The Murine Cyp1a1 Gene Is Expressed in a Restricted Spatial and Temporal Pattern during Embryonic Development*

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In adult mice the cytochrome P450 Cyp1a1 gene is not constitutively expressed but is highly inducible by foreign compounds acting through the aryl hydrocarbon (Ah) receptor. However, the expression profile of the Cyp1a1 gene in the developing embryo is not well understood. Using established transgenic mouse lines where 8.5 kb of the rat CYP1A1 promoter is cloned upstream of the lacZ reporter gene (1), we describe the expression of the CYP1A1-driven reporter gene in all tissues throughout stages E7–E14 of embryonic development. In contrast to the absence of constitutive Cyp1a1 and lacZ transgene expression in tissues of the adult mouse, a constitutive cell-specific and time-dependent pattern of CYP1A1 promoter activity was observed in the embryo. This expression pattern was confirmed as reflecting the endogenous gene by measuring Cyp1a1 mRNA levels and protein expression by immunohistochemistry. The number of cells displaying endogenous CYP1A1 activity could be increased in the embryo upon xenobiotic challenge, but only within areas where the CYP1A1 promoter was already active. When reporter mice were bred onto a genetic background expressing a lower affinity form of the Ah receptor (DBA allele), transgene and murine Cyp1a1 protein expression were both attenuated in the adult mouse liver upon xenobiotic challenge. By comparison, constitutive CYP1A1 promoter activity in the embryo was identical in the presence of either the high or low affinity Ah receptor. These novel data suggest that the Cyp1a1 protein may play a role in murine development and that regulation of the Cyp1a1 gene during this period is either through the action of a high affinity Ah receptor ligand or by an alternative regulatory pathway.

The mammalian cytochrome P450 (CYP)1-dependent monoxygenase system plays a key role in the metabolism and disposition of environmental chemicals. Certain members of this multigene family also play a central role in the biosynthesis and/or degradation of steroid hormones, fatty acids, and cholesterol (2, 3). Many of the enzymes involved in foreign compound metabolism are highly inducible, and exposure to a chemical often results in the induction of single or multiple cytochrome P450s that increase the rate of metabolism and disposition of the agent involved (4). The paradigm for this is cytochrome P450 CYP1A1, which in adults is not constitutively expressed but is highly inducible in many different tissues by a wide range of environmental chemicals such as the cigarette smoke components known as polycyclic aromatic hydrocarbons, the polychlorinated biphenyl mixture Aroclor 1254, and dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (5, 6).

CYP1A1 expression during embryonic development remains poorly characterized (7–10). It has been suggested that CYP1A1 mRNAs have a short half-life that contributes to the difficulty in reliable detection (11). Targeted deletion of the Cyp1a1 gene in mice resulted in no obvious phenotype, which implies that the protein has no essential developmental role; however, it was postulated that the structurally and functionally homologous catalytic Cyp1a2 might fulfill the functions of Cyp1a1 (11). The double knock-out has not been reported. It is generally agreed however, that CYP1A1 transcription is elevated in the developing liver upon exposure to a wide range of xenobiotics (12–15).

The mechanism of Cyp1a1 gene regulation involves the binding of the foreign compound to a cytoplasmic receptor, the aryl hydrocarbon (Ah) receptor, which then dissociates from the heat shock protein-90, allowing it to enter the nucleus where it dimerizes with the transcription factor Ah receptor nuclear translocator (ARNT), to activate the promoters of responsive genes (for review, see Ref. 16). Interestingly, ARNT (also known as hypoxia inducible factor-1β) can also heterodimerize with hypoxia inducible factor-1α, the transcription factor that mediates cellular responses to hypoxia (17) and to a number of other basic helix-loop-helix proteins (18, 19), although the function of these alternative heterodimers in Cyp1a1 gene expression has not been elucidated.

The expression of cytochrome P450s in developing embryos is important, as P450s may mediate the teratogenic effects of chemicals and influence important developmental pathways. This is particularly the case for Cyp1a1, which is highly active in the metabolism of many mutagenic and toxic compounds and in the metabolism of retinoic acid (21, 22).

To study the function of the CYP1A1 gene in more detail using a method independent of mRNA stability, we have previously exploited a transgenic mouse line where 8.5 kb of...
the rat CYP1A1 promoter is used to drive the expression of the reporter gene, lacZ. Using this model in previous studies, we have demonstrated that the transgene, in a number of different mouse lines, is expressed in a manner indistinguishable from that of the endogenous Cyp1a1 protein in the uninduced state and upon challenge with polycyclic aromatic hydrocarbons in adult transgenic mice (1). In this current study we use these models to investigate the constitutive and xenobiotic-inducible expression of the Cyp1a1 gene in the developing mouse embryo. In contrast to the adult mouse, we show that the CYP1A1 promoter is constitutively active in a cell-specific manner at certain stages of development. Furthermore, the CYP1A1 promoter is apparently only inducible by xenobiotics in those cells and at those times at which constitutive expression is also found.

