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Comparison of Oxidative Stress Response Parameters in Newborn Mouse Liver versus Simian Virus 40 (SV40)-Transformed Hepatocyte Cell Lines

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ABSTRACT. Induction of approximately one dozen genes and/or enzyme activities in liver of the untreated newborn c^{14CoS}/c^{14CoS} mouse—when compared with the c^{ch}/c^{14CoS} heterozygote or the c^{ch}/c^{ch} wild-type—is the result of enhanced levels of reactive oxygenated metabolites originating from a block in the tyrosine degradation pathway. Oxidative stress activates genes via the electrophile response element, whereas dioxin activates genes via the receptor-mediated aromatic hydrocarbon response element. Here, we compared several parameters in *14CoS/14CoS* versus *ch/ch* newborn mouse liver with that in simian virus 40 (SV40)-transformed hepatocyte lines that had been derived from newborn liver. We showed in this study that: (a) NADP(H):quinone oxidoreductase and UDP glucuronosyltransferase 1A6 mRNA levels were increased in both the (untreated) *14CoS/14CoS* newborn liver and cell line; (b) aldehyde dehydrogenase 3A1 mRNA was increased by both oxidative stress and dioxin in hepatocyte cultures, but was not detectable in liver of the intact mouse; (c) the glutathione *S*-transferase GSTA1, GSTP1, GSTA3, and GSTM1 mRNA levels were increased by oxidative stress in *14CoS/14CoS* newborn liver, but these transcripts were either low or undetectable in the cell lines; (d) GSTA1 mRNA was up-regulated by the absence of cytochrome P450 1A1 (CYP1A1) activity (i.e. the *Gsta1* gene is a member of the aromatic hydrocarbon [*Ah*] battery); and (e) GSTP1 mRNA was not up-regulated by the absence of CYP1A1 activity (i.e. *Gstp1* is not a member of the [*Ah*] battery). The *14CoS/14CoS* and *ch/ch* hepatocyte established cell lines were transformed with SV40, which expresses large T antigen; this gene product is known to bind to, and interact with, several cell cycle regulatory proteins such as p53 and the retinoblastoma protein–E2F complex. It is therefore likely that differences in the oxidative stress responses between the *14CoS/14CoS* newborn liver and the immortalized hepatocyte cell line might be explained by the presence of large T antigen in the established cell line. BIOCHEM PHARMACOL **59**;6:703–712, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. oxidative stress; [*Ah*] gene battery; mouse genetics; NAD(P)H:quinone oxidoreductase; UDP glucuronosyltransferase; CAAT enhancer binding protein-α; aldehyde dehydrogenase; glutathione *S*-transferases; cDNA probes; Northern hybridization; SV40-transformed cell culture lines; large T antigen; cell cycle; dioxin; β-naphthoflavone; Ah receptor

The c^{14CoS} mouse line has a radiation-induced 3800-kilobase deletion on chromosome 7 that includes the *c* (tyrosinase; albino) locus. The untreated c^{14CoS}/c^{14CoS} homozygote dies during the first day postpartum for unknown reasons, whereas the c^{ch}/c^{ch} wild-type and the c^{ch}/c^{14CoS} heterozygote remain viable [reviewed in Refs. 1–3]. Because the *14CoS/14CoS* homozygote dies during the first 24 hr after birth—rendering these animals difficult to study—it was felt that immortalized liver cell lines, derived from newborn livers, would be more convenient as an experimental model system. SV40¶-transformed hepatocyte cul-

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¶ *Abbreviations: Nqo1* and NQO1, mouse NAD(P)H:quinone oxidoreductase (also called NMO1, quinone reductase, DT-diaphorase) gene and enzyme; *Ugt1a6* and UGT1A6, mouse UDP glucuronosyltransferase 1A6 gene and enzyme; *gadd* and GADD, growth arrest and DNA damage-inducible genes and proteins; *Cebpa* and C/EBPα, mouse CAAT enhancer-binding protein-α gene and protein; *Aldh3a1* and ALDH3A1, mouse cytosolic aldehyde dehydrogenase-3 gene and enzyme (formerly *Ahd4*, AHD4); *Gsta1* and GSTA1, mouse glutathione *S*-transferase (Ya; class α) gene and enzyme; *Gstp1* and GSTP1, mouse glutathione *S*-transferase (class π) gene and enzyme; *Gsta3* and GSTA3, mouse glutathione *S*-transferase (Yc) gene and enzyme; *Gstm1* and GSTM1, mouse glutathione *S*-transferase (class mu, μ) gene and enzyme; *Ahr* and AHR, mouse Ah receptor gene and protein; ARNT, Ah receptor nuclear transporter; D2.B6-Ahr^{b1}, congenic mouse line homozygous for the C57BL/6J *Ahr*

tures have therefore been derived from the livers of these *14CoS/14CoS*, *ch/14CoS*, and *ch/ch* newborn mice [4]. In contrast to the *ch/14CoS* heterozygote and *ch/ch* wild-type, the *14CoS/14CoS* exhibits elevated mRNA, enzyme activities, and/or other parameters from among the following: NQO1 but not CYP1A1 [5]; three *gadd* genes [6]; superoxide dismutase, glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities [7]; UGT1A6 and ALDH3A1 mRNAs [8]; an amount of protein binding to the EPRE DNA motif [8]; the Group II Ca^{2+}-dependent secreted 14-kDa form of phospholipase A_2, increased arachidonic acid release, and elevated prostaglandins D_2, E_2, and $F_{2\alpha}$ [9]; and enhanced levels of NADPH and GSH concentrations [10]. All of these findings are consistent with a striking oxidative stress response in the untreated *14CoS/14CoS* homozygote. This oxidative stress in the *14CoS/14CoS* mouse has been shown to be caused by a disruption in the fumarylacetoacetate hydrolase (*Fah*) gene located at the proximal edge of the chromosome 7 deleted region [11, 12]. Absence of the enzyme FAH, which participates in the tyrosine degradation pathway, causes an accumulation of reactive oxygenated metabolites (ROMs), thereby leading to endogenous ROM-mediated oxidative stress in the untreated *14CoS/14CoS* newborn liver as well as in the hepatocyte cell line [8, 11].

