EXPRESSION OF THE RAT CYP2A3 GENE IN TRANSGENIC MICE

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ABSTRACT:

Rat CYP2A3 and mouse CYP2A5 are predominantly expressed in the olfactory mucosa. CYP2A3 is also expressed in the lung at a low level, whereas CYP2A5 is expressed in several additional tissues. To better understand the transcriptional regulation of the CYP2A genes, transgenic mice were generated with a full-length CYP2A3 gene fragment containing 3.4 kilobases of the 5′-flanking region. CYP2A3 mRNA was detected in the brain and olfactory bulb in four transgenic mouse lines, in the olfactory mucosa in three lines, and in kidney, liver, lung, and small intestine in two lines. Thus, the expression of the CYP2A3 transgene mimicked the tissue distribution pattern of mouse CYP2A5 rather than that of rat CYP2A3. Furthermore, the levels of CYP2A3 mRNA were very low in all lines examined, suggesting that more distal regulatory regions may be involved in the abundant expression of the CYP2A3 genes in the olfactory mucosa.

Rat CYP2A3 and its orthologs, mouse CYP2A5, human CYP2A6, and human CYP2A13, are cytochrome P450 enzymes highly active in the metabolism of many xenobiotics, including several known chemical carcinogens (Fernandez-Salgueiro and Gonzalez, 1995; Honkakoski and Negishi, 1997; Su et al., 2000). Despite high similarities in their amino acid sequence and substrate specificity, however, these enzymes differ in tissue distribution. CYP2A3 and CYP2A5 are abundantly and preferentially expressed in the olfactory mucosa (Su et al., 1996). CYP2A3 is also expressed in the lung at a low level and at trace levels in the breast and esophagus but not in the liver or other tissues (Kimura et al., 1989; Su et al., 1996; Hellmold et al., 1998; Gopalakrishnan et al., 1999), whereas CYP2A5 is expressed in many additional tissues, including the brain (unpublished results), liver, kidney, and small intestine (Su et al., 1998). In humans, CYP2A6 is predominantly expressed in the lung, whereas CYP2A13 is selectively expressed in the nasal mucosa and other parts of the respiratory tract (Su et al., 2000).

The difference in tissue distribution of rat CYP2A3 and mouse CYP2A5 could be due to differences in their promoter sequences or to the availability and nature of relevant transcription factors. An alignment of the available sequences of the 5′-flanking regions of the rat CYP2A3 and mouse Cyp2a5 genes revealed an 85% identity. In the present study, transgenic mice were generated with a full-length CYP2A3 gene, with 3.4 kilobases (kb) of the 5′ flanking region and 1.5 kb of the 3′ flanking region (Ueno and Gonzalez, 1990). The tissue distribution and the levels of transgene expression were determined in multiple transgenic lines. The results suggest that more distal regulatory regions may be important for the abundant expression of the CYP2A2 genes in the olfactory mucosa and that the species differences in CYP2A2 distribution in other tissues may not be due to differences in the promoter sequences. These findings provide a foundation for future studies on the mechanisms of tissue-selective gene expression in the CYP2A subfamily.

Materials and Methods

Generation of CYP2A3 Transgenic Mice. A 13.0-kb CYP2A3 gene fragment containing 3.4 kb of the 5′ flanking region, all exons and introns, and 1.5 kb of the 3′-flanking region was obtained by a PucI and SacI double digestion of a CYP2A3 genomic clone (Ueno and Gonzalez, 1990). Transgenic mice were produced at the Transgenic/Knockout Mice Core Facility of the Wadsworth Center (Albany, NY) using standard procedures (Hogan et al., 1994). The transgene construct was purified, and approximately 2 pl of the DNA fragment (2 μg/ml) was microinjected into the pronuclei of fertilized eggs from the C57BL/6 strain or the B6/CBA strain. The eggs were then cultured to the two-cell stage, reimplanted into the oviducts of pseudopregnant B6/CBA F1 mice, and allowed to develop to term. The transgenic mice were crossed with wild-type C57BL/6 mice to generate F1 and F2 heterozygous offspring, which were used for analysis of transgene expression.