MATERIALS AND METHODS

Reagents—TCDD was a kind gift from Dr. A. G. Smith, University of Leicester, United Kingdom. 3-Methylcholanthrene was supplied by Sigma-Aldrich, and Aroclor 1254 was a kind gift from Dr. H. R. Glatt of the Institute for Nutritional Research (DIFE), Potsdam, Germany. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

Production of Transgenic Embryos—(C57BL/6 × CBA) male mice carrying the transgene pAHR11β-galactosidase from the transgenic line D3/4 described previously (1) were mated with nontransgenic females. Mice were examined each morning for the presence of plugs. The positive identification of a plug was regarded as the first day of gestation (E1). Embryos were collected at 7, 9, 10, 12, 13, and 14 days post-conception (E7–E14).

Induction of Transgene Expression in Developing Embryos—Aroclor 1254 (500 mg/kg), in a suspension of Mazola brand corn oil, was used to cross the transgenic line D3/4 described previously (1) were mated with nontransgenic females. Mice were examined each morning for the presence of plugs. The positive identification of a plug was regarded as the first day of gestation (E1). Embryos were collected at 7, 9, 10, 12, 13, and 14 days post-conception (E7–E14).

Analysis of lacZ Activity—After the selected gestational time point, mothers were culled by cervical dislocation, and embryos were collected, dissected from embryonic sacs for E9 and later stages, and then stained as white mounts for lacZ activity essentially as described previously (21). Animals were analyzed in transverse 10-μm paraffin wax sections from rump to crown. Older embryos (E13–E14) were re-analyzed by serial 10-μm-frozen cryosections as described previously for adult tissue analysis (1) to control for reagent penetration. A minimum of six embryos at each stage was used from at least three litters. Non-transgenic litter mates were co-analyzed at all ages to ensure that the lacZ expression observed was pAHR11β-galactosidase-driven.

Breeders of Transgenic Mice to Express the Low Affinity Ah(−/−) Receptor—(C57BL/6 × CBA) transgenic mice expressing the high affinity Ah gene were bred with DBA/2 mice naturally expressing the variant low affinity Ah gene. The Ah receptor (Harlan, Bicester, UK) were generated for four generations. For the purposes of this study, Ahk and Ahk′ mouse strains are described as responsive Ah(−/+), respectively. Offspring of pAHR11β-galactosidase transgenic mice were selected over non-transgenic mice after each round of breeding using Southern analysis as described previously (1). At each breeding stage, the transgenic offspring were crossed with DBA/2 mice. After three generations, transgenic mice were screened by single-stranded conformation polymorphism (SSCP) assays (see below) for the expression of the variant Ah(−/−) receptor. Transgenic mice positive for the Ah(−/−) receptor were bred with non-transgenic DBA/2 mice to produce a breeding colony of mice homozygous for Ah(−/−) containing the pAHR11β-galactosidase transgene.

Identification of Ah Receptor Alleles—Genomic DNA was isolated from mouse tail biopsies following the procedure described by Sambrook et al. (22) and used as a PCR template. A region of exon 10 of the Ah receptor from either (C57BL/6 × CBA) or DBA/2 wild-type mice was amplified using the primers 5′-GCTCCACGTCAGCCCAAAG-3′ (1304–322 bp) and 5′-CCAATTTGCTCATGTTTCA-3′ (1499–1517 bp) to yield a product of 213 bp. PCR amplification consisted of 94°C for 4 min (one cycle), 59°C for 30 s, 72°C for 30 s, and 94°C for 30 s (34 cycles), followed by 59°C for 5 min and 72°C for 5 min (1 cycle). PCR products were then used in SSCP reactions. Briefly, PCR product was diluted in SSCP loading dye (95% [w/v] formamide, 0.05% [w/v] bromphenol blue, 0.05% [w/v] xylene cyanol FF, and 20 mM EDTA, pH 8) and heat-denatured at 95°C for 5 min. Samples were then analyzed on a 12% non-denaturing polyacrylamide gel at 4°C. The products of SSCP analysis were visualized by silver staining of the gel as described previously (23, 24). Control banding patterns observed in DNA from wild-type (C57BL/6 × CBA) and DBA/2 mice were compared with banding patterns observed in test animals. Initially PCR products were directly sequenced to confirm the specificity of this assay.

Induction of Transgene Expression in Transgenic Ah(−/−) and Ah(−/−) Mice—Transgenic Ah(−/+) and Ah(−/−) adult mice were intraperitoneally injected with vehicle, 3-methylcholanthrene (3-MC, 75 mg/kg), or TCDD (75 μg/kg) dissolved in Mazola corn oil. Doses were based on previous experiments (1, 25). Animals were culled by cervical dislocation 3 days post-induction, and the liver was snap-frozen in liquid nitrogen.

