Endocrine, *vol. 23, nos. 2–3, 219–228, March–April 2004* 0969–711X/04/23:219–228/$25.00

Estrogen Modulates Developmentally Regulated Gene Expression in the Fetal Baboon Liver

Miriam D. Rosenthal,[1] Eugene D. Albrecht,[2] and Gerald J. Pepe[1]

[1]Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA; and [2]Departments of Obstetrics/Gynecology/Reproductive Sciences and Physiology, Center for Studies in Reproduction, University of Maryland School of Medicine, Baltimore, MD

Although estrogen plays a central integrative role in regulating key aspects of placental and fetal endocrine development in the primate, our understanding of the regulation of maturation of the fetal liver is incomplete. In adults, estrogen modulates several aspects of hepatic function. Therefore, the current study determined whether fetal hepatic gene expression development was modulated by estrogen. mRNA differential display was used to identify genes whose expression was altered in fetal livers obtained on d 165 of gestation (term = d 184) from baboons that were untreated or treated on d 60–164 with the aromatase inhibitor CGS 20267 (2 mg/d; sc), which suppressed estrogen levels in the fetus by >95% ($p < 0.01$). As confirmed by Northern blot, the mRNA levels (ratio to 18s RNA) of metallothionein I (MT-I), porphobilinogen deaminase (PBG-D), and cytochrome P450 2C8 (CYP 2C8) in the livers of estrogen-deprived fetuses were 5-, 12-, and 3-fold higher ($p < 0.05$) than respective values of untreated fetuses. Moreover, mRNA levels of MT-I and PBG-D, expressed as a ratio to 18s RNA, were 3-fold and 26-fold higher ($p < 0.05$) on d 60–100 of gestation than on d 165 and in the adult. In contrast, CYP 2C8 mRNA increased 10-fold between d 100 and 165 and was not further altered in adult liver. Immunohistochemistry confirmed expression of MT-I in hepatocytes. Erythropoietic cells, normally present in the fetal baboon liver on d 100 but not on d 165, were also detected on d 165 in animals treated with the aromatase inhibitor. Thus, upregulation of PBG-D mRNA in estrogen-deprived baboons may reflect prolongation of the erythropoietic role of the fetal liver. In summary, these results indicate that the normal developmental change in MT-I, PBG-D, and CYP 2C8 mRNA expression in baboon fetal liver with advancing gestation are dependent on increased secretion of estrogen into the fetus. We suggest, therefore, that estrogen regulates normal development of the primate fetal liver.

Key Words: Estrogen deprivation; fetal liver; gene expression; erythropoiesis; metallothionein; cytochrome P450 2C8.

Received January 22, 2004; Revised March 5, 2004; Accepted March 8, 2004.

Author to whom all correspondence and reprint requests should be addressed: Gerald J. Pepe, PhD, Department of Physiological Sciences, Eastern Virginia Medical School, PO Box 1980, Norfolk, VA 23501-1980. E-mail: pepegj@evms.edu

Introduction

The fetal liver is a metabolically active organ, which plays a key role both in maintenance of life *in utero* and subsequent survival of the neonate. Although the liver undergoes a complex pattern of both morphological and biochemical maturation during fetal development, our understanding of the factors involved in this important process is incomplete. In adulthood, the human and nonhuman primate liver is responsive to several secretogogues including estrogen *(1,2)*, glucocorticoids *(3–5)*, and thyroid hormones *(6)*. Estrogen regulates gene expression of cholesterol 7α-hydroxylase *(7,8)*, ceruloplasmin *(9)*, angiotensinogen *(10)*, liver clotting factors *(11)*, and apoprotein A-1 *(12)* and also modulates hepatic lipid homeostasis *(13)*. Recent studies suggest that aromatase-deficient mice develop hepatic steatosis and exhibit impaired expression of genes for fatty acid oxidation *(14)*, while humans with an aromatase deficiency show altered lipid homeostasis *(15,16)*.

Our laboratories have shown that estrogen plays a central integrative role in regulating key aspects of placental and fetal development during primate pregnancy, including placental low-density-lipoprotein (LDL) uptake and P450 cholesterol side-chain cleavage enzyme (P450scc) expression. Furthermore, regulation of placental expression of the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes by estrogen serves to control cortisol production and secretion into the fetus, which in turn regulates development of the fetal pituitary–adrenal axis *(17,18)*.

Because estrogen modulates the adult liver, we hypothesized that the increase in placental estrogen secreted into the fetus during pregnancy is crucial to fetal liver development *in utero*. Therefore, we utilized mRNA differential display to identify genes whose expression was altered in the baboon fetus deprived of estrogen during the second half of

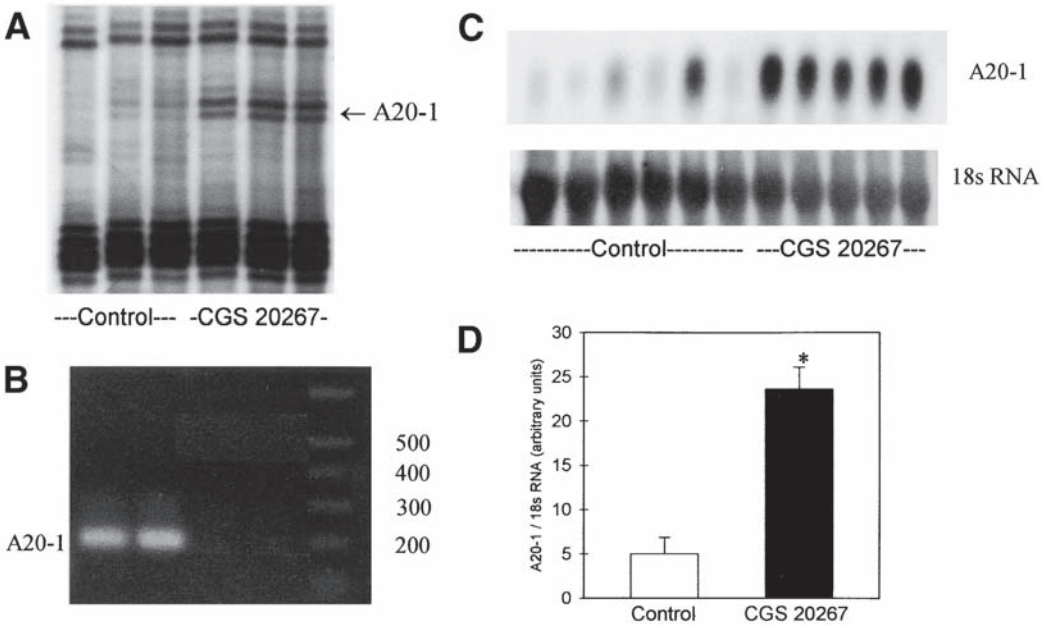

Fig. 1. Differential expression of A20-1 in baboon fetal liver from untreated animals and animals treated with CGS 20267. (**A**) Amplification of d 165 fetal liver RNA from untreated animals ($n = 3$) and animals treated from d 30 to 165 with CGS 20267 ($n = 3$) with anchored primer A and arbitrary primer 20, showing candidate band A20-1. (**B**) Agarose gel electrophoresis of the reamplified band A20-1. (**C**) Northern blot of total RNA (20 μg/lane) obtained from fetal liver from untreated baboons ($n = 6$) and from baboons treated with CGS 20267 beginning on d 30 ($n = 3$) or 60 ($n = 2$) of gestation. The membrane was then stripped and probed for 18s RNA, as described in Methods. (**D**) Mean (±SE) of ratios of the band recognized by A20-1 to 18s RNA as analyzed by phosphoimaging (arbitrary units). *Value differs from the control at $p < 0.0007$ (by unpaired t test).

gestation. The present report characterizes three such genes, two of which are expressed in hepatocytes and one that is expressed in erythropoietic cells, and also examines developmental changes in their expression in fetal livers from untreated baboons between early, mid, and late gestation.