Intriguingly, some of the parameters listed above—observed in *14CoS/14CoS* but not in the *ch/14CoS* heterozygote or *ch/ch* wild-type—occur in both newborn liver and the SV40-transformed hepatocyte cultures, whereas other of the parameters listed above occur in either newborn liver or the hepatocyte cell line. In the present study, we compared NQO1, UGT1A6, C/EBPα, GSTA1, GSTA3, GSTP1, and GSTM1 mRNA levels expressed in the intact mouse versus the immortalized cell lines. We found that the expression of some genes is up-regulated in both the intact animal and cells in culture, whereas other parameters are seen in one but not the other. Finally, we speculate on reasons why these differences exist between the intact mouse and liver-derived cell lines.

MATERIALS AND METHODS

Mouse Colony and Treatment of Animals

In 1986, male and female *ch/14CoS* breeders were generously given to us by Liane B. Russell (Oak Ridge, TN), and the breeding of *ch/14CoC* heterozygotes has been carried out in the Nebert mouse colony since then. The three genotypes are readily identified from their phenotype from about gestational day 10 up to and following birth: the *14CoS/14CoS* is albino (no eye pigmentation) and dies during the first 24 hr postpartum; the wild-type *ch/ch* is viable and has dark eye pigmentation and chinchilla coat color; and the *ch/14CoS* heterozygote is also viable and has intermediate (gene dose) eye pigmentation and dilute brown coat color. In some experiments, the pregnant *ch/14CoS* mother was treated intraperitoneally with TCDD [20 μg/kg in *p*-dioxane (500 μL/kg)] on the last day of gestation; controls received the vehicle alone. This treatment is known [3, 5] to cross the placenta and up-regulate the expression of a number of genes in fetal or newborn liver. C57BL/6J (B6) and DBA/2J (D2) inbred mice were purchased from The Jackson Laboratory. The D2.B6-*Ahr*^{b1} and B6.D2-*Ahr*^{d} congenic lines [13, 14] have been developed and maintained in the Nebert mouse colony since 1975; these mice (4-week-old sexually immature females) were treated intraperitoneally with either BNF (200 mg/kg) or the vehicle alone (25 mL corn oil/kg) 12 hr before killing.

Cell Culture Conditions

The development and characterization of *ch/ch*, *ch/14CoS*, and *14CoS/14CoS* SV40-transformed cell lines, derived from newborn mouse liver, have been described in detail [4]. The cells were immortalized with SV40 *tsA* mutants; because the transforming large T antigen encoded by the A gene is inactivated at elevated temperatures, the transformed phenotype of *tsA* mutant-immortalized cell lines can be reversed simply by a shift in temperature. At the permissive temperature of 34°, the hepatocytes exhibit a transformed phenotype and express low levels of liver-specific genes, whereas at the non-permissive temperature of 39°, these cells lose their malignant phenotype and express high levels of many liver-specific genes. Two wild-type clones, 20B and 48B, have been named *ch/ch-1* and *ch/ch-2*, respectively; one heterozygote clone, 113B, and one deletion homozygote line, 128, have been named *ch/14CoS-1* and *14CoS/14CoS-1*, respectively [4]. In this report, only cell lines 20B and 128 were used and will hereafter be referred to as *ch/ch* and *14CoS/14CoS*, respectively. The *14CoS/14CoS* and *ch/ch* hepatocyte lines are routinely grown at 34° in 75-cm^2 flasks containing 15 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 0.1% gentamycin, and 26 mM $NaHCO_3$, under 95% air:5% CO_2. These two cell lines, in exponential growth (about 75% confluent), were used for all studies. The phenotypes of elevated *Nqo1* and *Aldh3a1* gene expression in the *14CoS/14CoS* line, compared with that observed in the *ch/ch* wild-type cell line, have remained stable for more than 12 years. In some experiments, we used wild-type mouse hepatoma Hepa-1c1c7 cells, the CYP1A1 metabolism-deficient *c37* mutant, the ARNT-defective *c4* mutant, and the Ah receptorless (containing <10% of functional AHR levels) mutant [reviewed in Ref. 15]. In cultures treated with TCDD, 20 nM concentrations (first dissolved in *p*-dioxane) were

allele in >99% DBA/2J background; B6.D2-*Ahr*^{d}, congenic mouse line homozygous for the DBA/2J *Ahr* allele in >99% C57BL/6J background; TCDD, dioxin or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BNF, β-naphthoflavone; SV40, simian virus 40; 1X SSC, 0.15 M NaCl, 0.015 M sodium citrate; AHREs, aromatic hydrocarbon response elements; EPRE, electrophile response element; pRB, retinoblastoma protein; p53, *TRP53* gene product; and CYP, cytochrome P450.

supplied for 6 or 12 hr, followed by harvesting; controls received the vehicle *p*-dioxane alone.

RNA Extraction and Northern Blots

RNA was extracted by the acid guanidinium thiocyanate method [16]. Total RNA (10 or 20 μg) was separated in formaldehyde–agarose gels and transferred to Nytran membranes. Prehybridizations and hybridizations were carried out at 42° in a solution containing 50% deionized formamide, 6X SSC, 2.5X Denhardt's solution, 0.5% SDS, and denatured salmon sperm DNA (0.1 mg/mL). After hybridization for 16–20 hr, the filters were washed twice in 2X SSC and 0.1% SDS for 10 min at room temperature and then twice in 0.1X SSC and 0.1% SDS for 30 min at 45°. The filters were then exposed for 1–5 days, depending on signal intensity, to Kodak XAR-5 film at −70° with intensifying screens. Probes included the mouse NQO1 [17], mouse UGT1A6 [18], rat C/EBPα [19], mouse ALDH3A1 [20], rat GSTA1 3′-specific fragment [21], rat GSTP1 [22], mouse GSTA3 [23], and mouse GSTM1 [24] cDNAs; details of these clones are available in the references cited. A 1.8-kb fragment of pC/EBPα was removed with an *Eco* RI/*Hind* III digestion. The GSTP1 probe was an *Eco* RI 700-bp cDNA carried in a pUC18 vector. The GSTA3 probe was a polymerase chain reaction-amplified 660-bp *Eco* RI/*Hind* III segment. The GSTM1 probe was a 350-bp Nco I fragment of pPROKμ1. The densities of the 18S and 28S rRNA bands on ethidium bromide-stained gels were used as RNA-loading controls. The blots were then semi-quantitated by scanning densitometry of each of the mRNA bands, as a function of the mRNA to [18S + 28S] rRNA ratio [25]. This hybridization analysis of the RNA samples was usually repeated at least two additional times. Statistical analysis of the data was performed by Student's two-tailed *t*-test.