Extraction of mRNA and RT-PCR—E13 embryos were collected by time-mating of wild-type embryos. Embryos were decapitated, and heads and liver samples were snap-frozen in liquid nitrogen for Taq-Man RT-PCR. mRNA extraction was performed using RNA extraction columns (Qiagen Ltd.). RNA was treated with DNase 1 according to manufacturer’s instructions (Qiagen Ltd.). TaqMan PCR was carried out using the 7700 sequence detection system (Applied Biosystems). Primers and probes were specific for mouse Cyp1a1 (forward primer, 5′-TGCCCAC-CTGCTTAGGCTAA-3′; reverse primer, 5′-TGGCCCCCACATGCA-5′) and for the Cyp1a1-6-carboxyfluorescein-labeled probe (3′-ACAGCT- TCCAAATGGTTATGACG-3′). Cyp1a1 levels in unknown samples were calibrated against standard curves generated using cDNA isolated from the liver of a 3-MC-treated mouse. mRNA is expressed in arbitrary units compared to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. Glyceraldehyde 3-phosphate dehydrogenase levels in unknown samples were assayed using rodent glyceraldehyde 3-phosphate dehydrogenase primer and probes sets (Applied Biosystems) and calibrated against a six point standard curve according to manufacturer’s instructions.

Analysis of Cyp1a1 Expression by Western Blotting—Liver samples were homogenized (Ultra-Turrax T8, IKA Labortechnik) in four volumes of buffer containing protease inhibitors (1% Igepal, 25 μM Heps, pH 8, 0.4 mM KCl, 5 mM EDTA, 5 mM dithiobisulfoxyl fluoride, 10 μM MnF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μM benzamidine) and then incubated on ice for 15 min prior to centrifugation at 2000 × g for 10 min at 4°C. The supernatant was removed, and aliquots were taken for protein determination and further analysis. Protein concentrations were estimated using a Bio-Rad protein assay reagent according to manufacturer’s instructions. SDS-polyacrylamide gel electrophoresis and Western blotting were performed using standard methods (27). Briefly, total protein extracts (50 μg) were separated on 7.5% polyacrylamide gel and transferred to Immobilon-P transfer membrane (Millipore Corp.), and an anti-Cyp1a1 antibody (28) was used to detect the Cyp1a1 protein expression. The reporter gene expression was determined using a Bio-Rad protein assay reagent according to manufacturers’ instructions. SDS-polyacrylamide gel electrophoresis and Western blotting were performed using standard methods (27). Briefly, total protein extracts (50 μg) were separated on 7.5% polyacrylamide gel and transferred to Immobilon-P transfer membrane (Millipore Corp.), and an anti-Cyp1a1 antibody (28) was used to detect the expression of the reporter gene.

Analysis of Cyp1a1 Protein Expression by Immunohistochemistry—E12 embryos were immersed in 4% paraformaldehyde overnight prior to processing and embedding in paraffin wax using standard procedures. 10-μm paraffin wax sections were cut using a microtome on to poly-t-lysine-coated glass slides. Antigens were heat-retrieved by a 10-min treatment in boiling citrate buffer, pH 6, in a standard 800-watt microwave. Slides required additional treatment with an avidin-biotin blocking kit (Vector Laboratories, Peterborough, UK), performed according to the manufacturer’s instructions, to reduce nonspecific immunoreactivity due to endogenous biotin. The Cyp1a1 protein was identified using the same rabbit polyclonal anti-rat Cyp1a1 antibody (28) used in our Western blotting analysis. The Cyp1a1 antibody was then detected using biotinylated secondary reagents with standard ABC amplification according to the manufacturer’s instructions (Vector Laboratories, Peterborough, UK). Immunoreactivity was revealed with diaminobenzidine diluted in 10 mM imidazole for increased sensitivity to produce a brown, insoluble precipitate at immunopositive sites. Sections were counterstained in hematoxylin and mounted on coverslips.

RESULTS

We have described previously the production of transgenic mouse lines expressing lacZ reporter gene under the control of the rat CYP1A1 promoter to obtain information on the cell-specific regulation of CYP1A1 gene expression in adult mice (1). In this present study, we examine the expression of the reporter gene in two lines of transgenic mice after an
analysis of serial sections through embryos at successive stages in development from E7–E14 (Theiler stages 10–22). Each of the transgenic lines carry four transgene copies in tandem repeats at a single chromosomal location (1).

