

Transgenic Mouse Models of Human CYP3A4 Gene Regulation

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ABSTRACT

CYP3A4, the predominant but variably expressed cytochrome P450 of adult human liver, is subject to multifaceted constitutive regulation as well as transcriptional induction by a variety of structurally unrelated xenobiotics. Using transient transfections in HepG2 cells, we previously demonstrated the existence of a potent xenobiotic-responsive enhancer module located between -7.2 and -7.8 kilobases upstream of the CYP3A4 transcription start site. Induction is mediated by interaction of transcription factor binding sites in the XREM with the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). To determine the in vivo relevance of these findings and to establish a mouse model of human CYP3A4 regulation, we have generated transgenic mice carrying constructs comprising the upstream regulatory region of the human CYP3A4 gene linked to the *lacZ* reporter gene. Constitutive expression was observed in a developmental, tissue- and

cell-specific fashion that mirrors the human situation. In addition, robust hepatic and intestinal induction with a range of reagents known to activate PXR and/or CAR (e.g., dexamethasone, pregnenolone 16 α -carbonitrile, and phenobarbital) was observed. However, no expression or induction was apparent with a construct lacking upstream sequences beyond -3.2 kilobases. Histochemical staining for β -galactosidase activity revealed that dose-dependent increases in transgene levels were associated with a zonal expansion of *lacZ* expressing hepatocytes, suggesting that xenobiotic induction of CYP3A genes operates primarily through the recruitment of more cells committed to expression. In summary, CYP3A4/*lacZ* transgenic mice provide an in vivo model for the study of the molecular mechanisms involved in the regulation of a significant human drug metabolizing enzyme.

CYP3A4 is the predominant cytochrome P450 (P450) expressed in human liver, accounting for up to 60% of total hepatic P450 protein (Shimada et al., 1994). CYP3A4 is involved in the metabolism of an extensive range of endogenous substrates and xenobiotics, making a significant contribution to the termination of the action of steroid hormones (Brian et al., 1990), detoxification of bile acids (Araya and Wikvall, 1999), elimination of xenobiotics, and activation of several potent carcinogens (Nebert and Gonzalez, 1987). It has been estimated that in excess of half of all therapeutic drugs are metabolized in full or in part by this enzyme (Maurel, 1996).

CYP3A4 expression exhibits substantial interindividual variation that cannot be explained by genetic polymorphism (Lamba et al., 2002; Spurdle et al., 2002) and seems to result from the intrinsic transcriptional regulation of this gene.

Ten-fold or higher differences in hepatic mRNA expression and 20-fold differences in enzyme activity between healthy adults have been observed (Schuetz et al., 1994; Shimada et al., 1994; Maurel, 1996; Koch et al., 2002). This variability in expression of CYP3A4 has a significant impact on drug metabolism (Watkins et al., 1989). In the case of drugs with a low therapeutic index, such as agents used for cancer chemotherapy or organ transplantation, this variability has significant consequences and adds considerable complexity to clinical therapeutics. It is therefore evident that a basic understanding of how the CYP3A gene subfamily is regulated is of considerable relevance to clinical medicine as well as issues of endobiotic homeostasis.

CYP3A enzymes are subject to multiple levels of regulation. CYP3A4 is expressed in significant amounts in liver, small intestine, and colon (Kolars et al., 1994; Yokose et al., 1999). The mechanisms governing this tissue-restricted expression are poorly understood. Within a tissue, there is cell-specific expression of P450 enzymes. For example, within the hepatic lobule, most CYP3A expression is restricted to hepatocytes immediately surrounding cental veins (zone 3)

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ABBREVIATIONS: P450, cytochrome P450; PXR, pregnane X receptor; CAR, constitutive androstane receptor; kb, kilobase(s); XREM, xenobiotic-responsive enhancer module; PXRE, PXR-responsive element; PCN, pregnenolone 16 α -carbonitrile; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; ONPG, O-nitrophenyl- β -D-galactopyranoside; PCR, polymerase chain reaction.

(Yokose et al., 1999). Again, factors governing this zonal expression are not clear. Furthermore, some CYP3A genes demonstrate major developmental changes in man and some other species. CYP3A7, the major CYP3A isoform expressed in human fetal liver (Komori et al., 1989; Schuetz et al., 1994), undergoes a developmental switch in the first week of postnatal life, with CYP3A7 virtually disappearing concomitant with transcriptional activation of the CYP3A4 gene (Lacroix et al., 1997). A similar developmental switch has also been observed in the mouse (*Cyp3a16* to *Cyp3a11*) (Itoh et al., 1994; Sakuma et al., 2000).

Previous work has determined that the transcriptional induction of CYP3A4 by a range of structurally unrelated xenobiotics is mediated predominantly through the pregnane X receptor (PXR; also known as the steroid and xenobiotic receptor) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999; Moore et al., 2000b; Xie et al., 2000b), although the constitutive androstane receptor (CAR) may also play a role (Moore et al., 2000b; Xie et al., 2000b; Goodwin et al., 2002). Using transfected reporter gene constructs in a liver-derived cell line, we have found that a region within the 5'-flanking sequence of the CYP3A4 gene located between -7.2 and -7.8 kb upstream of the transcription initiation site is the predominant *cis*-acting element responsible for xenobiotic induction (Goodwin et al., 1999). This region has been termed a xenobiotic-responsive enhancer module (XREM). The XREM was shown to contain PXR-responsive elements (PXREs) and to work cooperatively with a PXRE arranged as an everted repeat with a six-base spacer (ER-6) located within the CYP3A4 proximal promoter. The proximal PXRE alone was found to have no inherent ability to mediate xenobiotic induction. The PXREs in CYP3A4 are also capable of interacting with CAR, indicating a likely interplay between these two nuclear receptors in CYP3A4 regulation (Goodwin et al., 2002).

