TOXICOLOGICAL SCIENCES 109(1), 41–49 (2009)
doi:10.1093/toxsci/kfp003
Advance Access publication January 23, 2009

Transgenic Expression of Aflatoxin Aldehyde Reductase (AKR7A1) Modulates Aflatoxin B_1 Metabolism but not Hepatic Carcinogenesis in the Rat

Bill D. Roebuck,*[,1] Denise N. Johnson,† Carrie Hayes Sutter,§ Patricia A. Egner,† Peter F. Scholl,†[,‡] Marlin D. Friesen,† Karen J. Baumgartner,* Nicholas M. Ware,* Sridevi Bodreddigari,§ John D. Groopman,† Thomas W. Kensler,† and Thomas R. Sutter§[,1]

*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755; †Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205; ‡Center for Food Safety and Nutrition, United States Food and Drug Administration, College Park, Maryland 20740; and §W. Harry Feinstone Center for Genomic Research, University of Memphis, Memphis, Tennessee 38152

Received August 29, 2008; accepted January 5, 2009

In both experimental animals and humans, aflatoxin B_1 (AFB_1) is a potent hepatic toxin and carcinogen against which a variety of antioxidants and experimental or therapeutic drugs (e.g., oltipraz, related dithiolethiones, and various triterpenoids) protect from both acute toxicity and carcinogenesis. These agents induce several hepatic glutathione *S*-transferases (GST) as well as aldo-keto reductases (AKR) which are thought to contribute to protection. Studies were undertaken in transgenic rats to examine the role of one inducible enzyme, AKR7A1, for protection against acute and chronic actions of AFB_1 by enhancing detoxication of a reactive metabolite, AFB_1 dialdehyde, by reduction to alcohols. The AFB_1 dialdehyde forms adducts with protein amino groups by a Schiff base mechanism and these adducts have been theorized to be at least one cause of the acute toxicity of AFB_1 and to enhance carcinogenesis. A liver-specific AKR7A1 transgenic rat was constructed in the Sprague-Dawley strain and two lines, $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$, were found to overexpress AKR7A1 by 18- and 8-fold, respectively. Rates of formation of AFB_1 alcohols, both in hepatic cytosols and as urinary excretion products, increased in the transgenic lines with $AKR7A1^{Tg2}$ being the highest. Neither line offered protection against acute AFB_1-induced bile duct proliferation, a functional assessment of acute hepatotoxicity by AFB_1, nor did they protect against the formation of GST-P positive putative preneoplastic foci as a result of chronic exposure to AFB_1. These results imply that the prevention of protein adducts mediated by AKR are not critical to protection against AFB_1 tumorigenicity.

Key Words: **aflatoxin; aldo-keto reductase; chemoprevention, hepatic carcinogenesis; hepatotoxicity; transgenic rat.**

Aflatoxin B_1 (AFB_1), a secondary metabolic product of the mold *Aspergillus flavus*, is a potent hepatotoxin and carcinogen in both experimental animals and humans (Kensler *et al*., 1999). In the liver, the metabolic activation of AFB_1 is mediated by microsomal cytochromes P450, CYP1A2, and CYP3A4, to form the AFB_1-8,9-*exo* and -8,9-*endo* epoxides (see Fig. 1) (Gallagher *et al*., 1996; Johnson and Guengerich, 1997). Only the AFB_1-8,9-*exo* isomer reacts with DNA, forming the N^7-guanine adduct and the derivative AFB_1 formamidopyrimidine adducts (Johnson and Guengerich, 1997). If not repaired, these DNA adducts or the abasic sites that remain from the spontaneous depurination of the N^7-guanine adduct can lead to DNA mutations. Both of the AFB_1 epoxides are substrates for glutathione *S*-transferases (GSTs), which catalyze the conjugation of the epoxide with reduced glutathione, thus mitigating the formation of DNA adducts (Eaton and Bammler, 1999). In addition to GSTs, other detoxication pathways may contribute to resistance to this mycotoxin. Both AFB_1 epoxides undergo rapid hydrolysis in water forming AFB_1 dihydrodiol. The AFB_1 dihydrodiol undergoes a base-catalyzed rearrangement to, and is in equilibrium with, AFB_1 dialdehyde (Johnson *et al*., 1996). AFB_1 dialdehyde can form Schiff base derived adducts with protein amino groups, particularly lysine (Groopman *et al*., 1980; Guengerich *et al*., 2002; Sabbioni *et al*., 1987) and these protein adducts have been implicated in the acute toxicity of AFB_1 (Ellis *et al*., 1993; Guengerich *et al*., 2002).

Aflatoxin aldehyde reductase was first identified as an ethoxyquin-inducible member of the nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)–dependent aldo-keto reductase (AKR) superfamily. This enzyme, now designated AKR7A1, was purified from rat liver and shown to catalyze the reduction of the protein reactive AFB_1 dialdehyde to the dialcohol product (Ellis *et al*., 1993; Hayes *et al*., 1993). Large increases in the levels of AKR7A1 protein have been observed in livers of rats following administration of ethoxyquin, dithiolethiones and other classes of inducers (Guengerich *et al*., 2001; Ireland *et al*., 1998; Knight *et al*., 1999). Aflatoxin aldehyde reductases, specifically rat AKR7A1

[1] Correspondence to either author: BDR, Tel: (603) 650-1676. Fax: (603) 650-1129. E-mail: Bill.D.Roebuck@dartmouth.edu; TRS, Tel: (901) 678-8391. Fax: (901) 678-2458. E-mail: tsutter@memphis.edu.

© The Author 2009. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oxfordjournals.org