Results

As previously reported *(19,20)*, baboons treated with CGS 20267 have mean maternal serum estradiol concentrations of 0.096 ng/mL throughout pregnancy, levels >95% lower than those in untreated controls, which increase from 1.0 ng/mL to >4.0 ng/mL between mid and late gestation. Estradiol levels in the fetus on d 165 of delivery in untreated baboons (0.59 ng/mL) were also reduced by >95% (0.04 ng/mL) in animals treated with CGS 20267. Successful pregnancies in the CGS 20267–treated baboons resulted in live fetuses that were not significantly different from untreated control baboons in body weight, gross morphology, or liver wet weight *(19)*.

Gene Expression in Fetal Liver

RNA isolated from fetal livers of three untreated and three CGS 20267–treated baboons in late gestation was analyzed by differential display, and differentially expressed cDNAs were eluted, reamplified, and cloned. Differential expression was then confirmed by Northern blot comparison of these samples as well as RNA from additional baboons that were untreated ($n = 3$) or treated with CGS 20267 ($n = 2$). Three of the positive cDNAs, designated A20-1, C28-1A, and C28-1B, were identified and are presented below in more detail.

Effect of Estrogen Depletion on Metallothionein I

Figure 1A shows the initial differential display for the cDNA A20-1 which was obtained utilizing anchored primer A (5'-AAGCTTTTTTTTTTA-3') and arbitrary primer 20 (5'-AAGCTTGTTGTGC-3'). The cDNA has an approximate size of 200 bp as determined by amplification and subsequent agarose gel electrophoresis (Fig. 1B). The differential display result was confirmed by Northern blot, utilizing the fetal hepatic RNA preparations from a total of five animals treated with CGS 20267 on d 60–165 and six controls. As seen in Fig. 1C, A20-1 identified one full-sized mRNA band of approx 500 bp, which was upregulated ($p < 0.0002$) 4.6-fold (Fig. 1D) in livers of CGS 20267-treated animals.

The sequence of A20-1 is shown in Fig. 2, with the initial primers underlined. Bases 6–91 showed 93% similarity (Blast-2 E value of E^{-27}) to the cDNA for a monkey metallothionein I *(21)*. This homology extends from the codons for the last three amino acids and the stop sequence through the length of the published sequence, which was reported to be incomplete at the 3' end. In humans, metallothioneins are

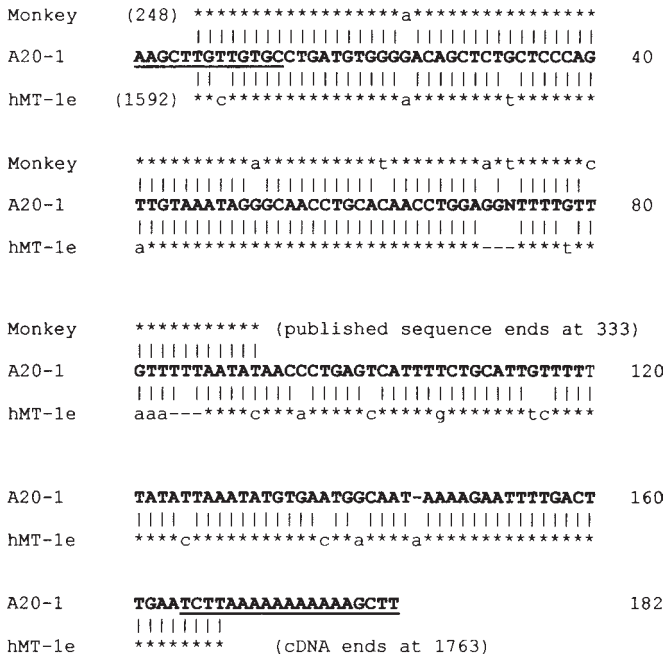

Fig. 2. Sequence of A20-1 and homology with published sequences for monkey metallothionein I and human metallothionein Ie. The cDNA for A20-1 was cloned and sequenced; sequences corresponding to the initial arbitrary and anchored primers are underlined. The sequence is aligned both with the published cDNA sequences for monkey (*Cercopothecidae* gen. sp.) MT-I (NCBI # V01533) and for hMT-Ie (NCBI # M10942). Sequence identity is shown with asterisks.

encoded by a family of genes, located on chromosome 16q13, that includes 10 functional MT genes *(22)*. A20-1 shows substantial homology to the human metallothioneins, hMT-Ie and hMT-Il, with Uniblast (www.tigem.it) E values of $6.7e^{-46}$ and $3.4e^{-32}$, respectively. Comparison with hMT-Ie (Fig. 2) indicates 91% homology of bases 9–70 of the baboon sequence with bases 1592–1656 of the human sequence for the complete gene. After a gap, including an apparent deletion in the baboon sequence relative to the human, there is 85% homology of bases 87–168 of the baboon sequence (up to the polyA tail) with bases 1727–1749 of the human sequence. Thus A20-1 is a metallothionein I, but precise characterization of the particular molecular species must await further studies of baboon isoforms.

Effect of Estrogen Depletion on Porphobilinogen Deaminase and Cytochrome P450 2C8

Figure 3 shows the initial differential display of a band designated C28-1, which was obtained utilizing anchored primer C (5'-AAGCTTTTTTTTTTTC-3') and arbitrary primer 28 (5'-AAGCTTACGGATGC-3'). When C28-1 was excised from the gel, reamplified, and cloned, clones of two slightly different sizes (approx 400 and 425 with inclusion of approx 120 bp of vector) were obtained (Fig. 3B). These clones, designated C28-1A and C28-1B, were utilized as probes for Northern analysis, comparing samples from near-term fetal livers of five CGS 20267–treated animals and six untreated baboons. As shown in Fig. 3C, C28-1A recognized a single band of approx 1.6 kb, which was upregulated ($p < 0.03$; Fig. 3D) 12-fold in fetal livers of CGS 20267–treated animals. C28-1B primarily recognized a band of approx 2 kb, which was upregulated ($p < 0.005$; Fig. 3E) approximately 2.5-fold in fetal livers from estrogen-deprived baboons.