RESULTS

Elevated NQO1 and UGT1A6 mRNA in Both the 14CoS/14CoS *Newborn Liver and Cell Line*

In the present study, we examined several genes in the [*Ah*] battery [reviewed in Refs. 15, 26, 27] and other closely related genes. Until the present study, it had been experimentally shown that the mouse [*Ah*] gene battery comprises at least five genes: two Phase I cytochrome P450 genes, *Cyp1a1* and *Cyp1a2*, and three Phase II genes. The three Phase II genes include: *Nqo1* [28], *Ugt1a6* [18], and *Aldh3a1* [20]. All five genes are transcriptionally up-regulated in the absence of a functional CYP1A1 enzyme, which causes an accumulation of the putative endogenous ligand [27, 29]; this up-regulation in the absence of CYP1A1 is the sole criterion for membership in the [*Ah*] gene battery [15, 26, 27]. In contrast, the three [*Ah*] Phase II genes, but not *Cyp1a1* or *Cyp1a2*, are induced by EPRE-mediated oxidative stress [8, 17, 18, 20]. In addition, all five of these genes are up-regulated by inducers, such as TCDD, that bind to and activate the AHR, following which the AHR–ARNT heterodimer binds to any of several AHREs in the 5′-flanking regulatory regions; use of TCDD shows a "clean" AHRE-mediated effect because it is virtually not metabolized, whereas use of the AHR ligand BNF can show a "mixed" AHRE- and EPRE-mediated effect because BNF is readily metabolized to electrophilic products causing oxidative stress. In this report, we show that the Phase II *Gsta1* gene is also a member of the [*Ah*] battery.

Figure 1 reveals that NQO1 mRNA levels were increased more than 15-fold in the *14CoS/14CoS* newborn liver and more than 5-fold in the *14CoS/14CoS* liver cell line compared with that in the *ch/ch* wild-type. UGT1A6 mRNA levels were elevated more than 2-fold in the *14CoS/14CoS* newborn liver and about 2-fold in the *14CoS/14CoS* liver cell line compared with that in the *ch/ch* wild-type. Consistent with previous reports [17, 18, 28], these data demonstrate that, although differences in fold induction (intact liver versus cell line) were seen, NQO1 and UGT1A6 mRNA levels were up-regulated in both *14CoS/14CoS* untreated newborn liver and the liver cell line as compared with that in *ch/ch*. The *Cyp1a1* gene appears to act as "captain" of the [*Ah*] battery [15, 26, 27]. Absence of a functional CYP1A1 enzyme leads to up-regulation of all members of the [*Ah*] battery. Figure 1 shows that NQO1 mRNA levels were elevated more than 100-fold in the *c37* mutant line as compared with that in the Hepa-1 wild-type *wt* line. UGT1A6 mRNA levels were elevated more than 2-fold in the *c37* mutant line compared with that in the *wt* line. These data confirm that there exists "cross-talk" between absence of the CYP1A1 enzyme and enhancement of both the NQO1 and UGT1A6 mRNA levels, which qualifies the *Nqo1* and *Ugt1a6* genes as members of the [*Ah*] battery [15, 26, 27].

A possible role of C/EBPα in causing the hypoglycemic death of the *14CoS/14CoS* homozygote has been suggested [30]. More recently, *Cebpa*(−/−) knockout mice were shown to exhibit delayed expression of two gluconeogenic enzymes in the neonatal period [31]. Moreover, *gadd45*, a gene known to be up-regulated in the untreated *14CoS/14CoS* homozygote [6], was recently shown to be controlled by *C/EBPα* [32]. For these reasons, we included C/EBPα mRNA measurements in Fig. 1; of interest, no differences between *14CoS/14CoS* and *ch/ch* were found in either newborn or the hepatocyte lines.

Elevated ALDH3A1 mRNA in the 14CoS/14CoS *Cell Line but Not in Newborn Liver*

In studies comparing untreated *c37* with Hepa-1 *wt* cultures, the *Aldh3a1* gene has been shown to also be a member of the [*Ah*] battery [20]. Figure 2 illustrates that increases in ALDH3A1 mRNA levels occurred in the untreated *14CoS/14CoS* but not the untreated *ch/ch* cell line, and that there was more than a 10-fold induction in the *14CoS/14CoS* line following TCDD treatment. However, we did not detect ALDH3A1 mRNA in the liver of