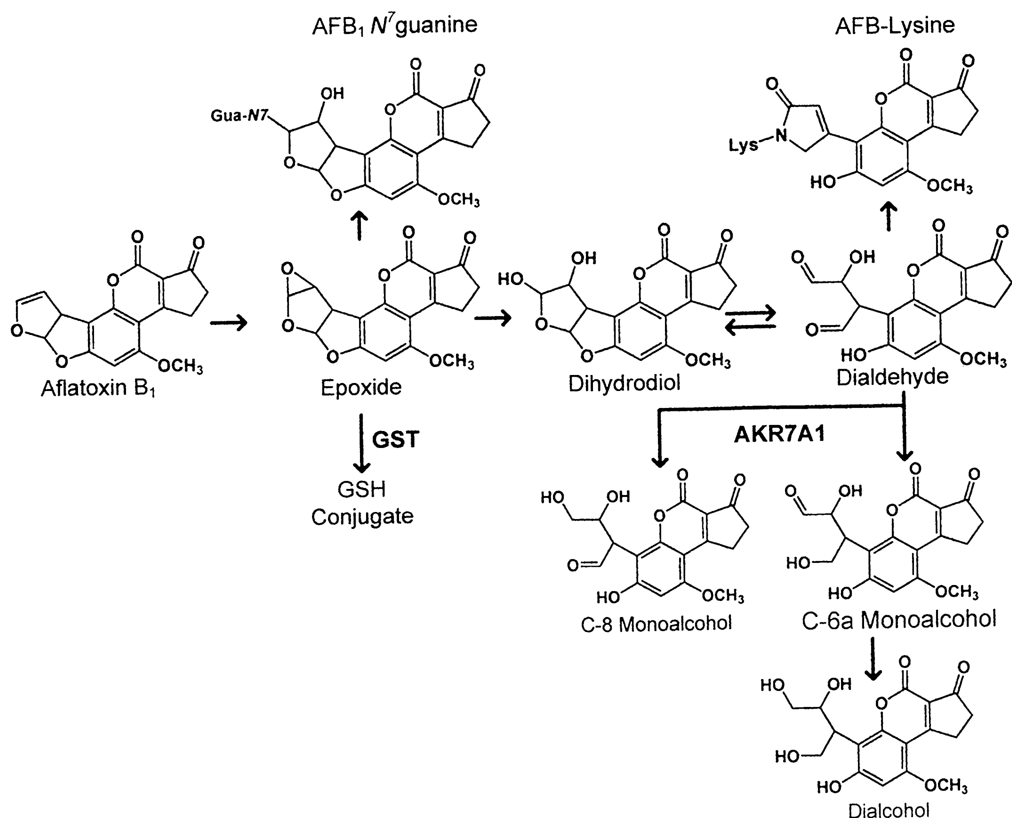

FIG. 1. Schematic of enzymatic and chemical conversion of AFB_1 to the reactive dialdehyde and its reduction by AKR7A1 to three possible aflatoxin alcohols.

(Ellis *et al.*, 1993; Guengerich *et al.*, 2001; Knight *et al.*, 1999), human AKR7A3 (Guengerich *et al.*, 2001; Ireland *et al.*, 1998; Knight *et al.*, 1999), and mouse AKR7A5 (Hinshelwood *et al.*, 2003), are known to catalyze the reduction of the reactive AFB_1-derived dialdehyde at similarly high rates of activity. In a study of rat AKR7A1 and human AKR7A3 enzyme kinetics, it was shown that the AFB_1 dialdehyde was preferentially reduced to a C-8 monoalcohol, corresponding to reduction of the C-8 carbon. Production of a C-6a monoalcohol occurred at a lower rate, and the formation of the dialcohol was not rapid in these reactions (Guengerich *et al.*, 2001).

Recently, we reported on the protection against the formation of AFB_1 dialdehyde protein adducts and cytotoxicity in cell culture by recombinant expression of cloned rat AKR7A1 and human AKR7A3 (Bodreddigari *et al.*, 2008). Because these results indicated a role of AKR in the protection against AFB_1-induced cytotoxicity *in vitro* and because rat AKR7A1 is highly induced by compounds such as oltipraz that are known protectors against the toxicity and carcinogenicity of AFB_1 in the rat (Liu *et al.*, 1988), a better understanding of this pathway *in vivo* is warranted. Induction of AKR7A1 may limit AFB_1-induced cytotoxicity *in vivo* by inhibiting the compensatory hyperplasia that occurs following cellular necrosis, thereby, attenuating several steps in AFB_1 carcinogenesis including events involved in initiation, promotion and progression (Roebuck, 2004). Based on these observations, we report here the development of a liver-specific AKR7A1 transgenic rat and studies to reveal the impact of AKR7A1 *in vivo* in hepatic AFB_1 metabolism, toxicity, and carcinogenicity.

MATERIALS AND METHODS

AKR7A1 transgenic rat. The DNA construct for expression of the rat AKR7A1 gene was produced using the cDNA clone of rat AKR7A1 (Knight *et al.*, 1999) and the liver-specific expression vector pLiv-7, kindly provided by Dr John Taylor, UCSF (Fan *et al.*, 1994; Yamanaka *et al.*, 1995). This vector was designed for liver-specific expression and contains sequences from the human *APOE* gene (Fan *et al.*, 1994). A schematic of the construct is shown in Figure 2A. The *Spe*I site in the AKR7A1 polylinker was opened by restriction endonuclease digestion. The linearized plasmid was filled in with T4 DNA polymerase and the plasmid was closed by blunt-end ligation. The AKR7A1 *Kpn*I–*Xho*I containing insert, 5′ AUG sequence towards the *Kpn*I site, was then purified from the resulting plasmid and ligated into the pLiv-7 vector that had been digested with *Kpn*I and *Xho*I. The resulting plasmid was purified and the orientation of the AKR7A1 insert verified by DNA sequence analysis using rat AKR7A1 specific primers, which were used to sequence into the pLiv-7 vector. The primer, 5′-ACGCGGAGCTGGAGGTCA-3′, annealed to the 5′ end of AKR7A1 and was used to sequence into pLiv-7. To confirm the orientation, a second primer, 5′-GTGGATGCCTTTGACCAAG-3′, which anneals to the 3′ end of AKR7A1, was used to sequence into pLiv-7. The *Sal*I–*Spe*I DNA fragment (pLivAKR7A1 transgene construct) was isolated by gel

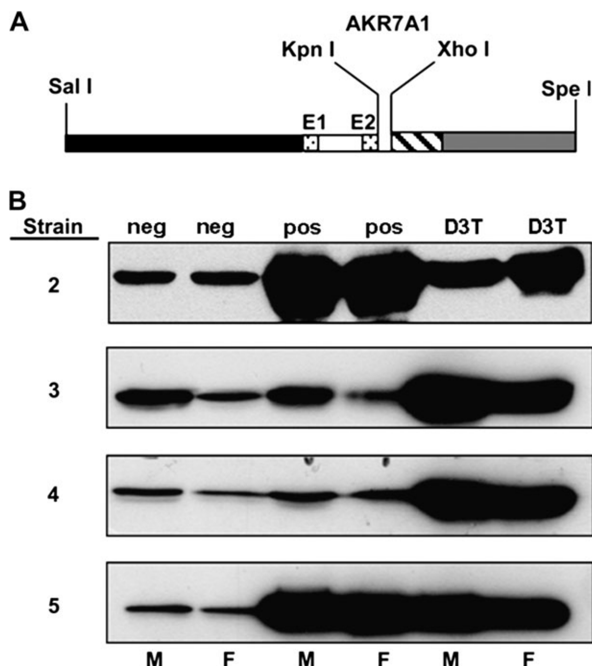

FIG. 2. Transgene expression of AKR7A1. (A) The DNA construct was designed for liver-specific expression using the pLiv-7 vector containing sequences from the human *APOE* gene: 3 kb of 5′-flanking region (black); the first exon (E1, dotted), first intron (open), and first six nucleotides of the second exon (E2, dotted); 0.25 kb of 3′-flanking region including the polyadenylation signal (dashed) and a 1.7-kb hepatic control region of the *APOE*/C-I gene locus (gray). The AKR7A1 cDNA was inserted into the polylinker region using the *Kpn*I and *Xho*I restriction sites. (B) Transgene expression of AKR7A1. Protein levels of AKR7A1 in samples of liver cytosol (30 μg protein/lane) from one male (M) and one female (F) of four separate AKR7A1 transgenic rat lines were analyzed by immunoblot. The samples, identified at the top, are nontransgenic genetic control animals (neg), transgenic animals (pos), and nontransgenic rats pretreated with 3*H*-1,2-dithiole-3-thione (D3T) a known inducer of AKR7A1.