Southern Blot Analysis. Genomic DNA was isolated from mouse tail biopsy (Hogan et al., 1994). Aliquots of 10 μg of DNA were digested with 100 units each of BamHI and PstI or with 100 units of SacI at 37°C overnight. The samples were subsequently fractionated by electrophoresis on 0.8% agarose gels, and transferred to nylon membranes. Hybridization and washing were performed as described previously (Su et al., 1996), using a 312-base pair (bp) CYP2A3 DNA probe derived from the proximal promoter region of the CYP2A3 gene (~254 to +58; Zhang and Ding, 1998). The intensity of the detected bands was quantified with a densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) for determination of transgene copy number.

Qualitative RNA-PCR Analysis. Total RNAs were isolated from various tissues of 2-month-old mice or the lung of an adult male Wistar rat (Charles River Laboratories, Wilmington, MA) using standard procedures. The RNAs were subsequently reverse transcribed, and the cDNA was amplified by PCR with an annealing temperature of 52°C. The primers were a 287-base pair (bp) pair to amplify the transgenic CYP2A3 gene. The amplified products were separated on a 1.5% agarose gel and stained with ethidium bromide to visualize the bands.
River Laboratories, Inc., Wilmington, MA) using TRIZol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized by reverse transcription (RT) from 2 μg of total RNA with use of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and an oligo-d(T)₁₅ primer (Applied Biosystems, Foster City, CA). For analysis of CYP2A3 transgene expression, PCR was carried out with a set of CYP2A3-specific primers (forward primer: 2A3UE34, 5'-tcgaggagatgtgagga-3'; reverse primer: 2A3LE67, 5'-tctctggtaggaagcatacttc-3'). The expected product was 510 bp in length. PCR mixtures contained 1X reaction buffer (Promega), 1 mM MgCl₂, 0.4 mM dNTPs, 0.4 μM each primer, 2.5 μl of a RT mixture, and 1.25 units of Taq DNA polymerase in a total volume of 25 μl. PCR was performed using a two-step protocol (30 s at 94°C and 45 s at 68°C) for 35 cycles.

For detection of the full-length CYP2A3 cDNA coding region, PCR was initially carried out with a set of CYP2A3-specific primers (forward primer: 2A35UTRF, 5'-gacgccctgtggaccttagcctcaatatcc-3'; reverse primer: 2A33UTRR, 5'-cacgaagctgctgcaacagc-3'). PCR mixtures contained 25 μl of Hotstart Master Mix (Qiagen, Hilden, Germany), 0.4 μM each primer, and 3 μl of an RT mixture in a total volume of 50 μl. PCR was performed at 95°C for 15 min to activate the Taq DNA polymerase, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. Nested PCR was then performed under the same condition as for the first-round PCR but with 1 μl of the reaction mixture from the initial PCR as the template and a set of CYP2A primers that do not distinguish CYP2A3 from CYP2A5 (forward primer: 2A3E1F, 5'-ctcgagctctctccggg-3'; reverse primer: 2A3E9R, 5'-cacaagttggagacacagtgc-3'). The nested-PCR products were subjected to restriction mapping with ClaI to confirm their identities.