BLAST analysis of the nearly complete sequence of C28-1A (data not shown) indicated 92% sequence identity (249/268, $E=2e^{-105}$) to the 3' untranslated region of human porphobilinogen deaminase (PBG-D), the third enzyme of the heme biosynthetic pathway. Two isoforms of PBG-D, expressed in erythropoietic and non-erythropoietic cells, respectively, are generated by transcription of a single gene from two different promoters (*23*). Because the two mRNAs are similar in size (approx 1.3 kB) and differ only at their 5' extremity, the isoform of PBG-D expressed in fetal baboon liver cannot be determined from the sequence of C28-1.

The sequence of C28-1B showed 88% sequence homology (identities = 214/242, $E=2e^{-74}$) to the published sequence for a cytochrome P450 2C from *Macaca fascicularis*. The human cytochrome P450 2C subfamily includes four genes, 2C8, 2C9, 2C18, and 2C19, clustered in a 500-kb region *(24)*, that are involved in the metabolism of endogenous lipids (e.g., epoxidation of arachidonic acid to dihydroxy-eicosatrienoic acids) as well as foreign compounds (e.g., mephenytoin) *(25,26)*. The sequence of C28-1B, isolated from fetal baboon liver, is highly homologous over its entire length (corresponding to the 3' 170 bp of exon 9 plus the entire 3' untranslated region, 205/239 identities, $E=2e^{-58}$) to hCYP2C8, but shows no significant nucleotide sequence homology to other members of the human CYP 2C subfamily.

Developmental Changes in Metallothionein I, PBG-D, and CYP2C8 mRNA

We then sought to determine whether the genes whose level of expression was increased in fetal livers from estrogen-deprived baboons were developmentally regulated. Fetal livers were obtained from fetuses of untreated baboons at early (d 60), mid (d 100), and late (d 165) gestation, and from adult female animals and total RNA was utilized in Northern blot analyses. As seen in Fig. 4, steady-state levels of mRNA for both metallothionein I (probed with A20-1) and PBG-D (probed with C28-1A) were highest early in gestation and lowest in adult samples. The level of expression (as normalized to the level of 18s RNA) observed at early and mid gestation was approximately twofold higher ($p < 0.005$) than that at late gestation, and sixfold higher ($p < 0.005$) than levels in the adult. Expression of PBG-D was 26-fold higher ($p < 0.0005$) on d 60–100 of gestation than at either d 165 of gestation or in the adult. In contrast,

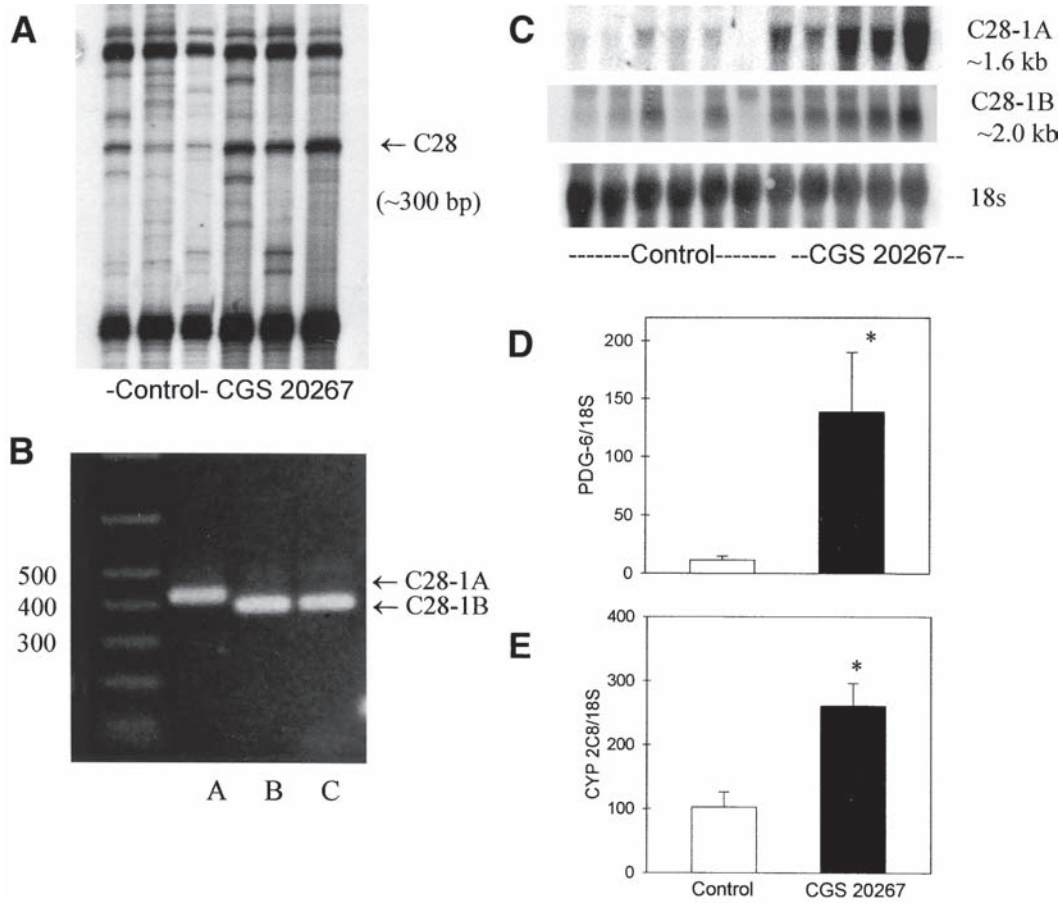

Fig. 3. Differential expression of C28-1A and C28-1B in baboon fetal liver from untreated animals and animals treated with CGS 20267. (**A**) Amplification of d 165 fetal liver RNA from control animals ($n = 3$) and animals treated from d 30 to 165 with CGS 20267 ($n = 3$) with anchored primer C and arbitrary primer 28 showing candidate band C28-1. (**B**) Agarose gel electrophoresis of the inserts obtained after cloning and colony PCR, indicating different clones with inserts of approx 420 bp (*A*) and approx 400 bp including approx 120 bp of vector. (**C**) Northern blot of total RNA (20 µg/lane) obtained from fetal liver from untreated baboons ($n = 6$) and from baboons treated with CGS 20267 beginning on d 30 ($n = 3$) or 60 ($n = 2$), probed successively for C28-1A, C28-1B, and 18s RNA. (**D**) Mean (±SE) of ratios of the band recognized by C28-1A to 18s RNA (arbitrary units) as analyzed by phosphoimaging (arbitrary units). *Value differs from the control at $p < 0.03$ (by unpaired t test). (**E**) Mean (±SE) of ratios of the band recognized by C28-1B to 18s RNA, as analyzed by phosphoimaging (arbitrary units). *Value differs from the control at $p < 0.005$ (by unpaired *t* test).

expression of CYP 2C8 was below detectable limits on d 60, then increased fourfold ($p < 0.05$) between d 100 and 165 and were not further altered in adult liver.