electrophoresis and further purified using a Qiaquick column (Qiagen Inc., Valencia, CA). Transgene specific PCR primers were synthesized: one primer to the AKR7A1 cDNA sequence, 5′-AATTTGAACCCCGGGAGAGGAAGA-3′, the other corresponding to the 5′ *APOE* sequence, 5′-ACGCGGAGCTGGAG-GTCACATC-3′. These primers produced a single PCR product corresponding to the transgene when the transgene was spiked at varying concentration into rat genomic DNA at ratios ranging from 1 to 10 gene copies per cell equivalent of DNA. The concentration of the pLivAKR7A1 DNA fragment was confirmed by electrophoresis versus plasmid DNA of a standard concentration and sent frozen to Xenogen Biosciences (Cranbury, NJ).

Xenogen Biosciences produced four founder rats containing the pLivAKR7A1 transgene. Briefly, zygotes from superovulated Sprague-Dawley rats were flushed from the oviduct and the transgene delivered by pronuclear microinjection. The injected zygotes were implanted into pseudopregnant foster mothers and the resulting founder pups were genotyped by PCR using the primers described above. Four transgenic rats (two males and two females) were produced and shipped to Dartmouth Medical School. These rats were bred to provide both transgenic positive and negative (genetic control) rats for subsequent experiments. Breeders were fed a commercial, natural ingredient diet. All rats used in experimental studies were fed the AIN-76A purified diet without the antioxidant ethoxyquin which is usually added to this diet. The latter diet was fed to maintain the hepatic drug metabolizing enzymes at relatively stable levels since the aflatoxins are activated and detoxified by a series of these enzymes. In all experiments, transgenic rats were matched with a genetic control, litter mate of the same sex. All experiments were approved by the Animal Care and Use Committee of Dartmouth College.

Estimation of integrated gene copy number and genotyping. Genomic DNA was isolated from rat livers using DNeasy tissue kit (Qiagen, Inc., Valencia, CA). PCR was performed using the Bio-Rad iCycler (Hercules, CA) and a forward primer to the AKR7A1 (FP)-5′-AATTTGAACCCCGGGA-GAGGAAGA-3′ sequence and a reverse primer (RP)-5′-ACGCGGAGCTG-GAGGTCACATC-3′ to the *APOE* sequence (IDT, Coralville, IA). The PCR mix consisted of 2× iQ SYBR Green supermix (2× reaction buffer, iTaq DNA polymerase, dNTPs, 6mM $MgCl_2$, SYBR Green I, and stabilizers) (Bio-Rad, Hercules, CA), 0.2μM FP, 0.2μM RP, and 300 ng of genomic DNA in a final volume of 50 μl. Amplification was carried out as follows: denaturation for 3 min at 95°C, 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. The gene copy number was calculated using 300 ng of genomic DNA and a standard curve containing 1, 3, 10, and 30 copies of cDNA (AKR7A1) and 300 ng of genomic DNA from a nontransgenic rat. The genotype for transgenic positive and negative rats was determined using the primers listed above.

Preparation of hepatic cytosol, immunoblot, and enzyme assay. Liver cytosolic proteins were prepared from untreated, D3T-treated (an oral gavage dose of 0.3 mmol/kg body weight on 5, 3, and 1 day prior to autopsy), or transgenic animals and analyzed by immunoblot as described previously (Egner *et al*., 2006; Knight *et al*., 1999). 9,10-Phenanthrene quinone reductase activity was determined by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6270$/M/cm), as described previously (Knight *et al*., 1999) in a 1 ml of reaction containing 25 μg of cytosolic protein and 30μM 9,10-phenanthrene quinone. When using AFB_1 dialdehyde as substrate, incubation mixtures consisted of 15 μl of hepatic cytosol, 50mM potassium N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonate) buffer pH 7.4, 5mM $MgCl_2$, 0.2mM NADPH, and 7μM AFB_1 dialdehyde in a total volume of 100 μl. Mixtures were incubated at 37°C, initiated by the addition of cytosol, and terminated after 3 min by addition of 10 μl of concentrated acetic acid. AFB_1 dialdehyde was formed by the dilution of AFB_1 diol into CHES buffer, pH 10. The postincubation mixtures were clarified by centrifugation (10,000 × *g*, 3 min) and 10 μl aliquots of the supernatants were diluted with 15 μl of high-performance liquid chromatography (HPLC) initial mobile phase prior to isotope dilution mass spectrometry analysis. Specific activity results are reported as pmol of AFB_1 alcohol metabolites formed/min/mg cytosolic protein and represent the mean ± SE from three independent experiments. Hepatic GST specific activities were determined spectrophotometrically by monitoring metabolism of the 1-chloro-2,4-dinitrobenzene substrate at 340 nm over 2 min as previously described (Habig *et al*., 1974). Cytosolic protein concentrations were determined by the Bio-Rad assay (Bio-Rad Corporation, Hercules, CA).

Aflatoxin metabolism. Urine was collected on dry ice over a 24-h period from male rats of transgenic positive and negative litter mates of approximately 5 weeks of age and 125 g body weight. This age and weight corresponded to those of rats in the carcinogenicity experiments (see below) when they received the first dose of AFB_1. AFB_1 was obtained from Sigma-Aldrich (St Louis, MO). Rats were gavaged with a single dose of AFB_1 (25 μg/rat) and housed singly in glass metabolic cages with access to water and diet *ad libitum*. The 24-h urine samples were stored at -80°C prior to analysis. Urine samples from animals were thawed and a 5-ml aliquot adjusted to pH 3.5 with acetic acid prior to centrifugation at 500 × *g* for 10 min. Samples were then spiked with 2 ng of $^{13}C_{17}$-AFB_1 dialcohol internal standard and extracted using 6-cm^3 Waters MCX Oasis SPE columns (Waters Corp., Milford, MA) equilibrated with 1 column volume of methanol followed by 1 column volume of water prior to loading the urine sample. AFB_1 alcohols were eluted from the SPE columns with 10 ml of 100% methanol and reduced to a final volume of approximately 50 μl using high-purity nitrogen. Samples were then diluted to approximately 600 μl with water and loaded onto an aflatoxin-specific preparative monoclonal antibody immunoaffinity column, as previously described (Egner *et al*., 2006). The immunoaffinity column consisted of a 2:1 mix of aflatoxin-lysine and