The aim of the present study was to determine the extent to which the 5'-flanking sequence of the CYP3A4 gene is capable of supporting the multifaceted regulatory features of CYP3A4 gene expression. This was accomplished by inserting a transgene encompassing 13 kb of the 5'-flanking sequence of CYP3A4 linked to a β -galactosidase reporter gene into mice. Examination of constitutive, tissue-restricted, developmental and inducible patterns of transgene expression was then undertaken. To determine the relative contribution of distal regulatory elements, the behavior of an additional transgene, extending to only -3.2 kb, was explored and compared with the longer XREM-containing transgenic construct.

Materials and Methods

Transgene Constructs. Two transgene constructs were synthesized containing the 5'-flanking sequence of the human CYP3A4 gene linked to an *Escherichia coli lacZ* reporter gene (Fig. 1). The first construct, designated -3.2CYP3A4/*lacZ*, contained the region of the CYP3A4 gene from the *HindIII* site at -3.2 kb relative to the transcription start site to nucleotide +53 base pairs downstream of the transcription start site. The second construct, designated -13CYP3A4/*lacZ*, included the region of the CYP3A4 gene from the *KpnI* site at -13 kb to +53 base pairs relative to the transcription start site. The latter construct includes the XREM region located between -7836 and -7208 bp. The DNA sequence of the CYP3A4 gene between -10468 and +906 has been previously determined and

deposited with the GenBank database under accession number AF185589. The *lacZ* reporter cassette (Goring et al., 1987) contained the coding region of the β -galactosidase gene flanked by a Kozak eukaryotic translational initiation sequence and a translational stop codon as well as the SV40 transcriptional termination and polyadenylation sequence. This cassette was inserted into the pGL3-Basic vector (BD Biosciences Clontech, Palo Alto, CA) from which the luciferase gene had been removed. Oligonucleotides containing *NotI* sites were inserted into the *KpnI* and *BamHI* sites of pGL3-Basic vector such that they flanked the entire transgene construct. The CYP3A4/*lacZ* transgene constructs were digested with *NotI* to remove vector sequences and purified on agarose gels before microinjection.

Generation of Transgenic Mouse Lines. Mice carrying the CYP3A4/*lacZ* transgenes were created by microinjection of the DNA constructs into the pronuclei of zygotes harvested from FVB/N strain mice. Microinjection and manipulation of embryos were carried out with the use of standard techniques. Transgenic founders were identified by Southern analysis of DNA extracted from tails of pups born after microinjection of the transgene construct. The probe used was a 3.0-kb DNA fragment derived from the *lacZ* reporter gene, labeled with [α -³²P]dCTP using the Mega Prime kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The copy number was estimated by comparison between the intensity of signals from transgenic mouse-tail DNA with standard amounts of purified transgene construct DNA loaded on agarose gels for Southern analysis. Stable transgenic mouse lines incorporating the -3.2 or -13CYP3A4/*lacZ* transgenes were bred from transgenic founders for all subsequent experimentation and analysis. The Animal Ethics Committee, Western Sydney Area Health Service, approved experimental protocols.

Administration of Xenobiotics to Mice. Male and female mice (8–10 weeks old) hemizygous for the -3.2CYP3A4/*lacZ* and -13CYP3A4/*lacZ* transgenes were used to determine the ability of a range of xenobiotics and hormones to activate expression of transgene-derived β -galactosidase. Mice were administered the following reagents and vehicles by single daily i.p. injection for 4 days: rifampin/corn oil, dexamethasone phosphate/H₂O, pregnenolone 16 α -carbonitrile (PCN)/2% Tween 20 in H₂O, phenobarbital/H₂O, clonazepam/2% Tween 20 in H₂O, and phenytoin/2% Tween 20 in H₂O. All reagents were supplied by Sigma Chemical Co. (St Louis, MO) except for dexamethasone phosphate, which was obtained from Faulding (Mulgrave, Australia), and PCN, which was obtained from Upjohn Co. (Kalamazoo, MI). The dose used for all xenobiotics to test for transgene induction was 100 mg/kg of body weight. Dose response studies using dexamethasone were carried out in the range of 1–100 mg/kg using male hemizygous transgenic mice.

Herbal remedies were commercially available preparations from Herbs of Gold Pty Ltd (Chatswood, NSW, Australia). The prepara-

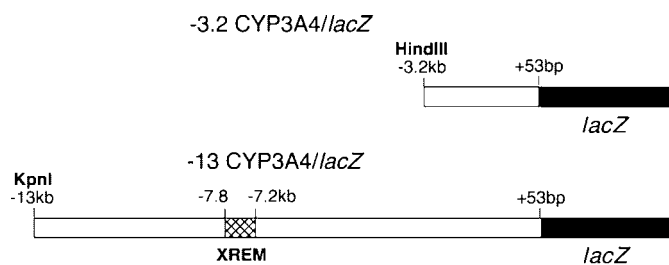


Fig. 1. CYP3A4/*lacZ* transgene constructs used to generate transgenic mice. The upstream regions of the human CYP3A4 gene are depicted as open boxes with the position of the XREM indicated by cross-hatching. The 5'-flanking region extending from +53 bp downstream of the transcription initiation site to a *HindIII* site at -3.2 kb was designated -3.2CYP3A4/*lacZ* and to a *KpnI* site at -13 kb as -13CYP3A4/*lacZ*. The coding region of the *E. coli lacZ* gene together with eukaryotic translational initiation and termination signals, transcription termination, and poly-adenylation sites are indicated by a filled box.

tions were used as supplied by the manufacturer: St. John's wort, 0.4 g of dried *Hypericum perforatum* herb/ml of 50% ethanol; echinacea, 0.2 g of *Echinacea purpurea* whole flowering plant/ml of 55% ethanol; brahmi, 1.2 g of dried *Bacopa monnieri* herb/ml of 50% ethanol. The herbal agents were administered orally by absorbing 100 μ l of each preparation into a single food pellet that was presented to individually housed line 9/4 male transgenic mice at 9:00 AM each day for 4 days. At this time, the normal mouse chow was removed until 5:00 PM to ensure the mice ingested the herbal-treated food pellet. Another group of mice was presented with pellets treated with 100 μ l of 50% ethanol as well as removal of normal mouse chow to control for any effects of food restriction. Mice were harvested and livers examined by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining, as detailed below.