For analysis of the 3'-untranslated region (UTR) of the transcripts of the CYP2A3 transgene, PCR was carried out with a forward primer (5'-ctcactatgtctccctgggtgcct-3') complementary to exon 9 of CYP2A3 mRNA and an anchored oligo-dT reverse primer (5'-ttttttttttttttttccccca-3'). PCR mixtures contained 1X reaction buffer, 2 mM MgCl₂, 0.4 mM dNTPs, 0.4 μM each primer, 2.5 μl of a RT mixture, and 1.25 units of Taq DNA polymerase in a total volume of 25 μl. PCR was performed for 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The 3'-UTRs of both CYP2A3 and CYP2A5 mRNAs would be amplified with this set of primers. The expected sizes of the PCR products derived from CYP2A5 and CYP2A3 mRNAs were 232 and 223 bp, respectively. The two products were distinguished by digestion of a 15-μl reaction mixture with 10 μl of Tsp509 I at 37°C for 1.5 h. Tsp509 I cuts the PCR product derived from CYP2A5 but not that from CYP2A3. PCR products were analyzed on agarose gels and visualized by staining with ethidium bromide. The uncut CYP2A3 band was gel-purified and used as a template in a nested PCR with the same primer set, generating enough material for definitive identification of the PCR products by DNA sequencing. The CYP2A3 band from the second round of Tsp509 I digestion was isolated for DNA sequencing.

**Real-Time Quantitative RNA-PCR Analysis.** Quantitation of CYP2A3 mRNA was carried out using a LightCycler (Roche Applied Science, Indianapolis, IN). RT was performed as described above. The CYP2A3-specific primers used were the same as described earlier. PCR mixtures contained 2 μl of FastStart DNA Master SYBR Green I (Roche Applied Science), 2 mM MgCl₂, 0.4 μM each primer, and 0.5 μl of a RT mixture in a total volume of 20 μl. PCR reactions were initiated with a denaturation/Taq activation step at 95°C for 6 min. For the detection of CYP2A3 mRNA, the reactions were performed for 40 cycles of 94°C for 2 s and 68°C for 35 s. For the detection of β-actin mRNA, the reactions were performed for 35 cycles of 94°C for 2 s, 60°C for 5 s, and 72°C for 20 s. For the detection of CYP2A5 mRNA, the reactions were performed with a pair of CYP2A4/5 primers (forward, 2A5L5: 5'-ggctccctctctcagatgg-3'; reverse, 2A5LE67: 5'-tctctggtaggaagcatacttc-3') at 94°C for 2 s, 58°C for 5 s, and 72°C for 35 s for 40 cycles. Detection of the fluorescence in each reaction was carried out at 3°C below the melting temperature (Tm) of the corresponding PCR product, which eliminates the interference from potential PCR primer dimers. The specificity of the PCR products was confirmed by melting-curve analysis. Quantification and melting temperature were analyzed using the LightCycler data analysis software (Roche Applied Science).

**Results**

**Characterization of the CYP2A3 Transgene.** A total of eight transgenic lines were generated, as summarized in Table 1. Presence of the CYP2A3 transgene was initially detected by PCR and then confirmed by Southern blot analysis after digestion of genomic DNA with BamHI and PvuI (not shown). The integrity of the transgene was verified by digesting genomic DNA samples with SacI, which yields a 10-kb fragment from the 13-kb transgene. The CYP2A3 probe detected the 10-kb band in genomic DNA samples prepared from six transgenic lines, but it detected a shorter band (about 7 kb) in line 1786 and 1854 (data not shown). Thus, the transgene was truncated in the latter two lines (which were not studied further), but it seemed to be intact in the other six lines. The copy numbers of the CYP2A3 transgene were determined using Southern blot analysis, with rat genomic DNA as a standard for quantification. CYP2A3 is a single-copy gene in the rat genome (Kimura et al., 1989). The copy numbers of the transgene varied from 1 to 12 among different transgenic lines (Table 1). As later observed, however, the transgene copy numbers were not correlated with the levels of transgene expression (data not shown).