Effects of Estrogen on Fetal Liver Gene Expression

We then determined effect of concomitant treatment of baboons with CGS 20267 and estradiol, in levels that replicated maternal estradiol concentrations and restored fetal estrogen to 30% of normal *(19,20)*, on steady-state mRNA levels of MT-I, PBG-D, and CYP2C8. Figure 5 shows aggregate data of fetal liver mRNA levels (normalized to 18s RNA) in late gestation from baboons untreated, treated with CGS 20267 alone, and treated with CGS 20267 plus estradiol. Administration of CGS 20267 and estradiol restored the high levels of both MT-I and PBG-D observed in animals treated with CGS 20267 alone, to values not significantly ($p > 0.05$) different from those in untreated animals. However, concomitant treatment with estradiol did not appear to decrease the expression of CYP2C8.

Effects of Estrogen Depletion on Erythropoiesis

As shown in Fig. 6, although fetal liver at mid gestation contained numerous clusters of erythropoietic cells, these cells were virtually absent in late gestation (d 165). In contrast, fetal liver from late gestation baboons treated with CGS 20267 retained numerous clusters of nucleated erythropoietic cells. Identification of erythropoietic cells was confirmed by positive staining with an antibody to fetal hemoglobin (Fig. 6).

Localization of Metallothionein in Fetal Liver

Immunochemical staining for metallothionein (Figs. 6D,E) both on d 100 and d 165 fetal liver from untreated baboons is specific to hepatocytes, with no staining observed in hema-

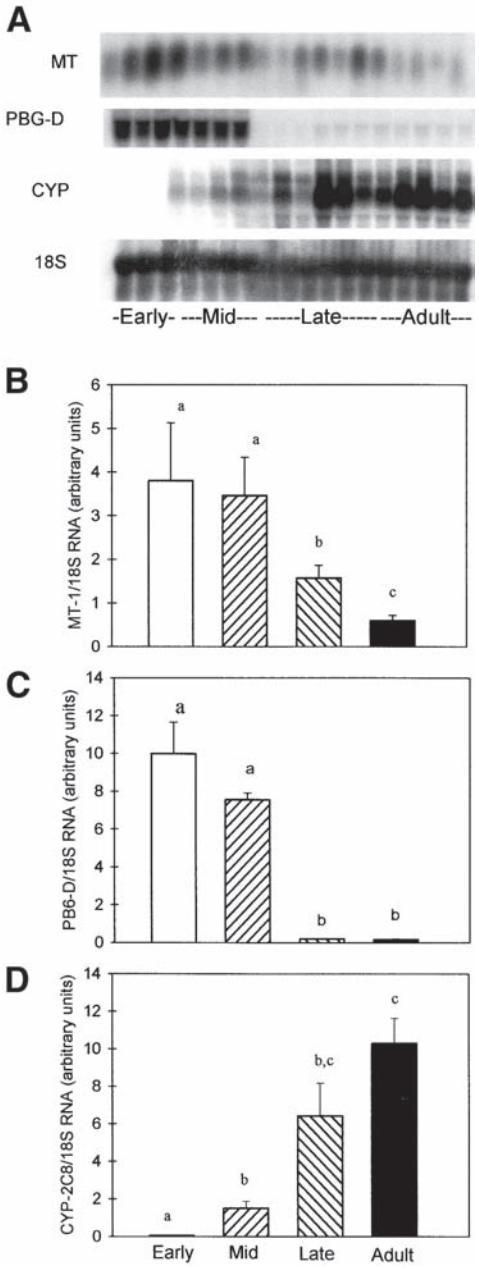

Fig. 4. Developmental expression of MT-I, PBG-D, and CYP2C8 mRNA in fetal and adult baboon liver. (**A**) Northern blot of total RNA (15 μg/lane) obtained from fetal liver at early (d 60), mid (d 100), and late (d 165) gestation (term = 184 d) and adult baboon liver. The membrane was probed successively with cDNAs to MT-I (A20-1), PBG-D (C28-1), CYP2C8 (C28-2), and 18s RNA, as described in Methods. (**B**, **C**, **D**) Means (±SE) of the respective ratios of MT-I, PBG-D, and CYP2C8 to 18sRNA, as analyzed by phosphoimaging (arbitrary units), in baboon fetal liver at early ($n = 3$), mid ($n = 4$), and late gestation ($n = 7$), and in adult liver ($n = 4$). Within each panel, mean values with different letter superscripts differ from each other ($p < 0.05$) as determined by ANOVA and post hoc corrected Bonferroni p values.

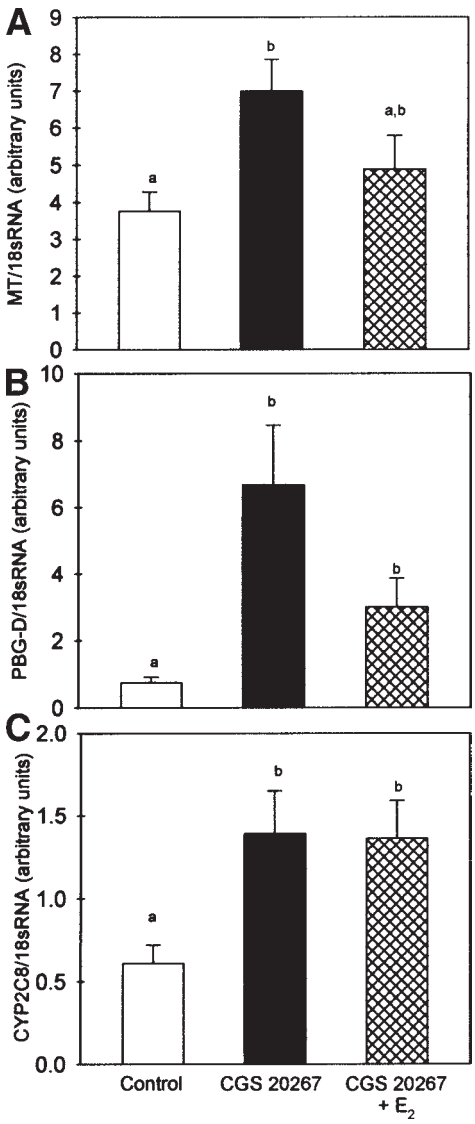

Fig. 5. Effects of daily treatment with either CGS 20267 alone or with CGS 20267 plus estradiol benzoate on gene expression in fetal baboon liver. Total RNA from fetal liver (d 165) from control animals ($n = 11$), animals treated from d 30 ($n = 3$), 60 ($n = 2$), or 100 ($n = 6$) with CGS 20267, and animals treated from d 30 ($n = 2$), 60 ($n = 1$), or 100 ($n = 3$) with CGS 20267 plus estradiol, were Northern blotted and analyzed as in Fig. 3. Data from multiple membranes were normalized utilizing reference samples that were spotted on each membrane of the series. Panels **A**, **B**, and **C** show the aggregate data (mean ±SE) of the respective ratios of MT-I, PBG-D, and CYP2C8 to 18sRNA, as analyzed by phosphoimaging (arbitrary units). Within each panel, mean values with different letter superscripts differ from each other ($p < 0.05$) as determined by ANOVA and post hoc corrected Bonferroni p values.

topoietic cells, vascular endothelial cells, fibroblasts, or Kupffer cells. Consistent with studies on fetal *(27,28)*, and regenerating liver *(29)*, significant immunohistochemical staining for metallothionein is observed in the nucleus as well as the cytoplasm. Examination of late gestation fetal liver (d 165) from baboons treated with CGS 20267 (Fig. 6F) indicates that metallothionein is similarly localized in both the nucleus and cytoplasm of hepatocytes.