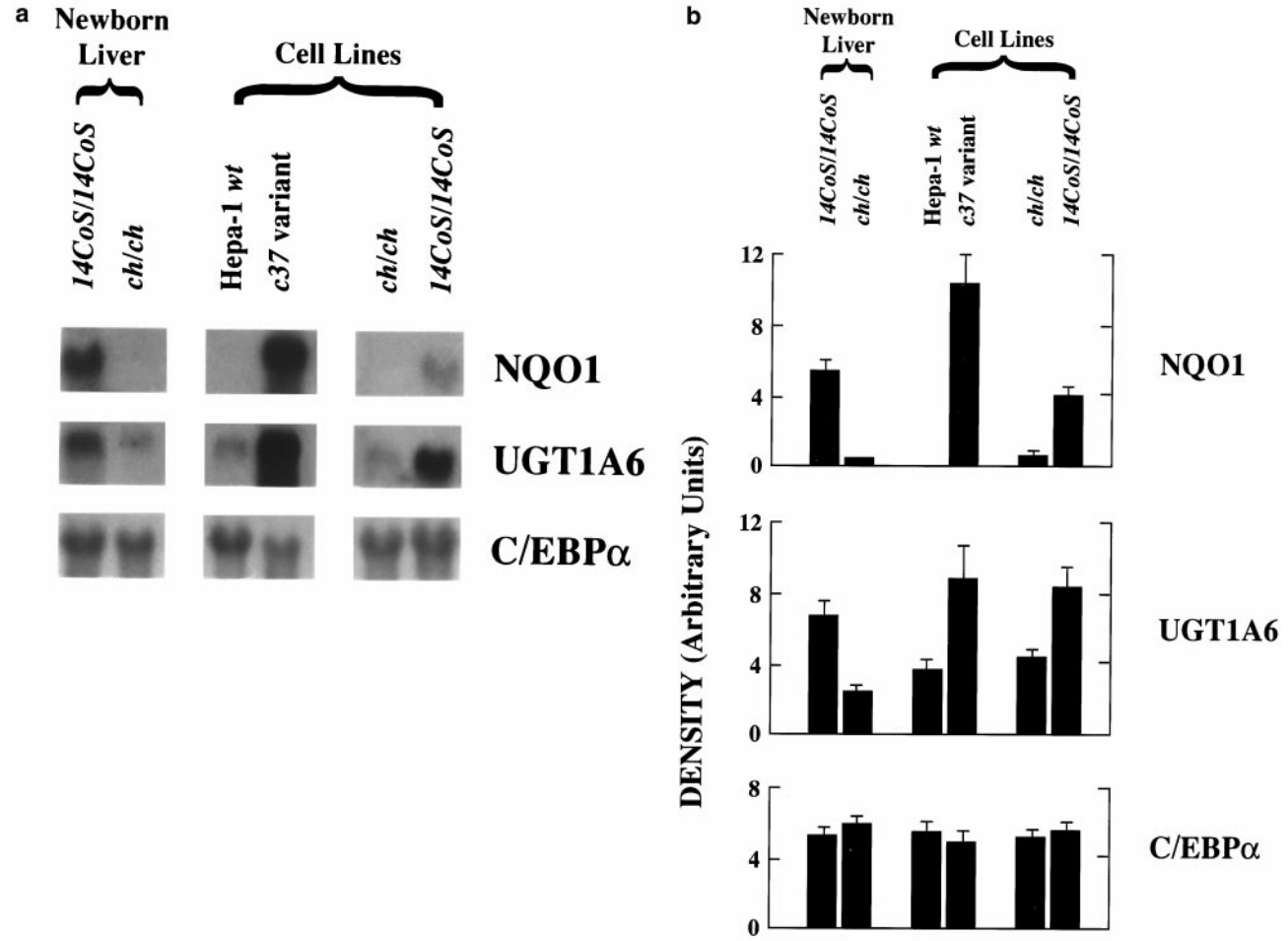

FIG. 1. (A) Northern blot of total RNA from *14CoS/14CoS* and *ch/ch* newborn liver and SV40-transformed hepatocyte cell cultures probed with NQO1, UGT1A6, and C/EBPα cDNA. Also included are total RNA samples from Hepa-1 *wt* and the CYP1A1 metabolism-deficient *c37* mutant cell line. (B) Histogram of experimental data in (A). The arbitrary units represent the semi-quantitative densitometry of the three different mRNA bands as a function of the mRNA to [18S + 28S] rRNA ratio. Bars and brackets denote means ± SD (N = 3 experiments).

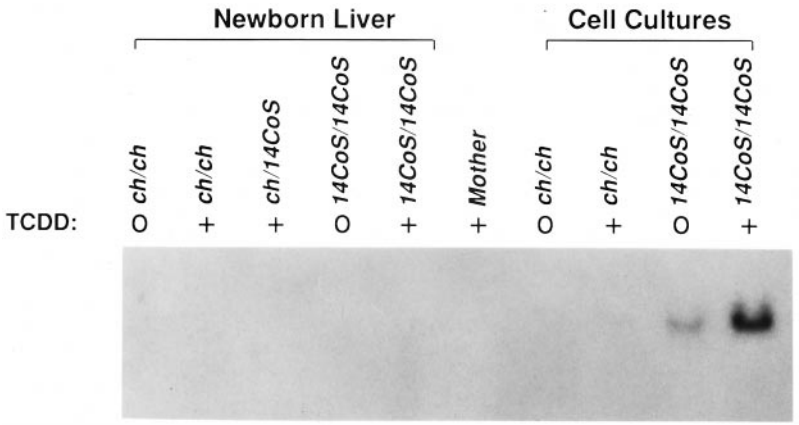

FIG. 2. Northern blot of total RNA from *ch/ch*, *ch/14CoS*, and *14CoS/14CoS* newborn liver and from the *ch/ch* and *14CoS/14CoS* liver cell lines probed with ALDH3A1 cDNA. TCDD treatment (+) for 12 hr is described in the Materials and Methods section; controls (o) received the vehicle alone. Following a 5-day exposure of the filter to the autoradiographic film (not shown), a small amount of ALDH3A1 mRNA was detectable in the TCDD-treated *ch/ch* hepatocyte line. Following even 20-day exposures of the filter to the autoradiographic film, however, no ALDH3A1 mRNA was found in liver of the control or TCDD-treated intact mouse.