**Expression of the CYP2A3 Transgene.** CYP2A3 transgene expression was examined using both qualitative and quantitative RNA-PCR approaches. Assays for detecting cytochrome P450 protein, such as immunoblot and enzyme activity assays, were not used because enzyme-specific antibodies, substrates, or inhibitors are currently not available. RNA blot analysis was also inadequate because of the low levels of transgene expression and the expected sequence homology between the transgene mRNA and the mouse CYP2A2 transscripts. The specificity of the PCR primers was first confirmed using CYP2A3 and CYP2A5 cDNA as positive and negative control templates, respectively (Fig. 1). Various tissues, including brain, heart, kidney, liver, lung, olfactory bulb, olfactory mucosa, small intestine, and spleen, were collected from each transgenic line. The expression of the CYP2A3 transgene was not detected in any of the tissues in two of the six transgenic lines studied (Table 1), presumably due to the integration of the transgene into a transcriptionally inactive area of the mouse genome. The results from the other four lines are listed in Table 2. The expression of the CYP2A3 transgene varied among different transgenic lines, most likely affected by the transgene integration sites. Interestingly, CYP2A3 mRNA was detected, in more than one line, in a number of tissues where CYP2A5 was coexpressed, including brain, heart, kidney, liver, lung, olfactory bulb, olfactory mucosa, and small intestine (see Fig. 1). In contrast, CYP2A3 mRNA was not detected in heart or spleen, where the constitutive levels of CYP2A5 mRNA were not correlated with the levels of transgene expression (data not shown).

The PCR method used for qualitative detection of CYP2A3 mRNA was designed to maximize specificity and sensitivity, but it only...
RNA-PCR was performed with the use of a set of CYP2A3-specific primers, as described under Materials and Methods. The samples analyzed include total RNAs from olfactory mucosa of an adult male Wistar rat (as a positive control; lane 1), olfactory mucosa of an adult male C57BL/6 wild-type mouse (as a negative control; lane 2), and brain (lane 3), heart (lane 4), kidney (lane 5), liver (lane 6), lung (lane 7), olfactory mucosa (lane 8), olfactory bulb (lane 9), small intestine (lane 10), and spleen (lane 11) of a 2-month-old male mouse of transgenic line 1482. The position of the expected PCR product from CYP2A3 mRNA is indicated by an arrow; it was clearly distinguished from the weak, diffused, nonspecific product that was visible in most samples (probably a primer dimer). The positive band in lane 7 is rather weak and may not reproduce well.

![Image](44x652 to 295x729)

**TABLE 2**

Qualitative analysis of the tissue distribution of CYP2A3 mRNA in transgenic mice

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
<th>Olfactory Bulb</th>
<th>Olfactory Mucosa</th>
<th>Small Intestine</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1482</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

+, detected; −, not detected.

amplifies cDNA sequences between exons 3 and 7. To determine whether the transgene transcript was processed correctly, we also performed experiments to evaluate whether all exons were present and whether the 3'-UTR was the same as that found in CYP2A3 mRNA from rats. In the first set of experiments, the CYP2A3 cDNA was detected by a nested PCR designed to amplify the entire coding region, from exons 1 to 9, of the transgene mRNA (data not shown). The identity of the PCR product was confirmed by DNA sequencing. More importantly, PCR products with a smaller size than that of the full-length coding region were not detected in any of the tissues examined, indicating that the transgene transcript was correctly spliced.

In the second set of experiments, the sequence of the 3'-UTR of the CYP2A3 transgene mRNA was determined with use of a forward PCR primer from exon 9 and an anchored oligo-dT reverse primer. As shown in Fig. 2, the PCR product corresponding to the 3'-UTR of CYP2A5, but not to the 3'-UTR of CYP2A3, was cut by Tsp509 I, as predicted from their sequences. With RNAs samples prepared from the transgenic mice, not all of the PCR products were cut by Tsp509 I, but sequence analysis of the residual, uncut PCR product confirmed that it was derived from CYP2A3 mRNA. Thus, the authentic polyadenylation signal of the CYP2A3 transgene was used. However, these results do not rule out the potential occurrence of alternatively polyadenylated CYP2A3 transcripts with a much longer 3'-UTR, which would not be detected under the PCR conditions used. The results in Fig. 2 also confirmed that the expression level of the transgene was quite low; the PCR product corresponding to CYP2A3 was much less abundant than that generated from the endogenous CYP2A5 mRNA in various tissues of the transgenic mice.