Developmental Transgene Expression. Male and female line 15/10 mice ($n = 2$ to 3 per group) were harvested at day 17 of fetal development as well as 3, 5, and 8 weeks after birth. Liver wedges and samples of small intestine were stained with X-gal and examined using a stereoscopic microscope for evidence of transgene activity.

Analysis of CYP3A4 Transgene and Endogenous Cyp3a11 Gene Expression. β -Galactosidase activity was visualized in slices and frozen sections of liver and other tissues by staining with X-gal. Tissues were fixed in 0.25% glutaraldehyde, 0.1 M phosphate buffer, pH 7.3, 5 mM EGTA, and 2 mM $MgCl_2$; washed in 0.1 M phosphate buffer, pH 7.3, 0.01% sodium deoxycholate, 0.025% Nonidet P40, and 2 mM $MgCl_2$; and stained by incubation at 37°C in wash solution supplemented with 1 mg/ml X-gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. Tissue slices were examined under a stereomicroscope (magnification, 20 $\times$) and tissue sections using a conventional microscope (magnification, 100 $\times$). Liver slices were scored for X-gal staining using a visual analog scale based on the radius of liver cells exhibiting positive staining extending out from central veins and intestinal slices on the intensity of villous staining. In addition, the extent of β -galactosidase activity was quantified in whole liver homogenates (100 mg of fresh tissue/ml of 0.25 M Tris-HCl, pH 7.3) using the *O*-nitrophenyl- β -D-galactopyranoside (ONPG) assay as described previously (Foster et al., 1988). After appropriate dilution, the homogenate was incubated with β -galactosidase assay reagent (0.1 M sodium phosphate buffer, pH 7.3, 1 mM $MgCl_2$, 50 mM β -mercaptoethanol, and 0.88 mg/ml ONPG) at 37°C, quenched by the addition of 1 M Na_2CO_3 , and the absorbance at 420 nm was determined. The units of β -galactosidase activity are given as A_{420} per milligram of protein per minute. The ONPG assay proved to be unsuitable for intestinal tissues and microscopic scoring alone was used.

Endogenous mouse Cyp3a11 mRNA expression was determined by real-time reverse transcriptase-polymerase chain reaction (PCR). RNA was extracted from liver using a commercially available reagent (TRIzol; Invitrogen, Inc., Carlsbad, CA). cDNA was synthesized from 5 μ g of total RNA using random hexamers and Super-

script II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. An aliquot of each cDNA synthesis reaction (1 μ l) was subjected to PCR amplification using a Prism 7700 real-time PCR platform (Applied Biosystems, Foster City, CA). Primers and TaqMan probe were as follows:

forward primer, bases 112–133, 5'-TGCTCCTAGCAATCAGCT-TGG-3'
reverse primer, bases 220–199, 5'-GTGCCTAAAAATGGCAGAG-GTT-3'
probe, bases 137–171, 5'-FAM-CCTCTACCGATATGGGACTCGTA-AACATGAACCTT-TAMRA-3'

The probe was designed to cross an intron-exon junction to avoid interference from genomic DNA. Additionally, primer and probe sequences were chosen to avoid detection of other Cyp3a subfamily members. Results were normalized against GAPDH determined using a commercially available TaqMan kit (Applied Biosystems). Cycle parameters for all PCR were: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Results

Four transgenic lines were generated using the construct containing the –3.2-kb sequence of the human CYP3A4 gene linked to *lacZ*. Transgene-derived β -galactosidase activity was not detected in liver, kidney, small and large intestine, spleen, brain, lung or skin tissue from adult mice for all four –3.2CYP3A4/*lacZ* transgenic lines, either constitutively or after treatment with xenobiotics (Table 1). In contrast, constitutive small intestinal transgene expression was readily detected in adults from two of the four lines carrying the –13CYP3A4/*lacZ* construct and consistently in the livers of one line (Table 1). Line 9/4 demonstrated low or absent constitutive expression in liver, with β -galactosidase detected only occasionally in isolated hepatocytes immediately adjacent to major blood vessels (Fig. 2A). Constitutive transgene expression was more pronounced in livers of line 15/10 mice, with patches of X-gal-staining cells macroscopically apparent on the cut surface of the liver (Fig. 2B). This appearance is caused by restriction of transgene expression to hepatocytes surrounding central veins (see below). The basal level of hepatic transgene expression in line 15/10 mice was consistently greater in male mice compared with female mice, as determined by visual assessment of X-gal-stained liver slices. Administration of inducing xenobiotics resulted in robust expression in a zone of cells surrounding central veins in both 9/4 (Fig. 2A) and 15/10 mice. Because the basal level of transgene expression in untreated mice in line 9/4 is

TABLE 1

Expression of –3.2CYP3A4/*lacZ* and –13CYP3A4/*lacZ* fusion genes in transgenic mice

Inducibility of transgene expression was determined by treatment with PCN (100mg/kg) administered by intraperitoneal injection daily for 4 days as described under *Materials and Methods*.