The CYP1A1 Reporter Is Constitutively Active during Early Development—In adult mice, the CYP1A1 gene is not expressed but is highly inducible by exposure to exogenous foreign compound inducers. However, as early as day 7 of development (E7), high levels of constitutive CYP1A1 promoter activity were observed in the embryo and extra-embryonic ectoderm at a very low level. Although a few more strongly labeled cells were located in the embryonic endoderm, the majority of the labeling was weak, especially in the embryonic mesoderm. Marked changes in lacZ staining occurred during rapid organogenesis between stages E8 and E9. During this period, a marked restriction in the expression pattern of the reporter was observed that became more apparent with advancing development. In contrast to observations in adult mice, surprisingly high constitutive activity was observed in the embryo and extra-embryonic ectoderm at a single chromosomal location (1).

In adult mice, the Cyp1a1 promoter was expressed in the hindbrain roof (Fig. 1C, sections i–iv). Interestingly, the myocardial cells in the left side of the chamber were more intensely labeled relative to other cells within the primitive heart (Fig. 1C, section i). Significant lacZ expression was also observed in the walls of the common atria and the sinus venosus (Fig. 1C, section i). CYP1A1 promoter activity was also found in the prospective hindbrain extending into the prospective midbrain, which, by comparison, had only single, weakly labeled, neuroepithelial cells (Fig. 1C, section i). A few individual cells were also labeled throughout the neural tube in the anterior hindbrain, although this was much weaker than that seen in the primitive heart and brain.

By E10, intense lacZ activity was observed in the thin roof of the anterior region of the hindbrain within all neuroepithelial cells, whereas in the lateral walls of the hindbrain a more heterogeneous staining pattern was observed (Fig. 1C, section ii). Promoter activity was observed in great numbers of neuroepithelial cells, possibly because of the increased cellular mass, and was of a similar intensity to that seen during earlier periods of development (Fig. 1C, sections i and ii). By E12, the number of lacZ-expressing cells had decreased, although intense staining was observed in the thin roof of the anterior part of the hindbrain that extended to the dorsal parts of the lateral walls, where the expression decreased ventrally. Labeling in the hindbrain continued to the E13 stage in a similar pattern but was now limited to fewer positive cells. In addition, weak labeling was observed in a number of cells in the choroid plexus, and this labeling was still apparent in reduced cell numbers at E14. There was generally no CYP1A1 promoter activity in the midbrain during early development, with the exception of single cells in the midline of the midbrain roof. By E13, however, the midbrain was intensely labeled at both the ventricular and intermediate layers (Fig. 1C, section iv), where stripes of intense transgene expression were observed; very strong labeling was observed in a column of cells adjacent to a column of histologically identical cells that were completely unlabeled (Fig. 1C, section iv, arrow). At the E14 stage, intense labeling was observed in the ventricular and intermediate layers of the hindbrain, but the overall number of expressing cells, as elsewhere, was dramatically reduced. Weak labeling was also observed in the trigeminal ganglion in E12–E14 embryos.

By E10, at the time of early organogenesis, the primitive left ventricle was heavily stained and labeling was now observed in the primitive right ventricle, although labeling in the left and right components of the differentiating common atrial chamber was very weak. By E12, activity was observed in the myocardial cells of the left ventricle and, to a lesser extent, in the right ventricle and the atria. In comparison with early stages of development, the number of cells involved was reduced. By E13 and E14 the same pattern was observed, although the left ventricle was more strongly labeled than the rest of the heart. At the E14 stage, an increase in activity was observed in the right and left atria, where previously no activity had been observed. Weak staining was measured in the dorsal aorta from E10–E14, whereas vessels such as the capillaries and lateral mesenchyme and other parts of the venous system showed no activity.

The first expression in the developing kidney was in the epithelium and the associated mesenchyme of a single nephric duct within the mesonephros of E10 embryos, (Fig. 1C, section v). By E12, the number of positive cells had increased and, at E13, all nephric ducts were now positive, although the intensity of the staining was noticeably reduced. By E14, labeling continued to be observed in the degenerating mesonephros but was no longer present in the epithelium or associated mesenchyme in the more advanced metanephros.

Labeling was first observed in the liver of E12.5 embryos, where a minority of individual cells were weakly labeled. By E13, the number of labeled cells had increased, and positive cells appeared in small clusters throughout the organ. The expression of the transgene was restricted to a short time frame, as no positive cells were observed in the liver of E14 embryos (data not shown).

Cells Exhibiting Constitutive CYP1A1 Promoter Activity Respond to Exogenous Inducing Agents—To determine whether exogenous agents that cross the transplacental barrier could induce the CYP1A1 promoter during murine development, we analyzed transgene expression in mouse embryos exposed for 7 days in utero to Aroclor 1254 (Fig. 1B, D, and sections i and ii). At E7, no induction of transgene expression was observed in the embryonic ectoderm and endoderm. However, as development proceeded, a marked induction of CYP1A1 promoter activity was observed within the major areas and during the developmental periods when constitutive CYP1A1 expression was observed (Fig. 1B, rows marked Hindbrain, Heart, Kidney, and Muscle). The administration of Aroclor 1254 did not extend the time windows when the CYP1A1 promoter was constitutively active in the developing embryo (Fig. 1B). Three embryonic stages (E7, E9, and E12) were studied in a second transgenic line (D4) and examined for lacZ expression, and an identical pattern to that described for the primary D34 transgenic line was observed (data not shown).