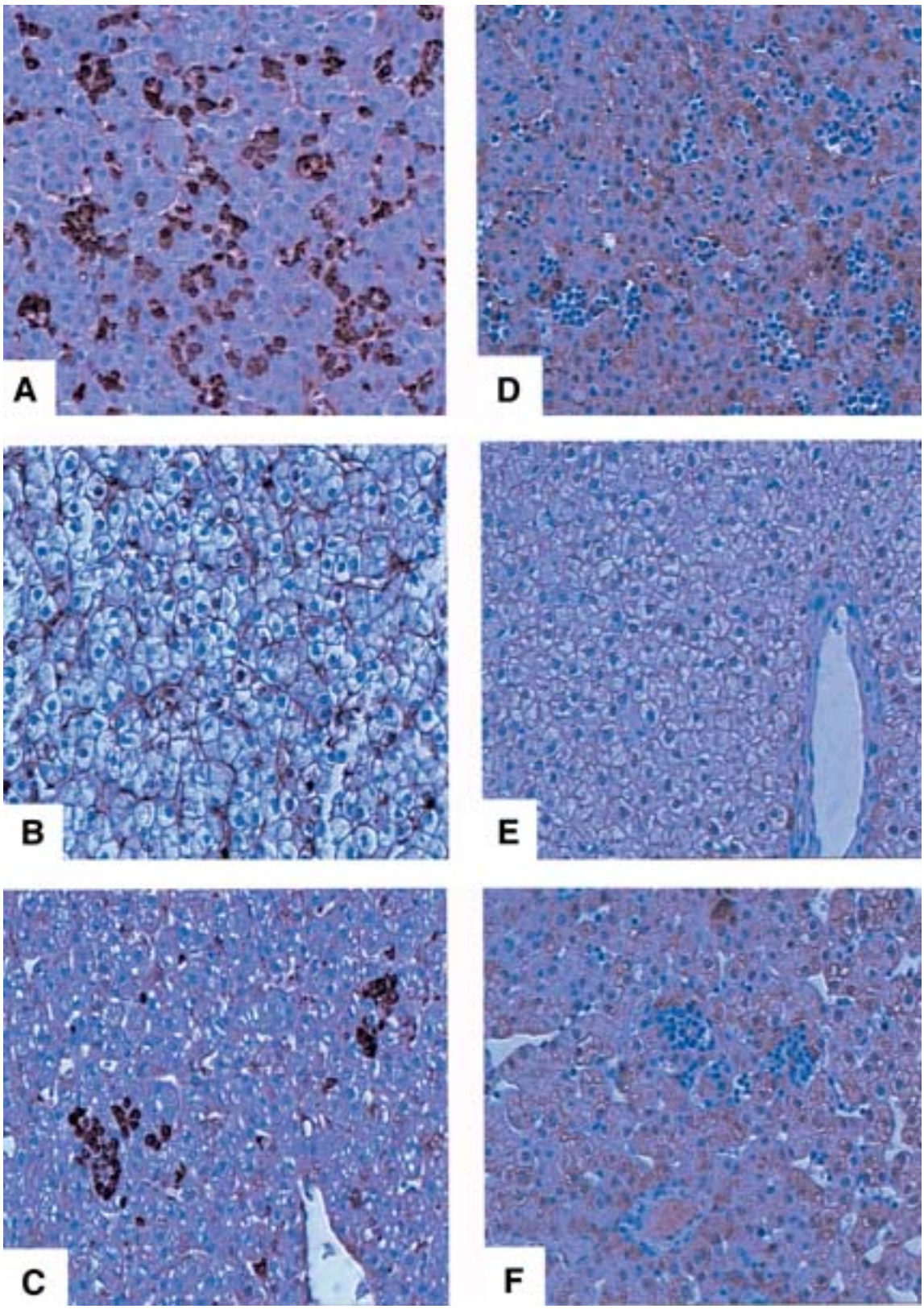

Fig. 6. Immunocytochemistry of fetal hemoglobin (**A–C**) and metallothionein (**D–F**) in fetal baboon liver. Fetal liver was obtained from untreated baboons on d 100 (**A,D**) and 165 (**B,E**) of gestation, and on d 165 from animals treated from d 100 to 165 with CGS 20267 (**C,F**). Original magnification = 200×.

Discussion

The present study demonstrates that the developmental pattern of gene expression in the baboon fetal liver is dependent on exposure to endogenous estrogen. Normally, in association with an increase in estrogen, fetal hepatic MT-I and PBG-D mRNA levels substantially declined between mid and late gestation, while expression of CYP2C8 increased with advancing gestation. Suppression of estrogen in the fetus by treatment with the aromatase inhibitor CGS 20267 prevented the decline in fetal liver steady-state mRNA levels of MT-I and PBG-D observed with advancing gestation. Moreover, MT-I and PBG-D mRNA levels in fetal livers from baboons treated concomitantly with CGS 20267 and estradiol were intermediate between those of untreated and CGS 20267–treated animals. We suggest, therefore, that estrogen suppresses fetal liver MT-I and PBG-D expression in the second half of primate pregnancy. In contrast, the progressive increase in fetal hepatic CYP2C8 expression with advancing gestation was enhanced by CGS 20267 treatment. It appears, therefore, that intrauterine estrogen has opposite effects on the temporal patterns of expression of different fetal hepatic genes although it remains to be determined whether the effects on mRNA are reflected by alterations in protein expression.