Transgene	Line	Copy No.	Liver		Small Intestine	
			Basal	Inducible	Basal	Inducible
–3.2CYP3A4/ <i>lacZ</i>	13	15	–	–	–	–
	24	>100	–	–	–	–
	31	80	–	–	–	–
	39	10	–	–	–	–
–13CYP3A4/ <i>lacZ</i>	13/5	70	–	–	–	–
	9/4	5	–/+	++++	+	+++
	9/7	50	–	+	–	–
	15/10	8	++	++++	++	+++

low or absent, induction is more apparent, making this mouse line particularly suitable for more detailed studies after xenobiotic administration. Extrahepatic transgene expression in mice from lines 9/4 and 15/10 was restricted to the gut, predominantly the mature enterocytes covering the villous processes of the small intestinal mucosa (Fig. 3), and the brain, being restricted to the ventral nucleus of the thalamus and regions of the hippocampus and choroid plexus (Fig. 4, A and B). Whereas treatment with xenobiotic inducers increased small intestinal transgene expression (Fig. 3), neither PCN nor phenobarbital treatment increased transgene expression in brain (data not shown). No transgene expression was observed in kidney, large intestine, spleen, lung, or skin, either constitutively or after treatment with inducing drugs.

The relative degree of induction for a range of xenobiotics was analyzed by determining the transgenic β -galactosidase activity in liver lysates of mice from line 9/4 using the ONPG assay (Fig. 5A). Dexamethasone and PCN were the most potent inducers, whereas rifampin activated the transgene to relatively modest levels. Phenobarbital, clotrimazole, and phenytoin were intermediate inducers. The induction profile of transgene activity in line 9/4 was strikingly similar to that observed for the endogenous *Cyp3a11* gene in the same mice (Fig. 5B), reflecting the known ligand-induced activation profile of the mouse PXR (Moore et al., 2000b). Administration of xenobiotics to line 15/10 mice induced transgene expression in a similar manner to that observed in line 9/4 (data not shown). A significant gender difference in hepatic transgene expression was found. Male mice exhibited a greater degree of induction than female mice for most xenobiotics (Fig. 5A). This was paralleled by the induction profile of the endogenous mouse *Cyp3a11* gene (Fig. 5B).

The activation of transgene expression in line 9/4 by dexamethasone was dose-dependent over the range 1 to 100 mg/kg (Fig. 6A). The higher transgene-derived β -galactosidase activity in liver homogenates from mice treated with increasing doses of dexamethasone was associated with an expanded zone of hepatocytes that were positively stained by X-gal (Fig. 6B). At low doses of dexamethasone, a ring of hepatocytes only 1 to 2 cells thick around the central vein expressed the transgene. With 100 mg/kg dexamethasone,

the zone of X-gal-positive hepatocytes increased up to a 10-cell radius, approximately midway between the central vein and the portal triad. A similar dose-dependent expansion of hepatocytes expressing the transgene was observed with the other xenobiotic treatments (data not shown).

To characterize the possible utility of *CYP3A4* regulatory transgenic animals for use in evaluation of alternate medications, mice from line 9/4 were treated with three commonly used herbal therapies; Brahmi, echinacea, and St. John's wort. The later preparation has been implicated in *CYP3A* induction and drug interactions in man. Hyperforin, a component of St. John's wort, has been shown to be a potent activator of human PXR (Moore et al., 2000a). The dose and route of administration of the herbal preparations was chosen to simulate the human situation. Although no induction of transgene expression was observed with either Brahmi or echinacea (data not shown), treatment with St. John's wort resulted in a substantial increase in hepatic X-gal staining, indicating induction of the $-13\text{CYP}3A4/lacZ$ transgene (Fig. 7).

Because *CYP3A4* is developmentally regulated, transgene expression was examined in liver and small intestine of male and female line 15/10 mice before and after birth to determine whether the -13 kb 5'-flanking sequence of *CYP3A4* would be sufficient to provide appropriate developmental responses. No transgene-derived β -galactosidase activity was observed in day 17 fetal liver or small intestine. In contrast, by 3 weeks after birth, both sexes demonstrated an adult pattern of small intestinal transgene expression, yet showed no hepatic expression. By 5 weeks, limited transgene expression was detectable in liver, increasing to adult levels by 8 weeks.

Discussion

The human *CYP3A* gene cluster at 7q22.1 spans 200 kb and consists of four functional genes and three pseudogenes (Gellner et al., 2001). The functional genes in telomeric to centromeric order are *CYP3A43*, *CYP3A4*, *CYP3A7*, and *CYP3A5*. *CYP3A43* demonstrates generally low levels of expression and is predominantly found in prostate (Gellner et

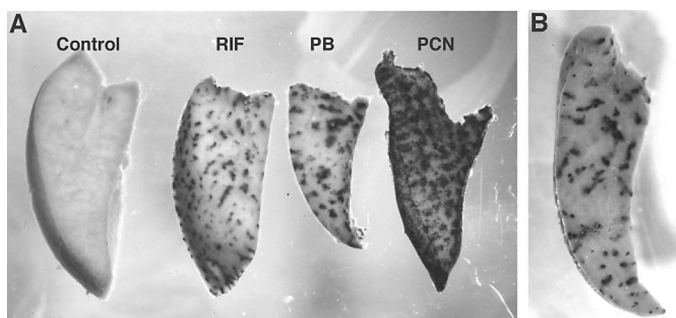


Fig. 2. Constitutive and xenobiotic-induced hepatic transgene expression. Female mice from line 9/4 harboring the $-13\text{CYP}3A4/lacZ$ transgene (A) were treated with various *CYP3A*-inducing drugs as described under *Experimental Procedures*. Hepatocytes exhibiting transgene expression are visualized as the darkly stained areas on the cut surface of the liver after X-gal treatment. RIF, rifampin; PB, phenobarbital; compared with corn oil-treated control mice (Control), which demonstrate little or no constitutive transgene expression. Constitutive hepatic transgene expression in female mice from line 15/10 (B) can be appreciated as the darkly stained areas on the cut surface of the liver.