aflatoxin-2B11 antibodies. The affinity column was washed with phosphate-buffered saline and water to remove nonspecifically bound materials and the AFB_1 alcohols were eluted from the immunoaffinity column with 6 ml of 70% dimethylsulfoxide/water (vol/vol) followed by another two volumes of water. The dimethylsulfoxide and water fractions were combined, diluted with water and then applied to a 3-cm^3 Varian Bond-Elut LRC C18 SPE column (Varian, Inc., Walnut Creek, CA). AFB_1 alcohols were eluted from the SPE with 5 ml of a 50/50 mixture of 1% acetic acid/methanol followed by 5 ml of 100% methanol and concentrated to approximately 40 μl under a nitrogen stream: 1 μl of final urinary extract was diluted with 24 μl of HPLC initial mobile phase prior to analysis. The AFB_1 dialdehyde metabolites (C-6a monoalcohol, C-8 monoalcohol, and dialcohol) were then quantified by isotope dilution tandem mass spectrometry as recently described (Johnson *et al*., 2008). Urinary creatinine levels were determined by Eagle Diagnostics Creatinine Direct Reagent Sets (De Soto, TX). Subsequent to urine collection, the rats were autopsied and liver samples were snap frozen. DNA was isolated and analyzed for AFB_1 N^7-guanine adducts by isotope dilution mass spectrometry as previously described (Egner *et al*., 2006). Plasma samples were processed and analyzed for AFB_1 lysine adducts by isotope dilution mass spectrometry as previously described (McCoy *et al*., 2005).

Acute aflatoxin toxicity. Hepatotoxicity was assessed by evaluating the classic phenomenon of bile duct proliferation in response to acute toxic doses of AFB_1 (Maxuitenko *et al*., 1994; Newberne, 1973; Roebuck and Maxuitenko, 1996). The quantification of AFB_1-induced bile duct proliferation has been described and used as a screening method for selecting chemoprotective dithiolethiones (Maxuitenko *et al*., 1996). At 7–8 weeks of age, rats received two doses of AFB_1 (500 μg/kg body weight, i.p.) on two successive days, followed on day three with two doses (100 mg/kg body weight, i.p.) of 5-bromo-2′deoxyuridine (BrdU) given 5 and 2 h prior to autopsy. Livers were fixed in formalin and processed by routine histological methods. Hepatic tissue sections (5 μm thick) were stained immunohistochemically for BrdU (BrdU *In-Situ* Detection Kit, BD Biosciences, San Jose, CA). Small bile ducts of less than 15 cells were evaluated by light microscopy to determine a BrdU labeling index. Approximately 500 bile duct cells were counted per rat liver and a bile duct labeling index was calculated.

Hepatic carcinogenicity. Historical data predicted (Busby and Wogan, 1984) and preliminary data (not shown) confirmed that transgenic rats derived from of the Sprague-Dawley rat strain were less sensitive to AFB_1 than the F344 rats for which extensive data exists regarding the induction of putative preneoplastic foci (Busby and Wogan, 1984; Roebuck and Maxuitenko, 1994; Roebuck *et al*., 2003; Yates *et al*., 2006). Therefore to have adequate numbers of putative preneoplastic foci (henceforth, termed foci) in livers for quantitative evaluation, the total dose of AFB_1 was doubled by extending treatment duration from two to four weeks and also permitting the foci to grow for two weeks longer than with F344 rats used in recent experiments (Roebuck *et al*., 2003; Yates *et al*., 2006). Throughout the experiments, both food and water were available *ad libitum* with only food being withdrawn for 12 h prior to autopsy to reduce the glycogen accumulation in the livers. At approximately 5 weeks of age and about 125 g body weight, AFB_1 treatment began. AFB_1 (25 μg/rat) was gavaged 5 days per week for 4 successive weeks and rats were autopsied 6 weeks later. The cumulative dose of AFB_1 was 500 μg AFB_1 per rat and the duration of the entire protocol was 10 weeks. From the left lateral hepatic lobe, multiple 2-mm-thick sections were cut by hand, fixed in acetone at 4°C, and embedded in paraffin. Hepatic sections (5 μm thick) were stained by standard immunohistochemical methods for expression of GST-placental isoform (GST-P) foci and the foci were identified and analyzed by light microscopy. As with previous analyses (Roebuck *et al*., 2003; Yates *et al*., 2006), the observed focal data of number of foci per unit tissue area examined and their focal transactional areas were first subjected to morphometric transformation resulting in the volume percent of liver occupied by GST-P positive foci, a parameter analogous to tumor burden. Details of this procedure have been published previously (Kensler *et al*., 1992; Maxuitenko *et al*., 1996; Yates *et al*., 2006).

Statistical analyses. Statistical analyses of the *in vivo* metabolism data were performed with Sigma-Plot software (SYSTAT Software, Inc., San Jose, CA) using one-way analysis of variance (ANOVA). The Holm-Sidak *post hoc* method was used for multiple pairwise comparisons. Focal data (volume %) and bile duct cell labeling indices were statistically analyzed using a two-way ANOVA (Stata Corp., College Station, TX).

RESULTS

Characterization of Transgenic Rat Lines

Four transgenic founder rats were produced using the APOE-AKR7A1 DNA construct to drive expression of AKR7A1 in the liver (Fig. 2A). Protein levels of AKR7A1 in hepatic cytosols prepared from the progeny of each founder are shown in Figure 2B. These immunoblots clearly show that lines $AKR7A1^{Tg3}$ and AKR $7A1^{Tg4}$ have less immunoreactive protein than lines $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$. The latter two transgenic lines exhibited similar or greater intensity for AKR protein than did the cytosols from the D3T-pretreated non-transgenic rats and were 18-fold and 8-fold greater than nontransgenic genetic control rats. Expression of AKR7A1 was similar between male and female rats for all transgenic lines. Measurement of 9,10-phenanthrene quinone reductase activity, a substrate for AKR7A1 (Knight *et al*., 1999), was used for initial functional characterization of the four transgenic lines. This analysis indicated that the $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$ lines exhibited elevated activities compared to nontransgenic rats; whereas, the $AKR7A1^{Tg3}$ and AKR $7A1^{Tg4}$ lines did not (data not shown). No discernable differences between male and female rats were seen in either the transgenic or nontransgenic lines with this assay. The copy number of AKR7A1 per genome was determined to be 25 for $AKR7A1^{Tg2}$ and 8 for $AKR7A1^{Tg5}$. More rigorous characterization of the catalytic capacity of the hepatic enzymes from the four rat lines was determined using AFB_1 dialdehyde as substrate. Shown in Figure 3A, hepatic cytosols from lines $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$, as well as from D3T-treated animals, produced C-6a monoalcohol, C-8 monoalcohol, and dialcohol. However, dialcohol represented between 90 and 95% of the product formed in all cases. None of the dialdehyde-derived alcohol metabolites were detected in hepatic cytosols from vehicle-treated, nontransgenic rats under the incubation conditions employed. Summing all alcohol metabolites, the specific activities (pmol alcohol metabolite/min/mg protein) were 418 ± 100, 49 ± 3, 42 ± 9 (mean ± SE, $N = 3$) for $AKR7A1^{Tg2}$, $AKR7A1^{Tg5}$ and D3T-treated nontransgenic animals, respectively. As a point of comparison, Figure 3B indicates that GST activity was not affected by transgene expression in either line, but was elevated twofold by D3T treatment, as has been observed previously (Roebuck *et al*., 2003). Collectively, the estimates of copy number, hepatic protein expression by Western blot, and assessment of specific activity indicate that AKR7A1 expression in line $AKR7A1^{Tg5}$ is comparable to that