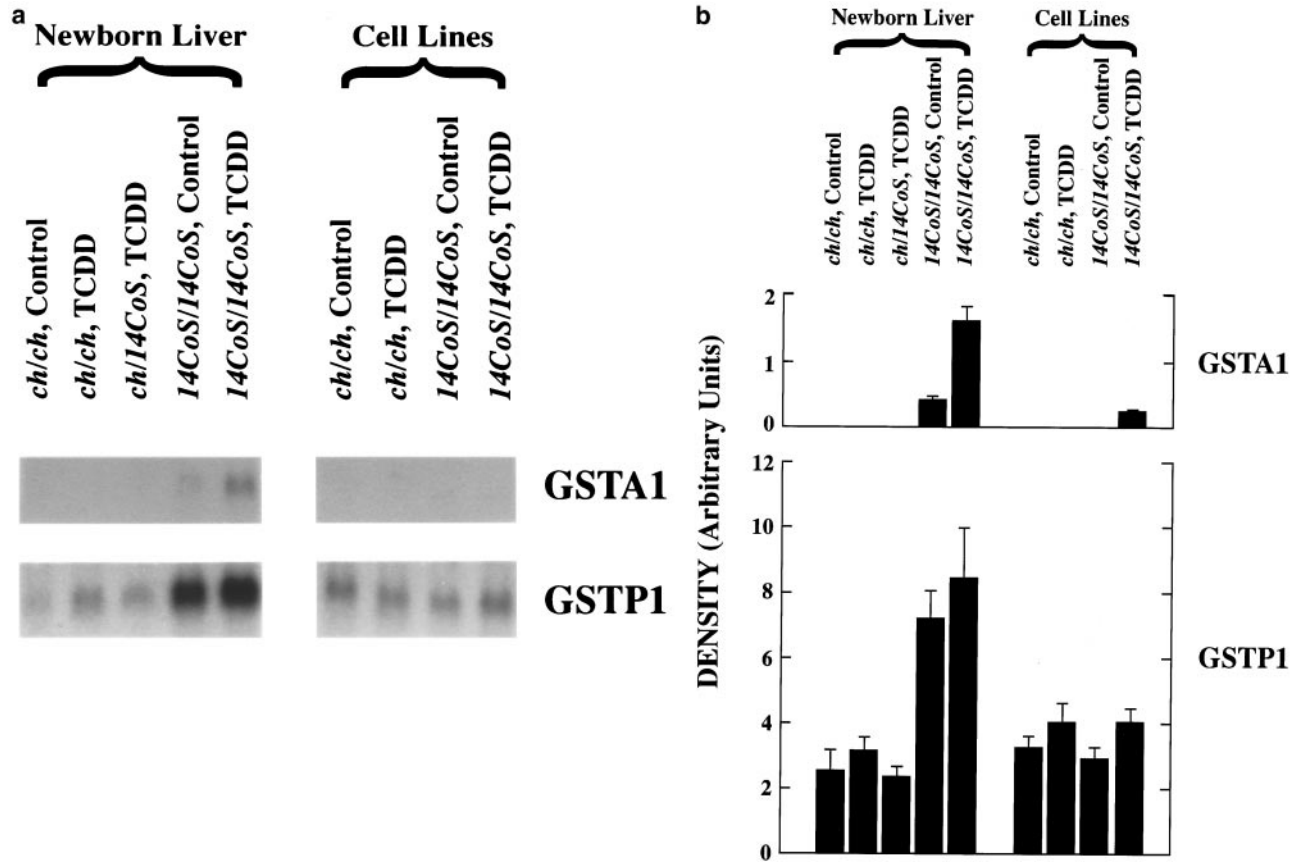

FIG. 3. (A) Northern blot of total RNA from *ch/ch*, *ch/14CoS*, and *14CoS/14CoS* newborn liver and from the *ch/ch* and *14CoS/14CoS* hepatocyte cell lines probed with GSTA1 and GSTP1 cDNA. Untreated samples (controls) are compared with TCDD-treated samples. Following a 5-day exposure of the filter to the autoradiographic film (not shown), a small amount of GSTA1 mRNA was detectable in the TCDD-treated *ch/ch* hepatocyte line. (B) Histogram of semi-quantitative densitometry data shown in (A). Bars and brackets denote means ± SD. (N = 3 experiments). Note different values on the ordinates.

the intact newborn or adult mouse. These findings suggest that, in established mouse liver cell lines, either an inhibitor of EPRE- and AHRE-mediated *Aldh3a1* gene expression is extinguished or an activator of *Aldh3a1* gene expression is turned on; alternatively, in the intact mouse, there exists either an inhibitor (e.g. methylation, silencing) of both EPRE- and AHRE-mediated *Aldh3a1* gene expression, or an activator of *Aldh3a1* gene expression is absent.

Elevated GSTA1 and GSTP1 mRNA in 14CoS/14CoS *Newborn Liver but Negligible in the Cell Line*

Figure 3 shows that GSTA1 mRNA was increased in the untreated *14CoS/14CoS* newborn liver compared with that in *ch/ch*, and that GSTA1 mRNA was further induced in *14CoS/14CoS* newborn liver about 4-fold by dioxin. GSTA1 mRNA concentrations were negligible in the hepatocyte cell lines. In contrast, GSTP1 basal mRNA levels were easily detectable in all newborn liver and cell line samples. Interestingly, GSTP1 mRNA was more than 2-fold greater in *14CoS/14CoS* than in *ch/ch* newborn liver, but this oxidative stress response was not seen in the hepatocyte cell lines. Moreover, TCDD did not induce GSTP1 mRNA in either the *ch/ch* or *14CoS/14CoS* newborn liver or the cell lines.

Elevated GSTA1, and not GSTP1, mRNA in the c37 *but Not the* c4 *or* c2 *Mutant Lines*

In the absence of CYP1A1 metabolism in the untreated *c37* mutant line, GSTA1 mRNA was present (although at low levels) (Fig. 4) compared with undetectable levels in the untreated wild-type *c4* or *c2* lines. These findings demonstrate that the *Gsta1* gene is a member of the [*Ah*] battery, although—for reasons not clear—*Gsta1* gene expression in established cell lines is weak. On the other hand, constitutive GSTP1 mRNA concentrations were very high and not significantly different in the nine lanes of Fig. 4, indicating that the *Gstp1* gene is not a member of the [*Ah*] battery. Moreover, GSTP1 mRNA was not induced by dioxin, while GSTA1 mRNA was induced more than 10-fold (Fig. 4). GSTA1 mRNA was not detectable in the TCDD-treated *c4* mutant line (ARNT-deficient) or *c2* mutant line (AHR receptorless).