CYP1A1 Promoter Activity Is a Legitimate Reporter of Cyp1a1 mRNA and Protein Expression—Although the temporal and spatial pattern of promoter activity in the pAHRIβ-galactosidase transgenic mice was conserved in different transgenic lines in the adult mouse (1) and now in the developing embryo, we investigated whether lacZ staining indeed reflected genuine Cyp1a1 expression. We used immunohistochemistry specific for mouse Cyp1a1 in E12 wild-type embryos to localize the protein in coronal embryonic sections. Similarly as in lacZ expression, we observed Cyp1a1 protein in the neuroepithelial cells of the hindbrain roof (Fig. 1C, section iii). The immuno-
staining and lacZ labeling were complimented by Cyp1a1-specific TaqMan RT-PCR. E13 embryos were selected for mRNA analysis because we observed high levels of CYP1A1 reporter activity in multiple brain regions, and microdissected embryos of this age would produce sufficient mRNA for analysis. TaqMan RT-PCR was used to assay the Cyp1a1 mRNA expression in regions where high (head) and low (liver) pAHR1β-galactosidase transgene expression were present in E13 wild-type unstimulated embryos. In parallel with the transgene expression, endogenous Cyp1a1 mRNA was observed at high levels in the developing head and at low levels in the developing liver (Fig. 2). Indeed the Cyp1a1 mRNA levels observed in the E13 embryonic head were comparable with those observed in adult rat liver 3 days after intraperitoneal injection of the archetypal xenobiotic 3-MC (Fig. 2).

**Generation of Transgenic Mice Expressing the Ah(−−) Receptor**—These embryonic studies were performed in (C57BL/6 × CBA) mice, which are a hybrid strain of mice generated from a combination of the C57BL and CBA inbred strains. The transgenic mice thus express both the A kb-1 and A kb-2 alleles of the Ah receptor that is known to exhibit a high degree of responsiveness to polycyclic aromatic hydrocarbons by inducing the expression of

**Fig. 1. Constitutive transgene expression displays temporal and spatial regulation in the developing mouse embryo.** Whole transgenic embryos 7–14 days (E7–E14) post-conception were analyzed for lacZ activity as described under “Materials and Methods.” A, a schematic representation of the temporal profile of constitutive transgene expression is shown in the form of bars for each tissue. The intensity of the blue coloration within the bars is proportional to the intensity of lacZ transgene activity over the period. B, tissues where constitutive transgene activity could be up-regulated by Aroclor 1254 are indicated. Crosses indicate lack of transgene inducibility, and check marks indicate that the number of cells expressing the transgene was increased. C, representative photographs of peak constitutive transgene expression (i–v) is shown as areas of intense blue labeling for the hindbrain and heart (section i, E8), along the length of the hindbrain roof and selected cells within the hindbrain walls (section ii, E9), in the midbrain (i, E13), and in the mesonephros of the developing kidney (section v, Day 12). Section iii is a high power (400×) photomicrograph of the hindbrain roof (region indicated in section ii) showing the distribution of the CYP1A1 protein (brown stain) at E9. D, representative photographs of Aroclor 1254-inducible expression in the hindbrain (section i, E9) and in the developing kidney (arrow) (ii, E12). Note the increase in number of lacZ-expressing cells.
CYP1A1 mRNA and protein expression.
RT-PCR in E13 embryos within regions where high (head) and low (liver) inducer, 3-MC.

Inducer, 3-MC.

adult rat liver 3 days after intraperitoneal injection of the inducer 3-MC.

developing liver. The unstimulated Cyp1a1 mRNA levels observed in developing liver were comparable with those observed in the E13 wild-type unstimulated embryos. Note that Cyp1a1 mRNA was observed at high levels in the developing head and at low levels in the developing liver. The unstimulated Cyp1a1 mRNA levels observed in the E13 embryonic head were comparable with those observed in adult rat liver 3 days after intraperitoneal injection of the Cyp1a1 inducer, 3-MC.