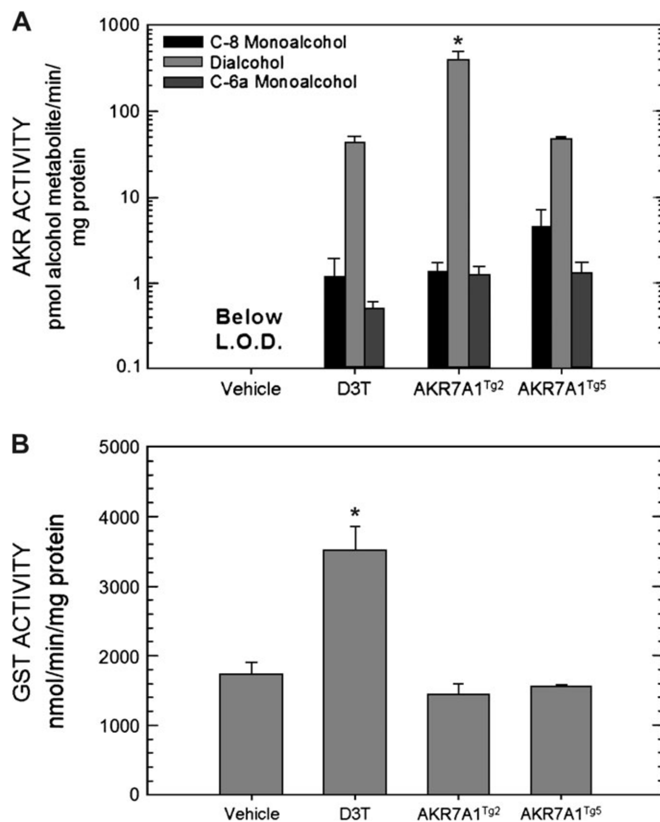

FIG. 3. (A) Formation of the C-6a monoalcohol, C-8 monoalcohol and dialcohol following incubation of hepatic cytosols with aflatoxin dialdehyde. Incubations were conducted for 3 min with AFB_1 dialdehyde susbtrate (7µM). All three AFB_1 alcohols could be detected in nontransgene hepatic cytosols under nonlinear incubation conditions (10µM substrate, 10 min). Metabolites were quantified by isotope dilution mass spectrometry. *$p < 0.05$, $AKR7A1^{Tg2}$ compared to $AKR7A1^{Tg5}$ by ANOVA. (B) GST activities in hepatic cytosols using chlorodinitrobenzene as substrate. *$p < 0.05$, D3T compared to vehicle treated by ANOVA. Values are mean ± SE ($N = 3$).

achievable with a pharmacologic intervention (i.e., treatment with D3T), while that in line $AKR7A1^{Tg2}$ is several fold higher.

In Vivo *Metabolism of AFB_1 Dialdehyde*

Of the three alcohols that could be formed from the AFB_1 dialdehyde intermediate (Fig. 1), only C-6a monoalcohol and the dialcohol were detected in rat urine samples. No substantial differences (data not shown) were observed for the urinary excretion of C-6a monoalcohol as a function of either transgene or D3T treatment, suggesting the *in vivo* formation of this metabolite is principally affected by other enzymes and/or extrahepatic tissues. Shown in Figure 4 are the levels of AFB_1-dialcohol, the major AKR7A1-derived metabolite from the C-6a monoalcohol, excreted during the 24 h following dosing with AFB_1. Line $AKR7A1^{Tg2}$ excreted 40-fold more dialcohol than their nontransgenic litter mates, while line $AKR7A1^{Tg5}$ excreted 12-fold more dialcohol. D3T-treated nontransgenic animals excreted 7.5-fold more of the dialcohol metabolite. These results are consistent with the characterization of AKR7A1 protein expression (see Fig. 2B) and AFB_1 alcohols produced *in vitro* by the hepatic cytosols preparations. Collectively, these results suggest the two transgenic lines provide a robust model to examine the impact of AKR7A1 on aflatoxin toxicity and carcinogenicity *in vivo*.

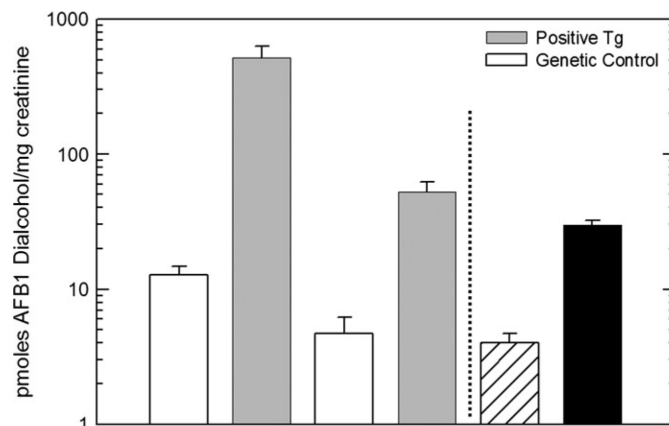

FIG. 4. Urinary excretion of AFB_1 dialcohol levels in AKR7A1 transgenic and D3T-treated nontransgenic rats. AFB_1 dialcohol was quantified by isotope dilution mass spectrometry as described in ''Materials and Methods'' and in reference 24. Values are mean ± SE ($N = 3$).

AFB_1 Toxicity

Without aflatoxin damage to the parenchymal cells, bile duct cells have a very low turnover and labeling index. The transgenic rats and their genetic controls had a labeling index of 0.3 ± 0.2 (mean ± SE, $N = 7$) which agrees well with historic controls (Maxuitenko *et al.*, 1996). As with the *in vivo* metabolism of AFB_1 described above, there were no statistically significant differences noted between male and female rats of either the transgene phenotype or their genetic controls; thus, these data were combined. For the $AKR7A1^{Tg2}$ line, there was no difference between the bile duct labeling index of the positive (26.9 ± 1.7, $N = 11$) and genetic control litter mates (25.6 ± 1.2, $N = 11$). A similar observation was made with the $AKR7A1^{Tg5}$ line, namely, the labeling indices of the transgenic positive rats (34.1 ± 2.2, $N = 16$) and genetic controls (32.4 ± 1.8, $N = 14$) did not differ statistically. The clear differences in extent of labeling between the two transgenic lines resulted because the experiments were undertaken at different times with different preparations of AFB_1 and BrdU. In response to induced hepatotoxicity by AFB_1, bile duct proliferation ensued and was not modulated by the presence of the additional AKR7A1 enzymatic activity in these rats. These results are in marked contrast to the protective effects from administration of dithiolethiones on this manifestation of AFB_1 toxicity (Maxuitenko *et al.*, 1996).