As reported previously *(19)*, maternal and fetal body weights and fetal organ weights including the liver were not significantly different in untreated and CGS 20267–treated baboons. However, although growth was not altered by lack of normal estrogen exposure *in utero*, the present study suggests that hepatic cellular organization and function may have been changed. Thus, in estrogen-deprived fetuses, it appears that there was a prolongation of the role of the liver as a site of erythropoiesis. In support of this suggestion, hepatic expression of PBG-D, an intermediate in the pathway of heme synthesis, was highest during early to mid gestation, indicative of the active role of the liver as the major organ of erythropoiesis during that time *(30)*. By late gestation fetal liver PBG-D expression declined, suggesting that the site of erythropoiesis had shifted from fetal liver to bone marrow. In contrast, steady-state PBG-D mRNA levels in late gestation fetal livers of CGS 20267–treated baboons remained elevated and similar to levels in untreated fetuses at mid gestation. In addition, histological examination indicated that livers from estrogen-depleted animals retained numerous clusters of nucleated, fetal hemoglobin positive, erythropoietic cells normally seen at mid gestation. Preliminary histologic studies also indicated that the extent of hepatic erythropoiesis at d 165 in animals treated with both CGS 20267 and estradiol, as with PBG-D mRNA levels, was intermediate between that of controls and of animals treated with CGS 20267 alone (Rosenthal, M.D. and Werner, A.L., unpublished observations). Although it is likely that factors in addition to and/or in conjunction with estrogen may influence fetal liver maturation, the inability of estrogen to fully restore fetal liver development most likely reflects the level of estrogen in the fetus. Thus, in our previous studies, we showed that, whereas maternal serum estradiol levels were restored to normal, treatment with CGS 20267 plus estradiol elevated fetal estradiol to levels which were only 30% of those of untreated animals *(20)*. Finally, whether erythropoietic cells are still observed in the neonatal period in animals deprived of estrogen *in utero* remains to be determined.

Metallothioneins are a family of small, cysteine-rich proteins that bind zinc, copper, and other heavy metals and are thought to be involved in regulation of zinc homeostasis and gene expression as well as heavy metal detoxification *(31)*. The developmental changes observed in the baboons of the present study are consistent with studies in humans *(27,32)* and other mammals *(33)* indicating that metallothionein I gene expression is higher in fetal and neonatal than adult liver. In the current study, expression of metallothionein was localized to the nuclei of hepatocytes and not detected immunochemically in vascular smooth muscle or erythropoietic cells in fetal livers from either control or CGS 20267–treated baboons. Unlike adult liver, where immunologically reactive metallothionein is primarily cytoplasmic, the extensive nuclear localization of metallothionein in the fetus *(27,28)* and in regenerating liver *(29)* may reflect a role for metallothioneins in the regulation of gene expression during development *(34)*. Indeed, in metallothionein-null mice hepatic gene expression is altered during development *(35)*. One mechanism for this regulatory role of MT may involve reversible exchange of zinc between metallothionein and the estrogen receptor zinc finger *(36)*. Thus, the changes in metallothionein expression in estrogen-deprived fetal liver may, in turn, modify other aspects of hepatocyte function.

The effects of upregulation of CYP2C8 in the fetal liver of aromatase-inhibited baboons also remain to be elucidated. Whereas human studies *(37)* have suggested that CYP2C8/9 becomes active after birth, our data indicate that in the baboon fetal hepatic expression of CYP2C8 mRNA is induced *in utero* in late gestation. CYP2C8 catalyzes the epoxygenation of arachidonate *(26)*, and is thus an endothelial-dependent hyperpolarizing factor synthase in coronary arteries *(38)*. In neonatal human liver, enhanced activation of CYP2C gene transcription is related to elevated production of 14,15- and 11,12-epoxyeicosatrienoic acids (EETs) *(39)*.

Although the present study has demonstrated changes in gene expression in fetal liver in estrogen-depleted baboons, the specific mechanisms for upregulation of MT-I, PBG-D, and CYP2C8 remain to be elucidated. While none of these genes has been shown to be directly estrogen responsive, MT-II has recently been shown to be upregulated in human tumor cell lines overexpressing ER-β *(40)*. In the mouse, the developmental change in MT-I gene expression is regulated primarily by glucocorticoids *(41)*, while human CYP2C8 has sites for regulation by hepatic transcription factors such as HPF-1 *(42)* as well as multiple 5" glucocorticoid-

responsive elements *(43)*. Recent studies have demonstrated that other genes, such as proteinase inhibitor 9, an inhibitor of apoptosis, are estrogen-inducible in human liver cells *(44)*. Preliminary studies using specific gene arrays also suggest that expression of genes in addition to MT-I, PBG-D, and CYP2C8 appear to be modulated by estrogen in baboon fetal liver (Rosenthal, M.D. and Pepe, G.J., in progress). Estrogen receptor β mRNA is expressed both in mid-gestation fetal human *(45)* and baboon (Rosenthal, M.D. and Billiar, R.B., unpublished observations) liver, suggesting that the fetal liver, like the adult, is an estrogen-responsive organ. Although preliminary studies suggest that fetal hepatic ERβ mRNA is not altered in estrogen-suppressed animals (Rosenthal, M.D. and Pepe, G.J., in progress), given the multiple effects of estrogen on other hormones, including glucocorticoids, during fetal development *(17)*, the observed changes in gene expression in aromatase-inhibited animals may, however, reflect indirect rather than direct effect of estrogen depletion on the developing liver.

In conclusion, the present study shows that administration of the aromatase inhibitor CGS 20267 to pregnant baboons and the resultant suppression of normal physiological levels of estrogen during fetal development have a striking effect on gene expression in the fetal liver. Although the consequences of these changes on hepatic function in adulthood remain to be determined, changes in hepatic gene expression in the absence of estrogen may reflect one mechanism by which the hormonal environment *in utero* programs *(46, 47)* subsequent physiological function.

Materials and Methods

Animals

Female baboons (*Papio anubis*) obtained from the Southwest Foundation for Biomedical Research (San Antonio, TX) and weighing 10–15 kg were housed individually in air-conditioned quarters. Females were paired with males for 5 d at the anticipated time of ovulation, as determined by menstrual cycle history and turgescence of the external sex skin *(48)*.

Fetal livers were obtained on d 60 (n = 3), 100 (n = 4), and 165 (n = 11) of gestation (term = 184 d) from untreated baboons and on d 165–170 of gestation from animals injected daily (n = 11) with the aromatase inhibitor CGS 20267 (4,4'-[1,2,3-triazyol-1-*yl*-methylene]-bis-benzonitrite, Letrozole; Novartis Pharma AG, Basel, Switzerland) *(49)* as described previously *(19)*. The initial dose of 0.1 mg/d (in sesame oil) was increased daily by 0.1-mg increments until a maximum of 2.0 mg/d. A second group received the same CGS 20267 treatment plus estradiol benzoate (0.1–2.0 mg/d sc in sesame oil) at doses designed to replicate the normal pattern of maternal serum estradiol concentrations. Drug treatments were initiated on either d 30 (n = 3), d 60 (n = 2), or d 100 (n = 6) of gestation. On d 60, 100, or 165–170 of gestation, baboons were anesthetized with isoflurane, maternal saphenous and umbilical venous samples were collected, and the fetus was delivered and euthanized with an overdose of sodium pentobarbital. The entire liver was weighed, and pieces stored in liquid nitrogen or fixed in 10% phosphate-buffered formalin. Liver was also available from adult nonpregnant female baboons, which had previously been euthanized.

Baboons were cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol employed was approved by the Institutional Animal Care and Use Committees of the Eastern Virginia Medical School and the University of Maryland School of Medicine.