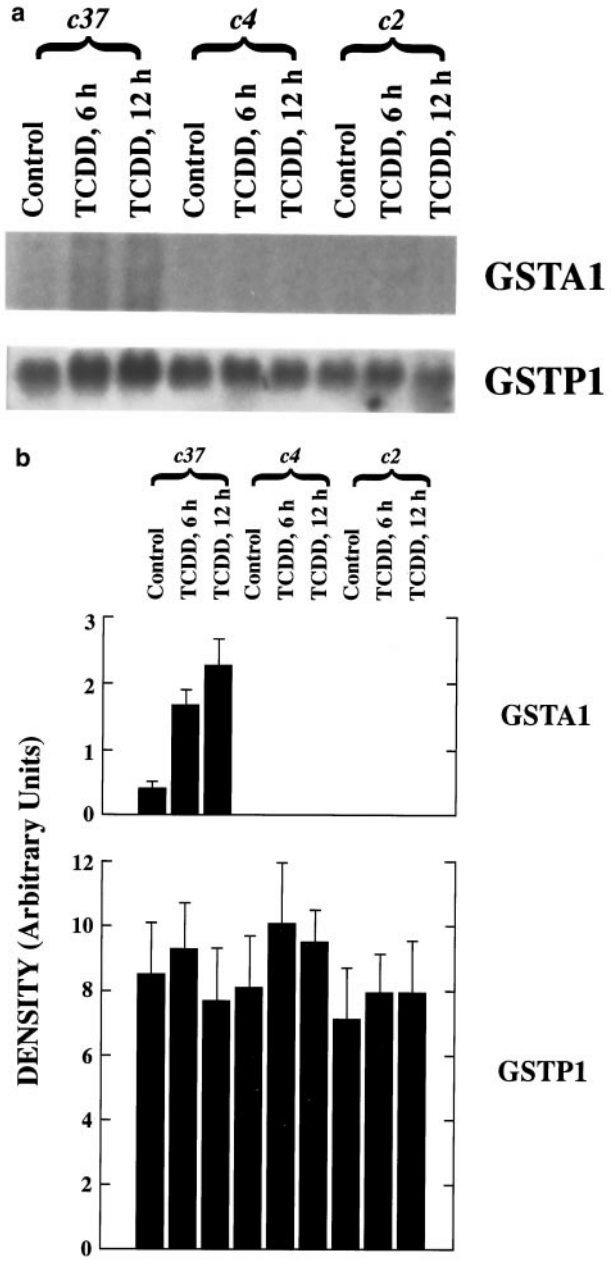

FIG. 4. (A) Northern blot of total RNA from the *c37*, *c4*, and *c2* variant lines probed with GSTA1 and GSTP1 cDNA. Controls (untreated) are compared with TCDD-treated samples. Hepa-1 wild-type (*wt*) data were similar to those of *c2*; following a 5-day exposure of the filter to the autoradiographic film, no basal *wt* or *c2* GSTA1 mRNA was found, but trace amounts of GSTA1 mRNA in TCDD-treated *wt* cells, but not *c2* cells, were detectable (not illustrated). (B) Histogram of semi-quantitative densitometry data shown in (A). Bars and brackets denote means ± SD (N = 3 experiments). Note different values on the ordinates.

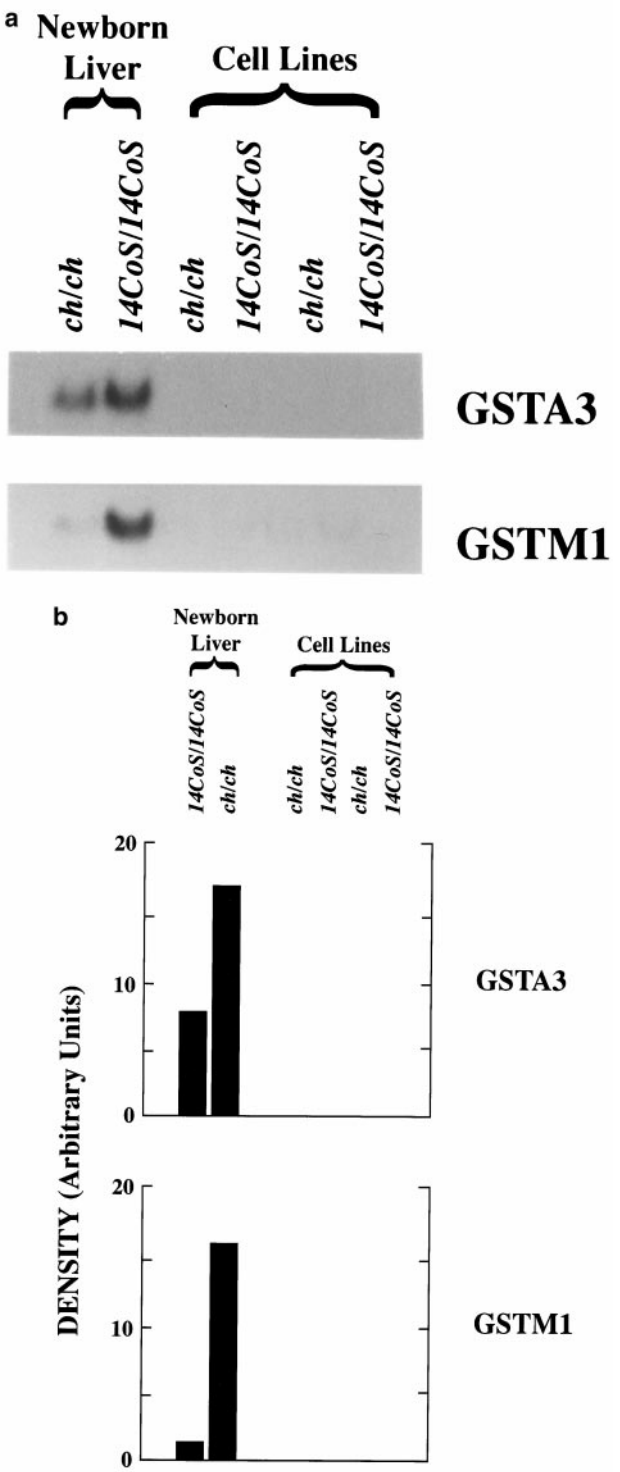

FIG. 5. (A) Northern blot of total RNA from *ch/ch* and *14CoS/14CoS* newborn liver and from the *ch/ch* and *14CoS/14CoS* liver cell lines (two independent experiments) probed with GSTA3 and GSTM1 cDNA. (B) Histogram of semi-quantitative densitometry data shown in (A).

Elevated GSTA3 and GSTM1 mRNA in 14CoS/14CoS *Newborn Liver but Negligible in the Cell Line*

The *Gsta3* gene [23] is chromosomally linked and in the same subfamily as the *Gsta1* gene, whereas the *Gstm1* gene [24] is in a different subfamily [33]. Figure 5 illustrates that GSTA3 and GSTM1 mRNA levels were increased more than 2-fold and greater than 15-fold, respectively, in the untreated *14CoS/14CoS* newborn liver compared with that in *ch/ch*. In the SV40-transformed cell lines, however, GSTA3 and GSTM1 mRNA levels were undetectable.

Thus, the same basic pattern was seen with all four glutathione *S*-transferase genes (*Gsta1*, *Gstp1*, *Gstm3*, and Gstm 1): a putative EPRE-mediated response to oxidative stress in *14CoS/14CoS* newborn liver, but negligible gene expression (except for *Gstp1*) in the immortalized cell culture lines.