AFB_1 Carcinogenicity

Using our well-characterized hepatic carcinogenesis model (Kensler *et al.*, 1992; Roebuck *et al.*, 2003; Yates *et al.*, 2006), AKR7A1 transgene afforded no protection against AFB_1-induced carcinogenesis (Fig. 5). With the exception of one rat (see below), there were no differences among any of the groups in the observed microscopic appearance of the GST-P positive foci. Irrespective of the sex of the rats or the transgenic line, $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$, the focal burden (volume %) of GST-P foci did not differ statistically between groups carrying the transgene and groups of their genetic control, litter mates. The less robust, morphometrically derived data of foci number per unit volume of liver and mean focal diameter showed no statistically significant difference between groups (data not shown). At variance with all other rats in these groups, one of 12 male, $AKR7A1^{Tg2}$ positive rats had an unusually high focal burden (74%) and mean focal diameter what was twice that of other rats, thus, largely accounting for the large variation in the male $AKR7A1^{Tg2}$ group. In the $AKR7A1^{Tg2}$ genetic controls, the focal volume was 5.50 ± 4.99 (mean ± SD); whereas, in the positive Tg2 group, the focal volume was 16.40 ± 19.64. There was not a statistically significant difference between these two groups ($p = 0.065$, *t*-test) and additionally, the higher volume percent was in the opposite direction for support of our initial hypothesis. An underlying explanation of the susceptibility of this one rat to AFB_1 is not obvious.

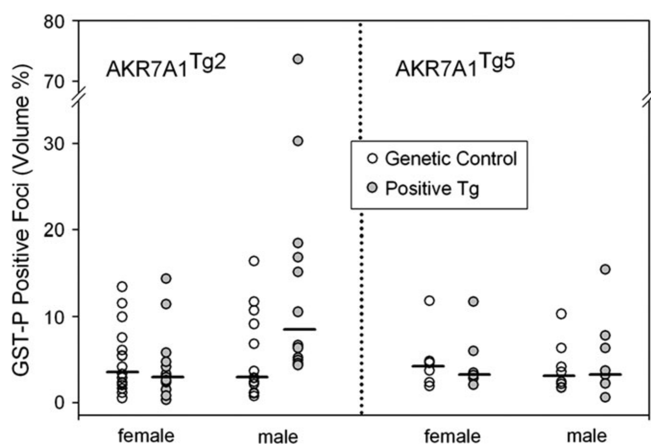

FIG. 5. Evaluation of the AKR7A1 transgene on AFB_1-induced GST-P positive foci formation. At 5 weeks of age, litter mates expressing AKR7A1 and genetic controls were orally gavaged with 25 μg AFB_1 5 days per week for 4 successive weeks. The livers were removed 6 weeks later, fixed in acetone, and immunohistochemically stained using an antibody recognizing GST-P. The number and size of the GST-P positive foci were measured by light microscopy and the volume % of GST-P positive foci (analogous to tumor burden) calculated. There were no statistically significant differences between AKR7A1 positive and genetic control. One of 12 rats with an unusually high focal burden (74%) accounted for the high group mean and large SE in the male $AKR7A1^{Tg2}$ group. These scatter plots are accompanied with median bars (*N* ranged from 12 to 16 rats per group for the $AKR7A1^{Tg2}$ and six to eight rats per group for the $AKR7A1^{Tg5}$ rats).

AFB_1 DNA and Protein Adducts

Further evaluation of the effects of transgene expression on aflatoxin toxicodynamics were assessed by quantification of biomarkers reflecting the internal dose and the biologically effective dose of aflatoxin, that is, aflatoxin-lysine adducts in serum and aflatoxin-N^7-guanine adducts excreted in urine, respectively (Fig. 6). Neither of the AKR7A1 transgenic rat lines *in vivo* showed alterations in excretion of the DNA adduct biomarker; an outcome concordant with earlier observations in which the gene was overexpressed in cells in culture without impact on DNA adduct burden (Bodreddigari *et al.*, 2008). By contrast, D3T, which is known to induce AKR7A1 in concert with many other Nrf2-regulated genes including GSTs, produced an 83% reduction in the urinary excretion of aflatoxin-N^7-guanine adducts. Line $AKR7A1^{Tg2}$ showed a significant 35% decrease in levels of aflatoxin-albumin adducts in serum following acute exposure to AFB_1. Line $AKR7A1^{Tg5}$, which has a several fold lower expression of the transgene, did not exhibit a statistically significant reduction in serum levels of the protein adduct. Additionally, no reductions in levels of this protein adduct biomarker were observed in the D3T-treated rats; however, D3T is known to reduce levels of aflatoxin-albumin adducts during chronic exposure to AFB_1 (Groopman *et al.*, 1992).

DISCUSSION

Since their discovery in the early 1960s, the hepatotoxicity and carcinogenicity of the aflatoxins have been extensively

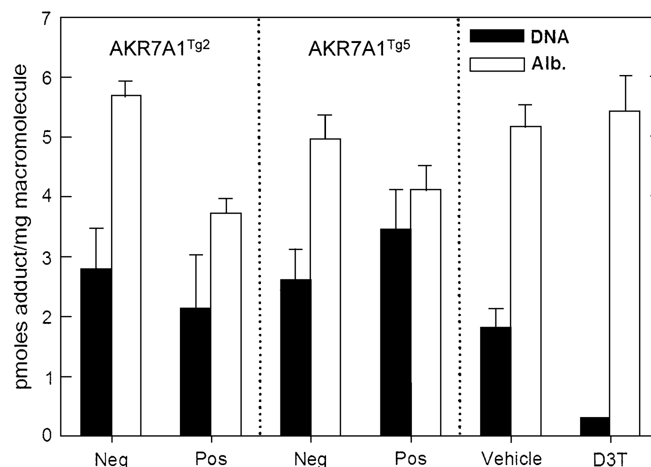

FIG. 6. Influence of AKR transgene on AFB_1 hepatic DNA and serum albumin protein adduct levels. $AFAR^{Tg2}$ positive animals had statistically significant lower levels of AFB_1-lysine adducts compared to their genetic control littermates ($p < 0.05$). D3T-treated animals had statistically significant lower levels of AFB_1 N^7-guanine adducts compared to vehicle treated ($*p < 0.05$). Quantification for data shown was by isotope dilution mass spectrometry. Statistical comparisons were achieved by ANOVA. Values are mean ± SE ($N = 3$).