Total RNA Isolation and Differential Display

Total RNA was extracted from liver as described previously *(50)*. Briefly, tissues were homogenized in 4 *M* guanidine isothiocyanate, 25 m*M* sodium acetate (pH 6.0), and 0.83% beta-mercaptoethanol at 4°C, and RNA was extracted with chloroform/isoamyl alcohol (24:1, v/v). The aqueous layer was forced through a 23-gauge needle, layered onto a 5.7 *M* cesium chloride gradient, and centrifuged at 179,000*g* for 21 h at 23°C. Aliquots of the total RNA (50 μg) were treated with DNase I (10 U; Sigma) for 30 min at 37°C and the RNA was then extracted with phenol/chloroform to remove protein contamination.

Differential display of mRNA was performed as described by Liang and Pardee *(51)* utilizing RNA image kits (GenHunter, Nashville, TN) according to the manufacturer's instructions. First-strand cDNA was synthesized in an MJ thermocycler using oligo (dT) primers with a single anchored nucleotide at the 3'-end. PCR reactions were then performed using the same anchored primers, H-AP arbitrary 13-mers, *Taq* DNA polymerase (Qiagen), and α-[^{33}P]dATP (2000 Ci/mmol, New England Nuclear). The amplified cDNA was then separated on a 6% denaturing polyacrylamide gel (Sequagel-6, National Diagnostics). The gel was dried without fixing and autoradiographed on Kodak Biomax film.

Retrieval, Cloning, and Sequencing of Differential Display Products

Bands corresponding to cDNAs of interest were excised from the dried gels. Each band was incubated in 100 μL 2X PCR buffer at room temperature for 10 min and then at 94°C for 90 min to release the PCR product. The cDNA was recovered by ethanol precipitation, redissolved in 10 μL of water, and 4 μL of the DNA reamplified by 22 cycles of PCR using the same primer set and temperatures as in the differential display. Gel-purified cDNAs were cloned using the GenHunter PCR-TRAP Cloning System (GenHunter). After checking for appropriate sized inserts by colony PCR and agarose gel electrophoresis, selected clones were sequenced using the Promega fmol DNA sequencing system and Lseq and Rseq primers within the cloning vector.

Sequences were compared with the National Center for Biotechnology Information nonredundant database using the BLASTN 2 program *(52)* and the Tigem (Naples, Italy) database using the HsUniGene cluster identifier program.

Northern Blot Analysis

Approximately 20 µg total RNA was denatured in 50% formamide, 2.2 *M* formaldehyde, and 20 m*M* 3-[*N*-morpholino]propane sulfonic acid, pH 7.0, and size fractionated by electrophoresis in a 1.0% agarose gel containing 2.2 *M* formaldehyde. RNA was transferred overnight by capillary action onto a nylon membrane (Magnagraph, MSS, Westboro, MA) using 20X SSC (1X SSC contains 0.15 *M* sodium chloride, 15 m*M* sodium citrate, pH 7.0), and immobilized by baking for 1 h at 80°C. For each differential display band of interest, cDNA was prepared by colony PCR and gel purification. Radiolabeled probes were prepared from 10 ng cDNA (obtained by colony PCR agarose gel electrophoresis and Quiaex II purification) by linear PCR amplification using an Ambion Strip-EZ PCR kit, 20 µCi [α-^{32}P]dATP (New England Nuclear), and the appropriate initial GenHunter anchored primer. Membranes were prehybridized for 2 h at 42°C in ULTRAhyb (Ambion, Austin, TX). ^{32}P-labeled cDNA probe (10^7 cpm/10 mL) was then added, and the membrane was hybridized overnight. Following hybridization, the membranes were washed three times (5 min each) at room temperature in 2X SSC containing 0.1% SDS, then twice (10 min each) at 50°C in 0.1X SSC, 0.1% SDS and visualized as described below. Prior to successive probing with a second cDNA probe, probes were degraded and removed from the membranes according to the Ambion Strip-EZ protocols. Size of mRNA transcripts was determined by loading one lane of each gel with Millenium Markers (Ambion); the corresponding portion of the resultant membrane was then probed with the corresponding marker template. In order to normalize message levels, the membranes were also probed for 18s RNA, using random labeling of a mouse cDNA probe (Ambion).

Quantification and Statistical Analysis

Binding of radiolabeled probe was visualized and quantified using phosphoimage analysis and the ImageQuant software. In some cases, data from multiple membranes were normalized utilizing reference samples that were applied to each membrane of the series. The membranes were then exposed (–80°C) for 4–96 h (depending on level of signal) to Fuji Medical X-ray film. Because mRNA levels (ratio to 18s RNA) of the various transcripts identified by differential display were similar in fetal liver samples obtained on d 165 of gestation from animals treated with CGS 20267 or CGS 20267 plus estradiol beginning on d 30, 60, or 100 of gestation, data from these respective treatment groups were pooled. Data were analyzed by unpaired *t*-tests or by ANOVA, with post-hoc comparison of the means utilizing adjusted *t* tests with *p* values corrected by the Bonferroni method.

Histology and Immunocytochemistry

Paraffin embedded sections of fetal liver were stained with hematoxylin and eosin and examined by light microscopy. Immunological detection of metallothionein was performed using peroxidase-blocked sections (4 µm) without microwave retrieval. Sections were blocked (30 min) in 5% normal goal serum (NGS; Vector Laboratories, Burlingame, CA) and incubated overnight with mouse monoclonal antibody to horse metallothionein (Zymed, S. San Francisco, CA). After washing, sections were incubated with biotinylated goat anti-mouse IgG (Vector) and biotin detected with an avidin peroxidase kit (Vector). Immunological detection of fetal hemoglobin was performed similarly, using sheep primary antibodies to human hemoglobin F (HgF, Bethyl Laboratories, Montgomery, TX; final concentration 5 µg/mL) and biotinylated rabbit anti-sheep IgG. After chromagen development, sections were washed, mounted, and photographed.

Acknowledgments

This work was supported from NIH Research Grant R01 HD-13294 (E.D.A. and G.J.P.) and by the Jeffress Memorial Trust (M.D.R.). The authors greatly appreciate the assistance of Mr. Jeffrey Stephens in performing the differential display and Northern blotting analyses, Reinhart B. Billiar, Ph.D. and Ms. Pam Brien in performing the immunocytochemistry, and Ms. Sandra Huband and Nicholas Zachos, Ph.D. in preparation of the figures. The authors greatly appreciate the supply of CGS 20267 generously provided by Norvartis Pharma AG, Basel, Switzerland.

