-react with CYP1A1 and 1A2, showed that both CYP1A1 and 1A2 proteins were induced by OT or UVT as well as by TCDD (Figs. 1 and 2). Further induction of both these proteins was observed in the cells that had been incubated with cicloheximide during the final 2 h of the inducer treatment. It should be pointed out that in earlier communication, we reported the presence of only CYP1A1 protein after treatment of Hepa-1 cells with oxidized tryptophan (Sindhu et al., 1996b). The discrepancy in these results is because in the earlier investigation after incubating the membrane with chemiluminescent substrate, we routinely exposed the membrane to the film for 1 to 2 min that, on development, yielded an intense CYP1A1 protein band. In addition, we were working on the generally held notion that CYP1A2 is either absent, or if present at all, it was thought to be below the levels of detection in liver-derived cell lines (Jaiswal et al., 1985; Xu and Bresnick, 1990). Because CYP1A1 and 1A2 proteins are barely separable on 10% acrylamide gels, longer exposure of the membrane to the film yielded only one intense band. This is confirmed by the presence of only one very intense band when MC-induced mouse liver microsomes were used as the standard (Sindhu et al., 1996b). During the course of our investigations on the super-induction of oxidized tryptophan-inducible CYP1A1 by cicloheximide, immunoblotting showed a very intense CYP1A1 protein band. The induction of CYP1A1 protein was so huge that we began controlling the time of the exposure of the membrane to the film at 10-s intervals. It was then that the presence of CYP1A2 protein was consistently observed. Therefore, in Sindhu et al. (1996b), the intense CYP1A1
protein band is comprised of both CYP1A1 and CYP1A2 proteins.

The primers for PCR amplification of CYP1A2-specific cDNA were determined by a computer program, HYBSimulator, as described (Mitsuhashi, 1996), and the selected sequences were the most specific ones with minimum chance of cross-hybridization against CYP1A1 or other known P-450 genes. Furthermore, the selected upstream sense primer is located on exon 4, whereas the downstream antisense primer was chosen from the junction of exon 6 and 7 to prevent amplification of genomic DNA, if any, that might contaminate the RNA preparations. RT-PCR resulted in the amplification of an expected 440-bp CYP1A2 cDNA (Fig. 4, top) and 338-bp β-actin cDNA (bottom), respectively. Treatment with OT or UVT caused an induction of CYP1A2 mRNA, whereas β-actin was not affected by oxidized tryptophan treatment. Restriction digestion of PCR-amplified CYP1A2 cDNA by AvaII yielded two expected fragments of 306 and 134 bp, respectively (Fig. 5). Finally, the PCR-amplified CYP1A2 cDNA was purified with QIAquick PCR purification kit (Qiagen) and sequenced. The observed CYP1A2 cDNA sequence had 100% homology with the reported sequence for mouse CYP1A2 cDNA.

Treatment of Hepa-1 cells with cycloheximide during the final 2 h of inducer treatment resulted in further induction of both CYP1A2 mRNA as well as the protein. Similar results were obtained with CYP1A1 (Sindhu and Kikkawa, 1999) suggesting that the induction and superinduction of these two isozymes occurs through a similar mechanism. These results are different from those reported by Teifeld et al. (1989) who showed that although Cyplal (→) null mutant mice develop normally but show deficient drug metabolism, Proc. Natl Acad Sci USA 93:1671–1676.


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