explored (Busby and Wogan, 1984; Eaton *et al.*, 1994; Kensler *et al.*, 1999; Roebuck and Maxuitenko, 1994). Generally, experimental hepatic carcinogenesis of aflatoxins in rats has involved either chronic exposure over 50–80 weeks in the diet (Wogan, 1974; Wogan *et al.*, 1974) or shorter exposures of usually 2–5 weeks by daily oral gavage (Kensler *et al.*, 1997; Roebuck *et al.*, 1991). With cumulative doses of approximately 100–170 μg AFB_1 per rat, long-term feeding resulted in a 100% incidence of hepatocellular carcinomas (HCC); whereas, the shorter exposure protocols have yielded fewer HCC in spite of the cumulative doses being greater. Central to this process is the metabolic activation of AFB_1 by CYP1A2 and CYP3A4 to yield two chemically reactive epoxides: AFB_1-8,9-*exo* and -8,9-*endo* epoxides (Gallagher *et al.*, 1996; Johnson and Guengerich, 1997). Only the AFB_1-8,9-*exo* isomer reacts readily with DNA, forming the N^7-guanine and its derivative AFB_1 formamidopyrimidine adducts (Johnson and Guengerich, 1997). If not repaired, these DNA adducts or the abasic sites that remain from the spontaneous depurination of the N^7-guanine adduct can rapidly lead to DNA mutations (Smela *et al.*, 2001). Several studies have shown strong correlations between N^7-guanine adducts and the putative preneoplastic GST-P positive foci (Kensler *et al.*, 1986; Kensler *et al.*, 1992; Roebuck *et al.*, 1991; Yates *et al.*, 2006). Kensler *et al.* (1986) observed that the first of a series of doses yielded more total DNA adducts than did subsequent doses. Possible explanations include that the ensuing toxicity from the initial doses of AFB_1 may induce protective mechanisms such as GSTs that result in reduced genetic damage from subsequent exposures or the inherent toxicity of AFB_1 may kill cells that have accumulated adducts thereby reducing the hepatic risk. There is an abundance of literature documenting AFB_1 as a genetic toxin, a generalized growth inhibitor of the liver, and a hepatotoxin able to engender regenerative hyperplasia (Busby and Wogan, 1984; Newberne, 1973; Roebuck, 2004).

In studies of aflatoxin carcinogenesis, the incidence of HCC and timing of their occurrence are both modulated by the dithiolethione oltipraz (Kensler *et al.*, 1997; Roebuck *et al.*, 1991). In short-term studies, protection against the development of hepatic foci have been demonstrated with a variety of chemicals: antioxidants (Kensler *et al.*, 1986), dithiolethiones (Kensler *et al.*, 1992; Roebuck *et al.*, 1991), and triterpenoids (Yates *et al.*, 2006). These same agents protect against the acute toxic effects of AFB_1 as adjudged by prevention of weight loss or maintenance of normal rates of growth, reduction in histological evidence of cellular damage, and mortality from liver failure. These chemoprotective agents all induce the enzymatic activities of both GST and AKR (Kensler *et al.*, 1986, 1992; Roebuck *et al.*, 2003; Yates *et al.*, 2006). Interestingly, the mouse is highly resistance to AFB_1 toxicity (Roebuck and Maxuitenko, 1994), an outcome that is considered to be accounted for predominantly by the high constitutive expression of GST in mouse liver (Eaton *et al.*, 1994).

While the role of AFB_1 N^7-guanine adducts in the initiation of cancer is well documented, the contribution of hepatotoxicity to this process is less clear. Overall, the mechanisms of cytotoxicity by AFB_1 are poorly understood. AFB_1 can form protein adducts particularly with lysine through a Schiff base reaction of the AFB_1 dialdehyde (Fig. 1), and several investigators have hypothesized that such protein damage may lead to hepatotoxicity (Ellis *et al.*, 1993; Guengerich *et al.*, 2002). However, several unknowns remain, including the critical protein targets that trigger cell death, the dose-response for these important reactions, and the mechanisms of cell protection against such attack. Several investigators have hypothesized that AKR7A1 activity is an important factor in this modulation and that elevated levels of AKR activity should prevent cytotoxicity and thereby attenuate carcinogenesis (Bodreddigari *et al.*, 2008; Ellis *et al.*, 1993; Guengerich *et al.*, 2002).

In a recent study (Bodreddigari *et al.*, 2008), we showed that recombinant *in vitro* expression of AKR7A1 afforded protection against AFB_1 dialdehyde cytotoxicity, resulting in a sixfold increase in the LC_{50} of the dialdehyde accompanied by a marked decrease in cellular protein adducts. To address the role of the dialdehyde metabolite in the cytotoxicity of AFB_1 and its modulation by AKR7A1 activity *in vivo*, a rat transgenic for AKR7A1 was constructed and characterized. Two transgenic lines, $AKR7A1^{Tg2}$ and $AKR7A1^{Tg5}$, with 25 and 8 inserted genomic copies, respectively, expressed enzymatically active AFR7A1. Furthermore, in the intact rats, the expected AFB_1 alcohols were formed in relation to the extent of AKR7A1 expression in the liver. However, only the highest expression line, $AKR7A1^{Tg2}$, exhibited reduced levels of serum albumin adducts. Importantly, the GST activity in the transgenic rat was at basal levels in these two transgenic lines, as were the level of N^7-guanine adducts, unlike what occurs with the use of chemoprotective agents. Together, these data confirm appropriate expression of active liver AKR7A1 protein at levels comparable or exceeding those found in response to treatment with chemoprotective agents. Unexpectedly, this metabolic phenotype did not translate into either protection against AFB_1-induced hepatic toxicity or diminution of foci. The high expression of AKR7A1 appears not to be relevant to levels achieved by treatment of rats with chemoprotective agents, indicating that *in vivo* neither the production of the AFB_1 dialdehyde nor its metabolism by AKR7A1 are critical for cytotoxicity or carcinogenicity.