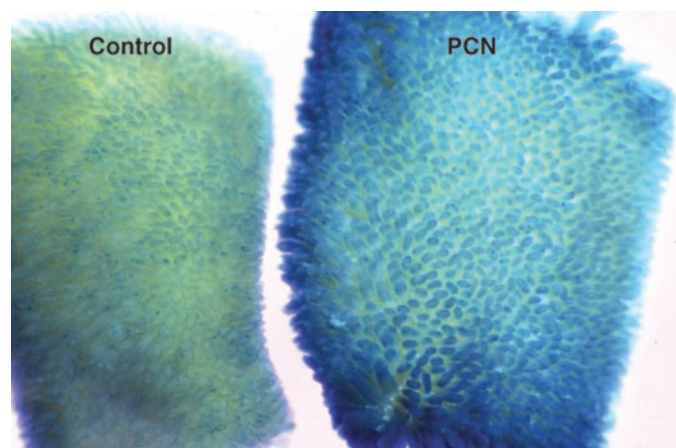


Fig. 3. Constitutive and xenobiotic-induced small intestinal transgene expression. Female mice from line 9/4 harboring the $-13\text{CYP}3A4/lacZ$ transgene were treated with corn oil (Control) or PCN as described under *Experimental Procedures*. Enterocytes exhibiting transgene expression are visualized as the blue-stained areas on the luminal surface of the intestine after X-gal treatment.

al., 2001). *CYP3A4* and *CYP3A5* are expressed mainly in liver and gut (Aoyama et al., 1989; Wrighton et al., 1990; Kolars et al., 1994), whereas *CYP3A7* is expressed predominantly in fetal liver (Komori et al., 1989) and gravid uterus and placenta (Schuetz et al., 1993). To date it has not been clear whether these genes are regulated independently or in part co-ordinately. The observations that *CYP3A4* and *CYP3A7* undergo reciprocal developmental regulation and that a high degree of correlation between *CYP3A4* and *CYP3A5* expression exists in liver (Lin et al., 2002) suggest a degree of co-ordinate regulation. However, in the present study, we have determined that the 5'-flanking sequence of *CYP3A4* is capable of directing expression in a cell- and

tissue-specific manner in the absence of other regions of the gene cluster. In addition, as we had previously observed in cell-based models, the *CYP3A4* 5'-flanking region is capable of conferring xenobiotic induction on reporter transgene expression so long as distal elements located between -13 and -3.2 kb are present. We have now extended these observations to show that these distal elements are also required for constitutive expression. Specifically, no transgene-directed β -galactosidase activity was detected either constitutively or after treatment with potent xenobiotic inducers in the four transgenic mouse lines carrying the shorter -3.2CYP3A4/*lacZ* construct.

Previous work from our laboratory has shown that a distal region within the *CYP3A4* 5'-flanking sequence, which we termed an XREM, was pivotal for the induction of the *CYP3A4* gene by xenobiotic ligands of the human PXR (Goodwin et al., 1999) and mouse CAR (Goodwin et al., 2002). The XREM contains both high- and low-affinity binding motifs for PXR-9-*cis*-retinoic acid receptor- α and CAR-9-*cis*-retinoic

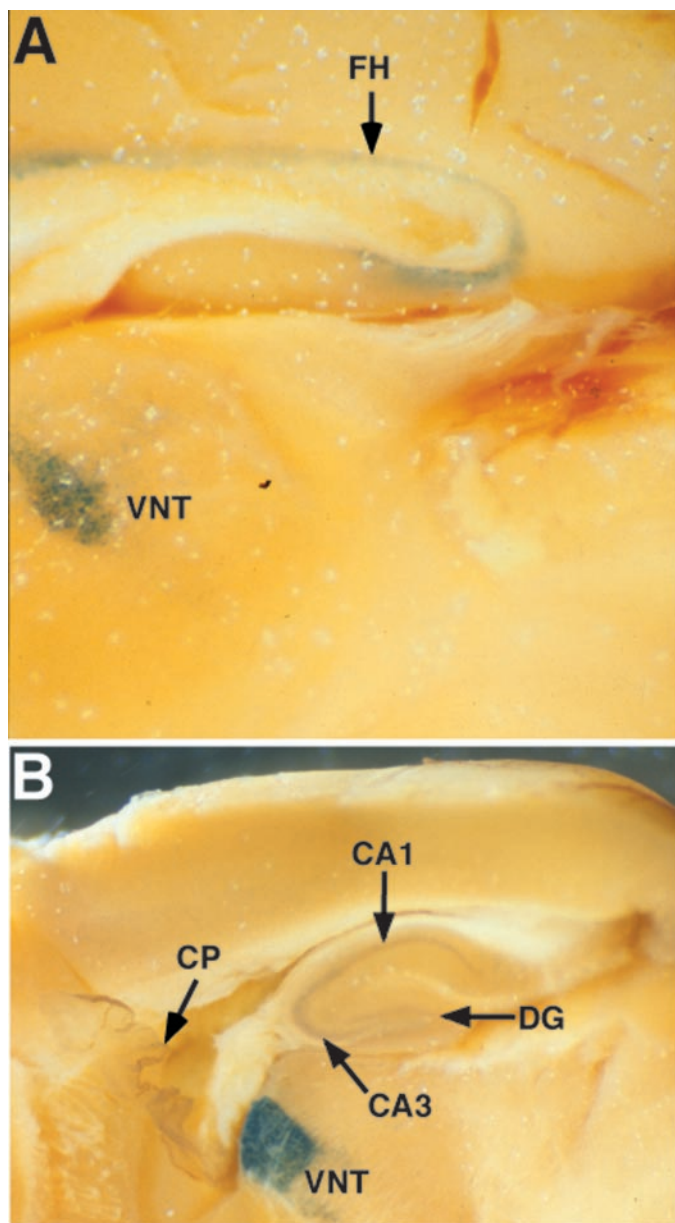


Fig. 4. Constitutive transgene expression in brain. Whole brain sagittal sections were examined after X-gal treatment in female mice from line 9/4. Cells exhibiting transgene expression are visualized as the blue-stained areas. A, midline sagittal section. B, sagittal section approximately 1 mm lateral to A. CA1, CA1 field of hippocampus; CA3, CA3 field of hippocampus; CP, choroid plexus; DG, granular layer of the dentate gyrus; FH, fimbria of hippocampus; VNT, ventral nucleus of thalamus.