CYP1A1 and related genes (29). Other inbred mouse strains such as DBA/2 express an Ah receptor with a low affinity for xenobiotic compounds and, thus, reduced expression of the CYP1A1 gene (29). Although 10 nucleotide differences have been detected in the Ah receptor between the responsive and non-responsive mouse strains, only two have been identified as resulting in different promoter activity, as evidenced by either the histological pattern or intensity of lacZ staining, was observed in the presence of either the high affinity Ah (++) receptor after either TCDD (Fig. 3C, section iv) or 3-MC (Fig. 3C, section v) treatment, with higher intensity in the animals treated with TCDD. On the other hand, animals expressing the Ah (--) receptor were unable to up-regulate transgene expression in response to 3-MC (Fig. 3C, section vi) but could respond to TCDD (Fig. 3C, section vii). Importantly, the level of cellular response to TCDD in Ah (--) expressing mice was dramatically lower than that observed for Ah (++) expressing mice (Fig. 3C, section vi versus section iv).

Constitutive Transgene Expression Is Independent of Ah Receptor Genotype—We investigated the effect of the Ah receptor genotype on CYP1A1 promoter activity in developing transgenic mice. Transgenic embryos homozygous for either Ah receptor type were analyzed for lacZ activity at two different embryonic stages associated with intense and distinctive patterns of constitutive transgene expression (Fig. 1, A and C), namely E9 and E12 (Fig. 3D). Despite serial sectioning from head to rump, essentially no difference in constitutive CYP1A1 promoter activity, as evidenced by either the histological pattern or intensity of lacZ staining, was observed in the presence of either the high affinity or the low affinity Ah receptor at these developmental stages (Fig. 3D). Intense lacZ reactivity could clearly be observed in a number of structures, including the heart, tail, and hindbrain.

DISCUSSION
We have described previously the use of the rat CYP1A1 promoter to obtain conditional regulation of gene expression in transgenic mice (1) and, more recently, in transgenic rats (31). The cell-specific pattern of transgene expression in adult mice was indistinguishable from the expression pattern of the endogenous CYP1A1 gene, making this a sensitive model for studying the transcriptional regulation of CYP1A1 (1). The expression of the CYP1A1 gene in developing embryos is controversial because of the lack of sensitive reagents for use in studies. Using our transgenic mouse lines (1) designed with the sensitive and stable lacZ reporter (32), we have made the intriguing discovery that the CYP1A1 promoter is constitutively active in certain embryonic tissues in a cell-specific fashion within restricted time windows during early development. This discovery was supported by the measurement of endogenous mouse Cyp1a1 mRNA and protein in the developing embryo. Furthermore, we demonstrate that the constitutive CYP1A1 reporter activity in development is conserved in different transgenic lines and may not be regulated through the classical Ah receptor pathway, which operates to induce Cyp1a1 expression in the adult mouse. Constitutive CYP1A1 phenotype, transgenic Ah(--) or Ah(+++) mice were challenged with 3-MC or TCDD, which result in known patterns of Cyp1a1 inducibility. Analysis of hepatic Cyp1a1 protein expression by Western blotting (Fig. 3B) confirmed that the Cyp1a1 induction profiles observed were characteristic of the different Ah receptor genotypes. Endogenous Cyp1a1 protein was not observed in the absence of an inducing agent regardless of receptor type (Fig. 3B, lanes 3 and 6). Induction of Cyp1a1 was observed, however, in Ah(+++) mice after 3-MC treatment but not in Ah(--) mice (Fig. 3B, compare lanes 2 and 5). By comparison, TCDD effectively induced the expression of Cyp1a1 in the presence of either form of the Ah receptor, although the response was more marked in the Ah(+++) mice (Fig. 3B, compare lanes 1 and 4). Transgene activity in the livers confirmed that the lacZ expression levels mirrored the expression of the endogenous Cyp1a1 protein (Fig. 3C). No transgene expression was detected in the livers of unstimulated mice expressing either type of receptor (Fig. 3C, sections i and ii). High levels of lacZ staining were observed in all hepatocytes of transgenic animals expressing the high affinity Ah(++) receptor after either TCDD (Fig. 3C, section iv) or 3-MC (Fig. 3C, section v) treatment, with higher intensity in the animals treated with TCDD. On the other hand, animals expressing the Ah(--) receptor were unable to up-regulate transgene expression in response to 3-MC (Fig. 3C, section vi) but could respond to TCDD (Fig. 3C, section vii). Importantly, the level of cellular response to TCDD in Ah(--) expressing mice was dramatically lower than that observed for Ah(++) expressing mice (Fig. 3C, section vi versus section iv).