**Discussion**

The levels of CYP2A3 transgene expression were very low in this study, despite the fact that the gene (including 3.4 kb of 5'-flanking region, 1.5 kb of 3'-flanking region, and all introns), instead of a cDNA, was included in the transgene construct. Furthermore, although both CYP2A3 and CYP2A5 are expressed predominantly in the olfactory mucosa in rats and mice, respectively (Su et al., 1996, 2000), this strong tissue preference was not reproduced by the CYP2A3 transgene in any of the transgenic lines analyzed. These observations imply that additional enhancers may be located further upstream or downstream of the gene fragment included in the transgene. Alternatively, although less likely, the putative olfactory mucosa-selective transcriptional activators may not be highly conserved to the extent that the mouse proteins would not activate the cognate regulatory element in the CYP2A3 transgene as efficiently as with the mouse Cyp2a5 gene.
An earlier study identified a nasal predominant transcriptional activating (NPTA) element in the 5' flanking region of the rat CYP2A3 gene (Zhang and Ding, 1998). The NPTA element was found to be essential for transcriptional activity of the CYP2A3 promoter in in vitro transcription assays, and it specifically interacted with proteins from the olfactory mucosal nuclear extracts but not with the proteins from the liver, lung, kidney, or brain. Moreover, the NPTA element is conserved in mouse Cyp2a5 and human CYP2A6 genes. Other evidence supporting the hypothesis that rat CYP2A3 and mouse Cyp2a5 genes may use the same cis-elements for transcriptional activation include the identical location of a TATA box in their proximal promoter region and the identical location of their transcription initiation sites, both at 24-bp upstream from the ATG translation start codon (Lindberg et al., 1989; Ueno and Gonzalez, 1990). However, further studies are needed to better define the role of the NPTA element in the regulation of the CYP2A genes, particularly in light of the implication that more distal regulatory regions may also be required for their transcriptional activation in the olfactory mucosa.

The species difference in tissue distribution of orthologous xenobiotic-metabolizing enzymes is one of the important factors to consider when risk assessment of chemical toxicity is made by extrapolating from metabolism and toxicity data obtained in animal.
of unique, mouse-specific regulatory elements in the ubiquitous expression of CYP2A5 in mice may be due to the presence of tissue-selective transcriptional repressors. Alternatively, the more ubiquitous expression of CYP2A5 in mice may be due to the presence of unique, mouse-specific regulatory elements in the Cyp2a5 promoter, which allows transcriptional activation of the Cyp2a5 gene in multiple tissues. However, the latter mechanism is unlikely if a mouse-like tissue distribution is achieved with the CYP2A3 gene in transgenic mice, as was found in this study, despite the low levels of transgene expression and the apparent impact of integration site on the transgene expression in different lines. Thus, the results of the present study suggest that the expression of CYP2A3 in rats and that of CYP2A5 in mice are probably controlled by similar cis-elements. Consequently, a more restrictive tissue distribution or different properties of the relevant transcription factors may at least partly account for the lack of CYP2A3 expression in rat liver and kidney.

Within the olfactory mucosa, the CYP2A proteins are expressed in Bowman’s glands and sustentacular cells but not in olfactory receptor neurons (Zupko et al., 1991; Chen et al., 1992; Walters et al., 1993). The cellular expression profile of the CYP2A3 transgene in the olfactory mucosa was not examined in this study because of difficulties in distinguishing CYP2A3 protein or mRNA from the endogenous CYP2A5. Of interest, a 3.6-kb 5′-flanking sequence of the mouse olfactory mucosa-specific Cyp2g1 gene was found to contain regulatory elements sufficient for olfactory mucosa-specific expression of a LacZ reporter gene in a recent study (Zhuo et al., 2001). However, the cellular expression patterns of the transgene failed to reproduce the pattern of the endogenous gene, which implicated the presence of additional regulatory sequences that are necessary for the correct cell type-selectivity within the olfactory mucosa. Additional transgenic studies with much longer upstream sequences are warranted. In addition, it will be interesting to determine whether the CYP2A and CYP2G genes are regulated under a common locus control region.

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References