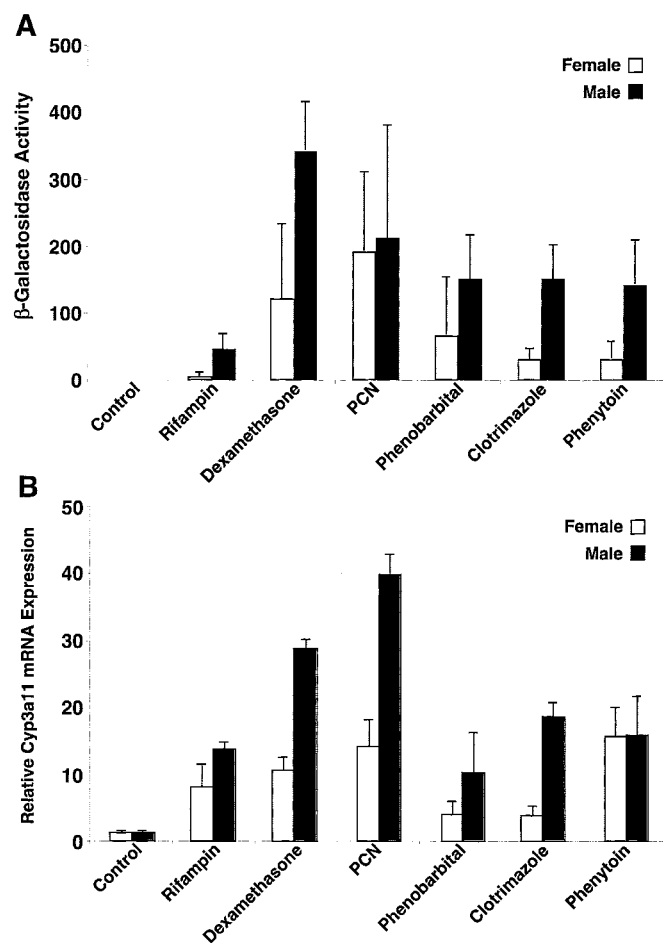


Fig. 5. Comparison of the xenobiotic induction profile of the -13CYP3A4/*lacZ* transgene with the endogenous mouse *Cyp3a11* gene. Transgenic mice from line 9/4 were treated with a range of xenobiotic chemicals as described under *Experimental Procedures*. A, transgene expression was assessed by determining β -galactosidase activity in total liver lysates using the ONPG assay. The units of β -galactosidase activity are given as A_{420} per milligram of protein per minute and expressed as mean $\pm$ S.D., $n = 3$ animals per treatment. B, hepatic expression of the *Cyp3a11* gene was examined in the same mice by real-time reverse transcriptase polymerase chain reaction and normalized for GAPDH RNA expression. The data are presented as -fold induction relative to Control for each sex and expressed as mean $\pm$ S.D., $n = 3$ animals per treatment.