CYP1A1 Expression in Development

FIG. 2. CYP1A1 promoter activity is a legitimate reporter of Cyp1a1 mRNA and protein expression. Cyp1a1-specific TaqMan RT-PCR in E13 embryos within regions where high (head) and low (liver) pAhIR1β-galactosidase transgene expression were present in E13 wild-type unstimulated embryos. Note that Cyp1a1 mRNA was observed at high levels in the developing head and at low levels in the developing liver. The unstimulated Cyp1a1 mRNA levels observed in the E13 embryonic head were comparable with those observed in adult rat liver 3 days after intraperitoneal injection of the Cyp1a1 inducer, 3-MC.
FIG. 3. Expression of the CYP1A1 promoter is modulated in Ah(+/+)
and Ah(−/−) transgenic mice treated with xenobiotic compounds. Interstrain breeding of the transgenic mice, as described under "Materials and Methods," enabled transgene expression to be examined on either an Ah(+/+)
or an Ah(−/−) genetic background. A, SSCP analysis of exon 10 of the Ah receptor was used to distinguish between Ah(+/+) and Ah(−/−) genetic backgrounds. Wild-type Ah(+/+) (lane 1) and Ah(−/−) (lane 2) mice were used for control banding patterns to allow the selection of Ah(−/−) transgenic mice (lanes 3 and 4). Note the presence of three distinct bands in Ah(+/+) mice and the presence of only two bands in the Ah(−/−) wild-type (lane 1) and Ah(−/−) transgenic mice (lanes 3 and 4). Arrows highlight bands of interest. B, adult Ah(+/+) or Ah(−/−) transgenic mice were treated with TCDD, 3-MC, or vehicle by intraperitoneal injection as detailed under "Materials and Methods." Livers were analyzed 3 days post-induction for native Cyp1a1 expression by Western blotting. The arrow represents the 45-kDa Cyp1a1 protein. Cyp1a1 is observed in the livers of transgenic Ah(+/+) mice that had been treated with TCDD or 3-MC, whereas Cyp1a1 is observed only after TCDD treatment in the transgenic Ah(−/−) mice. In the absence of inducing agent, no Cyp1a1 protein was detected in either mouse line. C, lacZ transgene activity subsequently analyzed in cryosections of the liver. Transgene expression was not observed in the livers of Ah(+/+) or Ah(−/−) transgenic mice treated with vehicle (sections i and ii, respectively) or in the livers of Ah(−/−) mice treated with 3-MC (section iii). By comparison, elevated lacZ activity was observed in the liver of Ah(+/+) mice after treatment with TCDD (section iv) or 3-MC (section v). Positive lacZ expression was observed only in the livers of Ah(−/−) after TCDD treatment, and the level of activity was greatly reduced compared with that in the liver of Ah(+/+) mice (section vi). D, transgenic Ah(+/+) and Ah(−/−) embryos (E9 or E12) were assayed for lacZ activity as whole mounts. Identical transgene expression was observed in embryos of the Ah(+/+) or Ah(−/−) type at both ages. hb, hindbrain; h, heart; t, tail.
reporter activity was inducible by Aroclor 1254 exposure in utero, but only within restricted time frames and in cell types already active during embryonic development.

Few reports of uninduced CYP1A1 expression have been described in vivo, although RT-PCR analysis of mouse embryos reveals constitutive CYP1A1 expression in craniofacial tissue (33). More recently, RT-PCR expression profiling of 40 P450 genes in embryonic and adult tissues showed that 70% of those studied were expressed at some point during embryonic development, with one-third of the expressed P450s belonging to the families CYP1–3 and Cyp1a1 expression being found only at E7 (34). However, in vitro studies using hepatocytes or keratinocytes have reported differentiation-dependent CYP1A1 expression (35–38). Likewise, our own studies show that the time points corresponding to the most intense levels of CYP1A1 promoter activity coincide with periods of active differentiation. For example, the most intense expression in the hindbrain was detected in the first stages of brain development at E8–E10, when the overall volume of the cephalic region is substantially increasing. In parallel with these observations, other tissues such as the heart and kidney displayed the most intense expression during early organogenesis, thereafter decreasing sharply with advancing development. The pattern of constitutive reporter expression was highly restricted, and most tissues exhibited no activity throughout the developmental stages studied. When tissues did show lacZ reporter activity, the magnitude of expression in specific cell types was tightly regulated within limited time windows. The absolute level of gene expression also appeared to be controlled at individual developmental stages. For example, weak and strong expression sites such as the liver and the midbrain (E13) were transcriptionally active at the same developmental stage.

The function of this tightly regulated pattern of CYP1A1 expression is currently unknown. However, the CYP1A1 protein is involved in the synthesis of retinoic acid from retinol (39, 40), and classical CYP1A1 inducing agents also increase the expression of retinoic acid (41). Furthermore, a retinoic-acid-responsive element is present within the promoter of the CYP1A1 gene and is differentially regulated by retinoid receptors (42). It is tempting to speculate that one endogenous function of CYP1A1 may be to control the levels of the signaling molecules, such as retinoid acid, involved in pattern formation during vertebrate development. However, Cyp1a1 gene-deficient (−/−) mice have been shown to develop normally (11), and this has been interpreted as demonstrating that the Cyp1a1 does not play an essential role during embryonic development (43). However, in such Cyp1a1 (−/−) mice the expression of Cyp1a2 (11) or other members of the cytochrome P450 family may be compensatory, which would explain the lack of a phenotype.