References

1 Archer, T. K., Tam, S. P., Deugau, K. V., and Deeley, R. G. (1985). *J. Biol. Chem.* **260,** 1676–1681.
2 Marschke, K. B. and Koritnik, D. R. (1987). *J. Steroid Biochem.* **26,** 443–450.
3 Cole, T. J., Blendy, J. A., Schmid, W., Strahle, U., and Schutz, G. (1993). *J. Steroid Biochem. Mol. Biol.* **47,** 49–53.
4 Schweizer-Groyer, G., Cadepond, F., Jibard, N., et al. (1997). *Biochem. J.* **324,** 823–831.
5 Vannice, J. L., Grove, J. R., and Ringold, G. M. (1983). *Mol. Pharmacol.* **23,** 779–875.
6 Feng, X., Jiang, Y., Meltzer, P., and Yen, P. M. (2000). *Mol. Endocrinol.* **14,** 947–955.
7 Bravo, E., Cantafora, A., Cicchini, C., Avella, M., and Botham, K. M. (1999). *Biochim. Biophys. Acta* **1437,** 367–377.
8 Chico, Y., Fresnedo, O., Lacort, M., and Ochoa, B. (1994). *Steroids* **59,** 528–535.
9 Middleton, R. B. and Linder, M. C. (1993). *Arch. Biochem. Biophys.* **302,** 362–368.
10 Gordon, M. S., Chin, W. W., and Shupnik, M. A. (1992). *J. Hypertens.* **10,** 361–366.
11 Farsetti, A., Misiti, S., Citarella, F., et al. (1995). *Endocrinology* **136,** 5076–5083.
12 Lamon-Fava, S., Ordovas, J. M., and Schaefer, E. J. (1999). *Arterioscler. Thromb. Vasc. Biol.* **19,** 2960–2965.
13 Djouadi, F., Weinheimer, C. J., Saffitz, J. E., et al. (1998). *J. Clin. Invest.* **102,** 1083–1091.

14 Nemoto, Y., Toda, K., Ono, M., et al. (2000). *J. Clin. Invest.* **105,** 1819–1825.
15 MacGillivray, M. H., Morishima, A., Conte, F., Grumbach, M., and Smith, E. P. (1998). *Horm. Res.* **49,** 2–8.
16 Morishima, A., Grumbach, M. M., Simpson, E. R., Fisher, C., and Qin, K. (1995). *J. Clin. Endocrinol. Metab.* **80,** 3689–3698.
17 Albrecht, E. D. and Pepe, G. J. (1999). *Placenta* **20,** 129–139.
18 Pepe, G. J. and Albrecht, E. D. (1995). *Endocrine Rev.* **16,** 608–648.
19 Albrecht, E. D., Aberdeen, G. W., and Pepe, G. J. (2000). *Am. J. Obstet. Gynecol.* **182,** 432–438.
20 Pepe, G. J., Ballard, P. L., and Albrecht, E. D. (2003). *J. Clin. Endocrinol. Metab.* **88,** 471–477.
21 Schmidt, C. J. and Hamer, D. H. (1983). *Gene* **24,** 137–146.
22 Stennard, F. A., Holloway, A. F., Hamilton, J., and West, A. K. (1994). *Biochim. Biophys. Acta* **1218,** 357–365.
23 Grandchamp, B., De Verneuil, H., Beaumont, C., Chretien, S., Walter, O., and Nordmann, Y. (1987). *Eur. J. Biochem.* **162,** 105–110.
24 Gray, I. C., Nobile, C., Muresu, R., Ford, S., and Spurr, N. K. (1995). *Genomics* **28,** 328–332.
25 Capdevila, J. H., Falck. J. R., and Harris, R. C. (2000). *J. Lipid Res.* **41,** 163–181.
26 Zeldin, D. C., DuBois, R. N., Falck, J. R., and Capdevila, J. H. (1995). *Arch. Biochem. Biophys.* **322,** 76–86.
27 Cherian, M. G. (1994). *Environ. Health Perspect.* **102(Suppl. 3),** 131–135.
28 Nartey, N. O., Banerjee, D., and Cherian, M. G. (1987). *Pathology* **19,** 233–238.
29 Tohyama, C., Suzuki, J. S., Hemelraad, J., Nishimura, N., and Nishimura, H. (1993). *Hepatology* 1**8,** 1193–1201.
30 Malhotra, K., Luehrsen, K. R., Costello, L. L., et al. (1999). *Nucleic Acids Res.* **27,** 839–847.
31 Andrews, G. K. (1990). *Prog. Food Nutr. Sci.* **14,** 193–258.
32 Klein, D., Scholz, P., Drasch, G. A., Muller-Hocker, J., and Summer, K. H. (1991). *Toxicol. Lett.* **56,** 61–67.
33 Webb, M. (1987). *Experientia Suppl.* **52,** 483–498.
34. Cherian, M. G. and Apostolova, M. D. (2000). *Cell Mol. Biol. (Noisy-le-grand)* **46,** 347–356.
35 Kimura, T., Oguro, I., Kohroki, J., et al. (2000). *Biochem. Biophys. Res. Commun.* **270,** 458–461.
36 Cano-Gauci, D. F. and Sarkar, B. (1996). *FEBS Lett.* **386,** 1–4.
37 Oesterheld, J. R. (1998). *J. Child Adolesc. Psychopharmacol.* **8,** 161–174.
38 Fisslthaler, B., Popp, R., Kiss, L., et al. (1999). *Nature* **401,** 493–497.
39 Treluyer, J. M., Benech, H., Colin, I., Pruvost, A., Cheron, G., and Cresteil, T. (2000). *Pediatr. Res.* **47,** 677–683.
40 Harris, H., Henderson, R., Bhat, R., and Komm, B. (2001). *Endocrinology* **142,** 645–652.
41 Quaife, C., Hammer, R. E., Mottet, N. K., and Palmiter, R. D. (1986). *Dev. Biol.* **118,** 549–555.
42 Klose, T. S., Blaisdell, J. A., and Goldstein, J. A. (1999). *J. Biochem. Mol. Toxicol.* **13,** 289–295.
43 Ged, C. and Beaune, P. (1991). *Biochim. Biophys. Acta* **1088,** 433–435.
44 Kanamori, H., Krieg, S., Mao, C., et al. (2000). *J. Biol. Chem.* **275,** 5867–5873.
45 Brandenberger, A. W., Tee, M. K., Lee, J. Y., Chao, V., and Jaffe, R. B. (1997). *J. Clin. Endocrinol. Metab.* **82,** 3509–3512.
46 Barker, D. J. (1999). *Ann. Med.* **31(Suppl. 1),** 3–6.
47 Seckl, J. R. (1998). *Clin. Perinatol.* **25,** 939–962.
48 Albrecht, E. D. (1980). *Am. J. Obstet. Gynecol.* **136,** 569–574.
49 Bhatnagar, A. S., Hausler, A., Schieweck, K., Lang, M., and Bowman, R. (1990). *J. Steroid Biochem. Mol. Biol.* **37,** 1021–1027.
50 Rosenthal, M. D., Albrecht, E. D., and Pepe, G. J. (1998). *J. Clin. Endocrinol. Metab.* **83,** 2861–2867.
51 Liang, P. and Pardee, A. B. (1992). *Science* **257,** 967–971.
52. Altschul, S. F., Madden, T. L., Schaffer, A. A., et al. (1997). *Nucleic Acids Res.* **25,** 3389–3402.