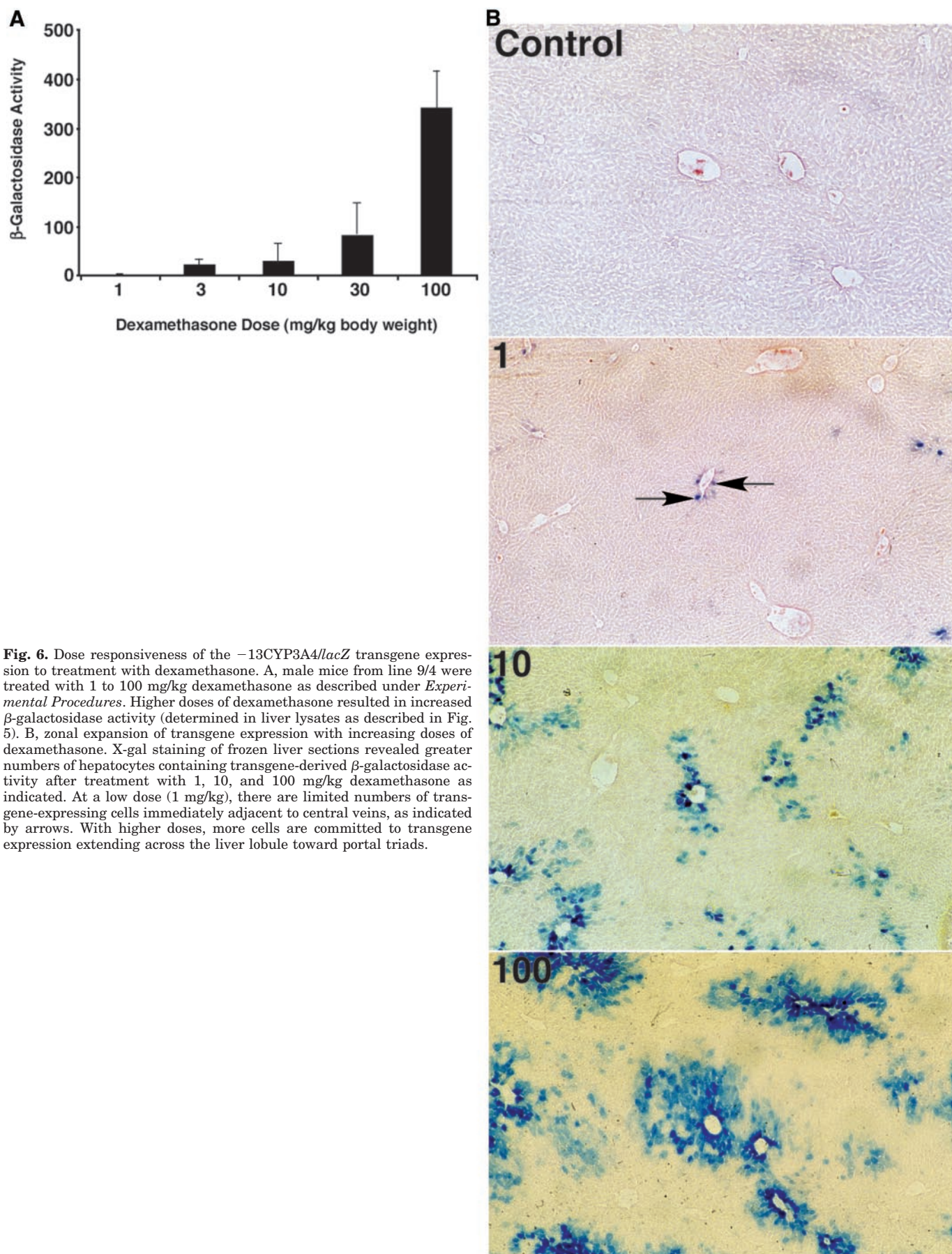


Fig. 6. Dose responsiveness of the $-13\text{CYP3A4}/lacZ$ transgene expression to treatment with dexamethasone. A, male mice from line 9/4 were treated with 1 to 100 mg/kg dexamethasone as described under *Experimental Procedures*. Higher doses of dexamethasone resulted in increased β -galactosidase activity (determined in liver lysates as described in Fig. 5). B, zonal expansion of transgene expression with increasing doses of dexamethasone. X-gal staining of frozen liver sections revealed greater numbers of hepatocytes containing transgene-derived β -galactosidase activity after treatment with 1, 10, and 100 mg/kg dexamethasone as indicated. At a low dose (1 mg/kg), there are limited numbers of transgene-expressing cells immediately adjacent to central veins, as indicated by arrows. With higher doses, more cells are committed to transgene expression extending across the liver lobule toward portal triads.

acid receptor- α heterodimers. In these previous experiments, a proximal PXRE, located between -172 and -149 bp, was unable to confer xenobiotic responsiveness, even in constructs containing 3.2 kb of the promoter sequence. In the present study, we used a transgenic approach to study the function of the *CYP3A4* gene 5'-flanking sequence in vivo. These results confirm that the proximal promoter region of *CYP3A4* lacks inherent transcriptional activity and that function is dependent on the integrity of elements located further upstream, beyond -3.2 kb. A previous study using a short *CYP3A4* 5'-flanking region construct (-179 to -35 bp) linked to a heterologous thymidine kinase promoter and a chloramphenicol acetyltransferase reporter gene found some induction after transient transfection into primary rat or rabbit hepatocytes treated with the potent inducing agents dexamethasone and rifampin, respectively (Barwick et al., 1996). However, our results show that the intact *CYP3A4* proximal promoter in the context of integrated transgenic DNA, which more closely resembles the natural genetic environment, is functionally silent.

The transgenic models described herein also provide important information concerning the cell- and tissue-restricted expression of *CYP3A4*. Several P450s, including *CYP3A4*, are expressed only in pericentral hepatocytes within hepatic lobules (Yokose et al., 1999). We found that the -13 kb *CYP3A4/lacZ* construct exhibited this cellular distribution, again demonstrating that the *CYP3A4* 5'-flanking region is capable of recognizing tissue- and cell-specific *trans*-acting factors responsible for this pattern of expression. After treatment with potent xenobiotic inducers of murine CYP3A expression the zone of transgene expression was found to expand outwards from central veins toward portal triads. Thus, the increased expression of *CYP3A* genes observed with xenobiotic

inducers is predominantly the result of recruitment of additional hepatocytes capable of expressing this gene rather than an increase in expression levels within hepatocytes that harbor constitutive activity.

The relative degree of -13 kb *CYP3A4/lacZ* transgene induction by xenobiotic compounds observed in this study reflects the known activation profile of the mouse PXR (Moore et al., 2000b). As expected, PCN and dexamethasone were the most potent inducers. Interestingly, rifampin treatment also resulted in easily detectable reporter gene activity. Rifampin is a relatively poor activator of mouse PXR; studies performed in CV-1 cells using transiently transfected receptor and *CYP3A4* reporter gene constructs show only a 1.5- to 2-fold induction after treatment with $10 \mu\text{M}$ rifampin (Moore et al., 2000b). The present findings suggest that the in vivo environment of the transgenic animal provides a more sensitive system compared with cell line-based models for studies of *CYP3A4* induction.

Some P450s, including *CYP3A* enzymes, are expressed in brain, although their functional role in this location is open to conjecture. A recent in situ hybridization study using a fluorescent cRNA probe to detect *CYP3A* in rat brain found considerable localized expression, particularly in regions of the thalamus and hypothalamus (Pai et al., 2002). The results of the present study are in excellent agreement with these previous findings, demonstrating that the mechanisms controlling cell-specific expression of *CYP3A* in the rodent brain are equally applicable to human *CYP3A* genes. The lack of induction of brain transgene expression by phenobarbital or PCN is in keeping with previous findings that *Cyp2b1* expression in rat thalamus and hypothalamus is also refractory to induction by phenobarbital (Upadhyaya et al., 2002). The reason for this lack of induction is unknown, although one possibility that deserves further exploration is that xenobiotic 'sensing' receptors such as PXR and CAR may be absent from these tissues.