Hepatic GSTA1 but Not GSTP1 mRNA Induction by TCDD in 4-week-old Mice

The negligible amounts of GSTA1 mRNA, not only in cell cultures (Figs. 3 and 4) but also newborn liver (Fig. 3), might be the result of a) special characteristics of established cell lines, b) developmental differences in *Gsta1* gene expression, and/or c) lack of specificity of the cDNA probe. We therefore examined 4-week-old sexually immature mice (Fig. 6). GSTA1 mRNA concentrations were increased almost 2-fold in the BNF-treated B6 inbred line and the D2.B6-*Ahr*^{b1} congenic line (both having the high-affinity AHR), but not in the BNF-treated D2 inbred line or the B6.D2-*Ahr*^{d} congenic line (both having the poor-affinity AHR). The lack of a more striking fold induction most likely reflects the relatively high basal GSTA1. In contrast, GSTP1 mRNA was constitutively expressed at very high levels and was not significantly induced by BNF. As mentioned above, use of BNF, because of its being metabolized to electrophilic products, gives a "mixed" response of both AHRE- and EPRE-mediated induction of GSTA1.

DISCUSSION

In the present study, we have examined several genes in the [*Ah*] battery and other closely related genes. Table 1 summarizes the findings of this report, as well as other *14CoS/14CoS* studies during the past decade. Some parameters are similar in both the newborn liver and cell lines, whereas others are seen in one but not the other model system. The *Cyp1a1* gene is known not to be up-regulated in the untreated *14CoS/14CoS* newborn or cell line [5].

NQO1 and UGT1A6 mRNAs were up-regulated in both *14CoS/14CoS* newborn liver and the established cell line, whereas C/EBPα mRNA was not different (Fig. 1). ALDH3A1 mRNA was up-regulated in the liver cell line and was dioxin-inducible, but *Aldh3a1* gene expression was not seen in liver of the intact mouse (Fig. 2). ALDH3A1 mRNA and activity is found, however, in the cornea, stomach, skin, and urinary bladder [35]. GSTA1 mRNA was not detected in the untreated *14CoS/14CoS* cell line, yet there was detectable up-regulation in the absence of CYP1A1 metabolism in untreated *c37* cells, as well as dioxin inducibility (Fig. 3). In newborn liver, GSTA1 mRNA was detectable in *14CoS/14CoS* and induced further by TCDD (Fig. 3).

GSTP1 mRNA was constitutively expressed at easily detectable levels in both newborn liver and the cell lines (Fig. 3). Interestingly, there was a large EPRE-mediated increase in GSTP1 mRNA in *14CoS/14CoS* newborn liver,

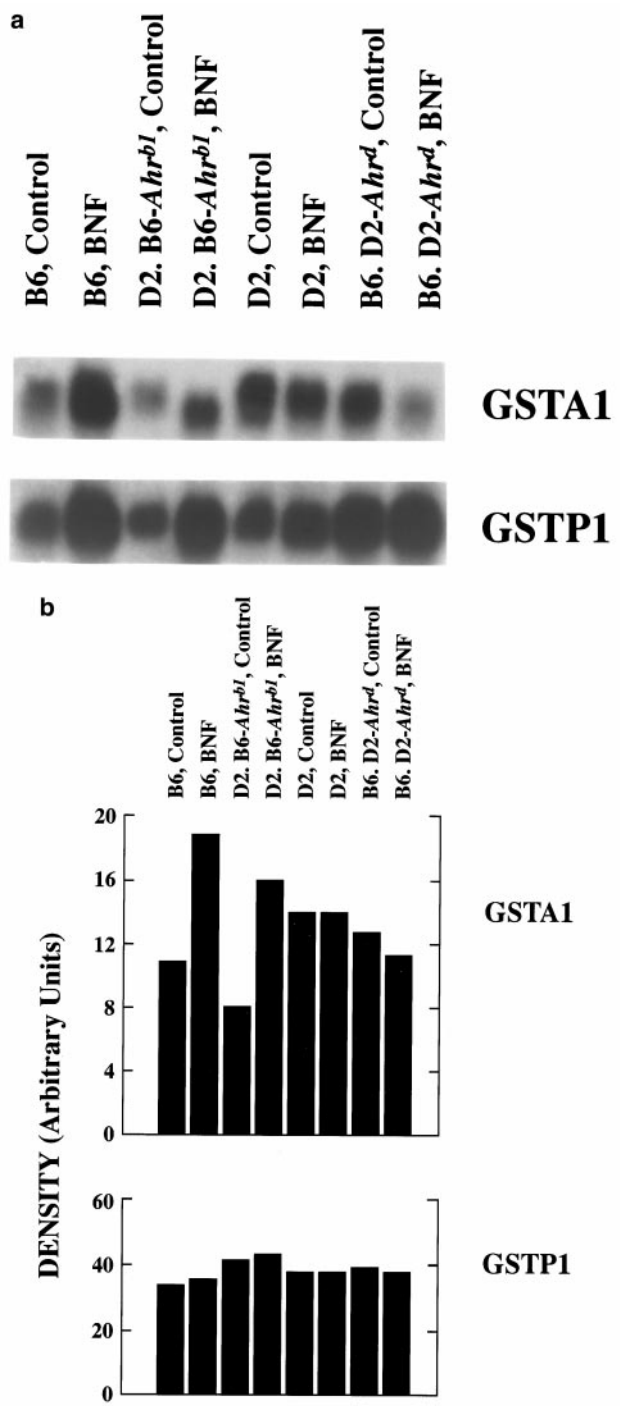

FIG. 6. (A) Northern blot of control and BNF-treated B6 and D2 inbred mouse lines and the D2.B6-*Ahr*^{b1} and B6.D2-*Ahr*^{d} congenic lines probed with GSTA1 and GSTP1 cDNA. Total RNA from livers of three 4-week-old female mice were combined for each of the eight samples. (B) Histogram of semiquantitative densitometry data shown in (A). Note different values on the ordinates.

and there was no further induction by TCDD; this was not observed in the *14CoS/14CoS* cell line. Similarly, GSTA3 and GSTM1 mRNA levels were up-regulated in the untreated *14CoS/14CoS* newborn liver but undetectable in the cell lines (Fig. 5). These data suggest that there are

TABLE 1. Parameters in untreated *14CoS/14CoS*, as compared with untreated *ch/14CoS* and *ch/ch*