The mechanisms that control the expression of the CYP1A1 gene during development are unknown. A CYP1A1 repressor protein has been identified in the gut (44) and may be operational in other tissues, providing the rigid control observed here. Interestingly, adjacent cells were either tightly repressed or highly active. For example, in the midbrain were observed stripes of expression, suggesting that the expressing and non-expressing cells are part of a well organized cell lineage of neuroepithelial cells. The boundary between the stripes was very sharp and did not contain cells with transitional levels of reporter gene expression, a phenomenon that has been described for gene expression in the gut (45, 46).

In an attempt to delineate the molecular mechanisms underlying the constitutive CYP1A1 expression pathway, we analyzed CYP1A1 promoter expression in adult and developing mice, expressing either a high affinity form of the Ah receptor (+/+), as in the primary transgenic lines, or, in transgenic mice, expressing a reduced ligand affinity form of the Ah receptor (−/−). Despite the native Cyp1a1 protein and the CYP1A1 transgenic promoter both displaying the expected phenotype in the Ah(−/−) transgenic mice with reduced Cyp1a1 inducibility to classical xenobiotics (47), the constitutively active CYP1A1 promoter activity was not affected in the developing Ah(−/−) transgenic mice. We conclude from these data that the conventional Ah receptor signal transduction pathway established for the inducible adult CYP1A1 gene may not be responsible for its expression during development or, alternatively, that the pathway is not sensitive to the affinity of the Ah receptor. Reports of heightened CYP1A1 transcription have been described in response to an Ah receptor antagonist (48), and non-Ah-receptor-mediated Cyp1a1 gene expression has been documented previously (49) and may be functional during development.

The Ah receptor and ARNT are expressed during embryonic development (50, 51) in a pattern consistent with the CYP1A1 promoter activity described herein and, thus, may be implicated in embryonic CYP1A1 expression. For example, the brain and heart exhibit the highest levels of Ah receptor and ARNT expression at E9/E10, diminishing with increasing development (50, 51). Both transcription factors have been shown to be essential for normal development; the Ah receptor is critical in the formation of the liver and vascular system (52), whereas ARNT is necessary for normal angiogenesis, because without it mice do not survive (53). Whether basic helix-loop-helix proteins, known to exhibit a promiscuous dimerization specificity (54), are involved in the transcriptional activation of the CYP1A1 gene during development is under investigation. Other basic helix-loop-helix proteins, for example AINT, have been reported during development and have the ability to dimerize with ARNT (19), whereas CH1 expression in the developing heart acts as a transcriptional repressor (55).

The classical Ah receptor signal transduction pathway for inducible CYP1A1 gene transcription relies on an agonist, usually an exogenous ligand, as the initiating factor (16). Whether an endogenous ligand not affected by Ah receptor ligand affinity regulates CYP1A1 gene expression in a cell-specific manner is a matter of intense debate. Ah receptor gene deletion studies have established an essential role for the receptor in development (for review, see Ref. 56). Recently, dietary components and endogenous low affinity Ah receptor ligands have been shown to be capable of inducing CYP1A1 transcription (57, 58). Whether these endogenous ligands or the Ah receptor are involved in the constitutive CYP1A1 expression during development remains uncertain.

As others have reported, we demonstrate the transplacental activity of CYP1A1-inducing agents on developing embryos (12–15). Importantly, upon challenge with Aroclor 1254, no additional sites of CYP1A1 expression were noted and no extension of the expression windows was observed. This would suggest that the CYP1A1 gene responds to exogenous ligands in the same tissue- and cell-specific manner as observed during development.

In summary, we have demonstrated for the first time that a cell-specific pattern of endogenous CYP1A1 promoter activity exists in developing mice that has a well defined temporal expression profile, which may not be regulated through the established Ah receptor signal transduction pathway. Identification of these developmental windows of expression in specific tissues will greatly advance the functional study of the expression of the CYP1A1 gene in the developing mouse. Furthermore, the transgenic mice will allow detailed study of the effects of natural and synthetic compounds on CYP1A1 expression in the developing embryo.
Acknowledgments—We thank Elizabeth Graham of the Developmental Biology Unit, Western General Hospital, Edinburgh, for help with tissue preparation, Dr. Michael Stubbins for assistance with the SSCP analysis, and Dr. Juan Wang for the design of the TaqMan primers and probes. We are also grateful to Ron Hines for the generous gift of a plasmid containing the CYP1A1 promoter sequence and to Kate Flint for the β-galactosidase gene. This manuscript was proof read by Dr. Richard Krinholtz.

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The Murine Cyp1a1 Gene Is Expressed in a Restricted Spatial and Temporal Pattern during Embryonic Development
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J. Biol. Chem. 2005, 280:5828-5835.
doi: 10.1074/jbc.M412899200 originally published online November 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M412899200

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