An intriguing aspect of human *CYP3A* regulation is the switch between *CYP3A7* (the predominant fetal form) and *CYP3A4* that occurs shortly after birth (Lacroix et al., 1997). *CYP3A7* is also expressed in the gravid uterus and the placenta (Schuetz et al., 1993). A plausible explanation for these patterns of expression is that *CYP3A7* may play a role in protecting the fetus from the high circulating concentrations of maternal steroid hormones that accompany pregnancy. The mechanism of *CYP3A4* developmental activation seems to depend on the integrity of transcription factor binding elements within the proximal promoter, particularly the proximal PXRE. Individuals with the *CYP3A7* allelic variant *CYP3A7*1C*, which contains a *CYP3A4* PXRE, demonstrate hepatic *CYP3A4* expression into adulthood (Burk et al., 2002). We have shown that the -13 kb *CYP3A4/lacZ* transgene is also subject to developmental activation, further establishing that the required *cis*-acting sequences are contained within the *CYP3A4* 5'-flanking region. The timing of transgene activation in the liver between 5 and 8 weeks is similar to the reported activation of the endogenous mouse *Cyp3a11* gene, which achieves adult levels of expression approximately 4 weeks after birth (Itoh et al., 1994; Sakuma et al., 2000).

In the present study, a degree of sexual dimorphism in the basal and xenobiotic-induced hepatic expression of the -13 kb *CYP3A4/lacZ* transgene was apparent. Before and after

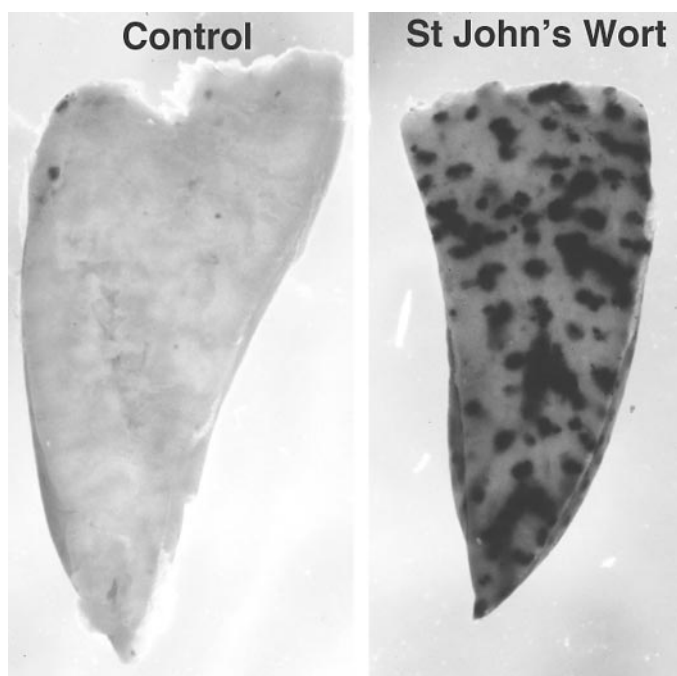


Fig. 7. Induction of the -13 kb *CYP3A4/lacZ* transgene by a herbal medication. Livers from male line 9/4 transgenic mice fed St. John's wort absorbed onto food pellets for 4 days show increased expression of the transgene compared with control mice. Hepatocytes exhibiting transgene expression are visualized as the darkly stained areas on the cut surface of the liver after X-gal treatment.

treatment with inducing agents, male mice exhibited higher levels of transgene activity than female mice. This was paralleled by the fold induction of the endogenous mouse *Cyp3a11* gene, which was greater in male mice than in female mice. It has previously been reported that estrogens may act as a negative regulator of *Cyp3a11* expression (Yamada et al., 2002), which may explain the sex-related differences noted in the present study. Additionally, sexually dimorphic patterns of growth hormone secretion are known to influence hepatic P450 gene expression in rodents (Mode et al., 1992), male-predominant expression being mediated through the transcription factor *Stat5b* (Park et al., 1999). Thus, sex steroids may act indirectly through their effects on growth hormone. In this respect, our previous observation that *CYP3A4* expression is modulated by growth hormone in primary cultures of human hepatocytes (Liddle et al., 1998) may be relevant to the sex differences noted in *CYP3A4* transgene activity.

Finally, the transgenic models described herein may have a useful role in therapeutic drug development. Drugs that exhibit P450 induction are challenging in that they may impact on their own metabolism as well as that of coadministered drugs. We have shown that mice carrying the -13 kb *CYP3A4/lacZ* transgene are useful for screening compounds for this unwanted activity, as exemplified by the mice fed St. John's wort. The main limitation of this model is the presence of the murine rather than the human xenobiotic 'sensing' receptors PXR and CAR. These receptors exhibit species-specific divergence in their ligand activation profiles (Moore et al., 2000b). However, others have already demonstrated the feasibility of humanization of PXR in mice (Xie et al., 2000a); therefore, there are no technical impediments to constructing mice with both humanized sensors and response transgenes that can be used for preclinical drug evaluation.

In summary, the present study demonstrates that the 5'-flanking sequence of the *CYP3A4* gene is functionally self-contained and capable of directing the multifaceted patterns of *CYP3A4* enzyme expression observed in man. Additionally, there is an absolute requirement for distal elements between -3.2 and -13 kb for transcriptional activation, consistent with previous observations concerning the functional importance of the XREM region. Additionally, regulatory transgenic mouse models of the type described herein should provide a useful platform for the characterization of the inductive properties of potential therapeutic drugs early in the development process, well before clinical studies are contemplated.

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