Parameter [Refs.]*	Newborn liver	Hepatocyte cell line	Proven member of [*Ah*] battery	Dioxin-inducible
NQO1 mRNA	Elevated	Elevated	Yes	Yes
UGT1A6 mRNA	Elevated	Elevated	Yes	Yes
C/EBPα mRNA	No difference†	No difference	No	No
ALDH3A1 mRNA	Undetectable	Elevated	Yes	Yes
ALDH3A2 mRNA [20]	No difference	No difference	No	Yes
GSTA1 mRNA	Elevated	Undetectable‡	Yes	Yes
GSTP1 mRNA	Elevated	No difference	No	No
GSTA3 mRNA	Elevated	Undetectable		
GSTM1 mRNA	Elevated	Undetectable		
GSH, GSSG levels [10]	Elevated	Elevated		
GSSG/GSH ratio [10]	No difference	No difference		
HNF-1α mRNA [34]	Decreased	Undetectable	No	No
GADD153 mRNA§	Elevated	Undetectable	No	No

*For parameters not measured in the present study, a reference is cited. ALDH3A2 is the microsomal aldehyde dehydrogenase-3 mRNA (formerly AHD3). HNF-1α, hepatocyte nuclear factor 1α.

†"No difference" means mRNA is easily detectable, but *14CoS/14CoS* levels are the same as *ch/14CoS* and *ch/ch* levels.

‡However, constitutive GSTA1 mRNA was elevated, and dioxin caused further induction in the CYP1A1 metabolism-deficient *c37* variant cell line.

§Same results with GADD54 and GADD45 mRNA [6].

EPRE motifs in the regulatory regions of the mouse *Gsta1*, *Gstp1*, *Gsta3*, and *Gstm1* genes which are capable of functioning in the intact animal but not in the immortalized cell lines.

Elevated GSTA1 mRNA in the *c37* cell line and its dioxin inducibility (Fig. 4)—similar to that seen for NQO1 [28], UGT1A6 [18] and ALDH3A1 [20]—confirms that expression of the *Gsta1* gene is up-regulated in the absence of CYP1A1 metabolism and that the gene is thus a member of the [*Ah*] battery [15, 26, 27]. In contrast, GSTP1 mRNA was expressed constitutively at high levels, was independent of CYP1A1 metabolism, and was not significantly induced by TCDD (Fig. 4).

Lastly, hepatic GSTP1 and GSTA1 mRNA levels were much higher in 4-week-old mice (Fig. 6) than in the newborn. Low GSTA1 but high GSTA3 mRNA levels have been reported in the adult mouse [23]. A careful developmental study of mouse liver *Gst* gene expression as a function of age from the neonate to the adult would be helpful.

mRNA Levels Not Necessarily Representative of Protein Levels

Increases in mRNA concentrations can be caused by both enhanced rates of transcription and mRNA stabilization. Although elevated mRNA levels usually suggest transcriptional up-regulation of the gene, this has not been demonstrated in the present study. Also, it should be emphasized that mRNA levels do not always reflect the level of a functional protein. Nevertheless, striking differences in basal mRNA levels, in the response to oxidative stress and in the induction by dioxin, were seen in our study. It should also be noted that Northern hybridization analysis is not as sensitive, or quantitative, as other techniques. "Negligible," or barely detectable, mRNA levels after a 5-day exposure of the membrane to an autoradiographic film would more easily be detected by a sensitive method such as the oligo–PCR assay [36]. Actual quantitation of mRNA levels would require the much more tedious procedure of solution hybridization [37].

Speculation on Differences between Liver in the Intact Animal and Established Cell Lines

If parameters of the oxidative stress response are detectable in *14CoS/14CoS* newborn liver but not seen in cell cultures, one possible explanation would be the phenomenon of "gene loss" or "extinction," when cells are placed in culture or cell lines are established [38–41]. For example, numerous drug-metabolizing enzyme activities are lost within hours of placing cells in culture. In fibroblast × hepatoma somatic cell hybrids, the tissue-specific extinguisher-1 locus (*Tse1* on mouse chromosome 11) can repress expression of several liver genes in *trans* [42]. The *Tse1* gene has been found to encode a regulatory subunit of cAMP-dependent protein kinase [43, 44]. The underlying mechanisms of extinction are thought to mirror, at least in part, the repertoire of regulatory mechanisms controlling mammalian cell differentiation [45]. We suspect that the major reason for differences between the *14CoS/14CoS* newborn liver and established hepatocyte cell line, however, reflects the fact that the established cell line has been transformed with an SV40 temperature-sensitive A255 mutant virus [4], which means that large T antigen is expressed at the permissive temperature of 34°. Large T antigen is known to interact with the growth suppressor proteins pRB and p53 and bind to the pRB–E2F complex at the permissive temperature of 34°, but these properties are lost upon a shift from 34° to 39° [46, 47]. Thus, although SV40 transforma-

tion prevents "replicative senescence" of cells in culture, large T antigen has strong effects on proteins that participate in the cell cycle, growth arrest, and apoptosis [47]. One possible future experiment would be to look for expression of all four GST mRNAs studied herein in *14CoS/14CoS* and *ch/ch* cells at the non-permissive temperature of 39°: would we now see G*st* gene expression that was not observed at the permissive temperature?

The dioxin-inducible [*Ah*] battery represents the cross-talk of CYP1A1, the AHR, and the expression of at least six genes [15, 26, 27]. Dioxin also causes chronic total-body oxidative stress [48]. The importance of the AHR in cell cycle regulation [49–52] and apoptosis [53–55] has just begun to be appreciated. A direct interaction has been shown to occur between the AHR and pRB [56]. Oxidative stress is known to be one of the major signals leading to apoptosis [57]. Consequently, if the AHR and C*yp1a1*, or any other of the genes in the [*Ah*] battery, participate in the cell cycle, then the expression of these genes might be altered in SV40-immortalized cell lines. Moreover, if large T antigen adversely perturbs pRB-mediated cell cycle progression and/or steps that lead to oxidative stress-mediated apoptosis, this could explain why some of the "oxidative stress response" parameters studied herein are strikingly different between *14CoS/14CoS* newborn liver and the SV40-immortalized hepatocyte cell line.

In summary, mRNA levels of several genes associated with the oxidative stress response, as well as induction by TCDD, were examined. Important differences were found in some of these parameters, comparing the *14CoS/14CoS* newborn liver with the 14CoS/14CoS cell line; we believe that these differences in gene expression can likely be explained by the presence of large T antigen in the SV40-transformed hepatocyte cell line.

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