Of interest to these observations are the results of another *in vitro* experiment where we expressed either human CYP1A2 or human CYP1A2 and rat AKR7A1 in AHH-1 lymphoblastoid cells (Bodreddigari *et al.*, 2008). In this experiment AFB_1 was metabolized by CYP1A2 within a cell that has negligible levels of GST activity. Coexpression of AKR7A1 significantly increased cell survival, but did not alter the level of DNA adducts formed at a dose of 3 ng/ml of AFB_1. Thus, at this dose, it appeared that cytotoxicity was being driven by the

AFB_1 dialdehyde and not by the CYP1A2-derived epoxide or other oxidative metabolites such as AFM_1. At higher concentrations of AFB_1, the AKR7A1-expressing cells exhibited a concentration-dependent decrease in the proportion of cells protected against cytotoxicity. This observation suggests that other factors are contributing to AFB_1-mediated cytotoxicity. Of interest to this discussion, independent studies have demonstrated the apparent saturation of mutation induced by exposure to AFB_1 in human lymphoblast cell lines (Crespi *et al.*, 1990; Kaden *et al.*, 1987). This plateau in mutant fraction is observed despite linear increases in the amount of AFB_1 adducts formed (Crispi *et al.*, 1990; Kaden *et al.*, 1987) and in both studies, it occurred at concentrations of AFB_1 exceeding 6 ng/ml. Having controlled for cell cycle and the potential for different subpopulations of cells, these studies support the concept of a disproportionate loss of cells exceeding some threshold of DNA damage. For example, apoptotic mechanisms activated at elevated levels of DNA damage could account for the concentration-dependent loss of protection afforded by AKR7A1. This idea is supported by an earlier *in vitro* study by Fields *et al.* (1999) where they expressed in hamster V79 cells either rat CYP2B1 or rat CYP2B1 and murine GST A3-3. In their study, coexpression of the GST resulted in a more than a 70% reduction in DNA and RNA adducts, and a 4.6-fold resistance to cytotoxicity (Fields *et al.*, 1999). These data indicate a role of the epoxide in mediating cytotoxicity. However, this study and those described above cannot be directly compared, as the cells expressing rat CYP2B1 had to be treated with much higher concentrations of AFB_1. Nonetheless, at doses of AFB_1 required to produce foci in Sprague-Dawley rats, it seems likely that the role of the epoxide is much greater than the role of the dialdehyde in the observed hepatotoxicity. In the past, the available analytical techniques were not sufficiently sensitive to carefully document the mass balance of the dose of AFB_1 to the liver and its metabolic fate. However, perhaps with the current techniques of HPLC with isotope dilution mass spectrometry (McCoy *et al.*, 2005), this important question can be more fully elaborated.

The rationale for using AKR7A1 for the development of hepatocyte-specific transgenic rats was predicted on the basis of the extraordinary levels of induction of this enzyme in rodent liver by chemoprotective agents and the observed protection against AFB_1 toxicity afforded by forced overexpression in cells in culture. Despite this strong evidence in support of the hypothesis that AKR7A1 could modulate susceptibility to aflatoxin carcinogenesis, especially when coupled with the pronounced phenotype of enhanced AFB_1 dialdehyde metabolism in the transgenic rats, no protective effect was observed. Clearly, *in vitro* and *in vivo* observations are not always concordant. This study again sounds a cautionary note against total reliance on reductionist approaches to complex systems. Whether hepatic GST induction is solely responsible for protection by inducers from hepatic cytotoxicity, or more likely multiple aspects of the Keap1-Nrf2 transcriptional program work in concert to protect the liver from AFB_1 is not known. However, with the hindsight afforded by this study, the focus of future studies on mechanisms of cancer chemoprevention using systems approaches might be more fruitful.

FUNDING

National Institutes of Health, National Cancer Institute (R01 CA 39416); and National Institute of Environmental Health Sciences (P30 ES 03819); and the W. Harry Feinstone Center for Genomic Research to T.R.S.

ACKNOWLEDGMENTS

We thank Dr John Taylor, UCSF, for the liver-specific expression vector pLiv.7 and Mr Eric York, Department of Pathology, Dartmouth Medical School for superb histological services.

REFERENCES

Bodreddigari, S., Jones, L. K., Egner, P. A., Groopman, J. D., Sutter, C. H., Roebuck, B. D., Guengerich, F. P., Kensler, T. W., and Sutter, T. R. (2008). Protection against aflatoxin B_1-induced cytotoxicity by expression of the cloned aflatoxin B1-aldehyde reductases rat AKR7A1 and human AKR7A3. *Chem. Res. Toxicol.* **21,** 1134–1142.

Busby, W. F., and Wogan, G. N. (1984). Aflatoxins. In *Chemical Carcinogens* (C. E. Searle, Ed.), pp. 945–1136. American Chemical Society, Washington, D.C.

Crespi, C. L., Steimel, D. T., Aoyama, T., Gelboin, H. V., and Gonzalez, F. J. (1990). Stable expression of human cytochrome P4501A2 cDNA in a human lymphoblastoid cell line: Role of the enzyme in the metabolic activation of aflatoxin B_1. *Mol. Carcinogenesis* **3,** 5–8.

Eaton, D. L., and Bammler, T. K. (1999). Concise review of the glutathione *S*-transferases and their significance to toxicology. *Toxicol. Sci.* **49,** 156–164.

Eaton, D. L., Ramsdell, H. S., and Neal, G. E. (1994). Biotransformation of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 45–72. Academic Press, New York.

Egner, P. A., Groopman, J. D., Wang, J., Kensler, T. W., and Friesen, M. D. (2006). Quantification of aflatoxin B_1-N^7-guaine in human urine by high-performance liquid chromatography and isotope dilution tandem mass spectrometry. *Chem. Res. Toxicol.* **19,** 1191–1195.

Ellis, E. M., Judah, D. J., Neal, G. E., and Hayes, J. D. (1993). An ethoxyquin-inducible aldehyde reductase from rat liver that metabolizes aflatoxin B_1 defines a subfamily of aldo-keto reductases. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10350–10354.

Fan, J., Wang, J., Bensadoun, A., Lauer, S. J., Dang, Q., Mahley, R. W., and Taylor, J. M. (1994). Overexpression of hepatic lipase in transgenic rabbits leading to a marked reduction of plasma high density lipoproteins and intermediate density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8724–8728.

Fields, W. R., Morrow, C. S., Doehmer, J., and Townsend, A. J. (1999). Expression of stably transfected murine glutathione S-transferase A3-3

protects against nucleic acid alkylation and cytotoxicity by aflatoxin B_1 in hamster V79 cells expressing rat cytochrome P450-2B1. *Carcinogenesis* **20,** 1121–1125.

Gallagher, E. P., Kunze, K. L., Stapleton, P. L., and Eaton, D. L. (1996). The kinetics of aflatoxin B_1 oxidation by human cDNA—Expressed and human liver microsomal cytochromes P450 1A2 and 3A4. *Toxicol. Appl. Pharmacol.* **141,** 595–606.

Groopman, J. D., Busby, W. F., Jr., and Wogan, G. N. (1980). Nuclear distribution of aflatoxin B_1 and its interaction with histones in rat liver in vivo. *Cancer Res.* **40,** 4343–4351.

Groopman, J. D., DeMatos, P., Egner, P. A., Love-hunt, A., and Kensler, T. W. (1992). Molecular dosimetry of urinary aflatoxin-N^7-guanine and serum aflatoxin-albumin adducts predicts chemoprotection by 1,2-dithiole-3-thione in rats. *Carcinogenesis* **13,** 101–106.

Guengerich, F. P., Arneson, K. O., Williams, K. M., Deng, Z., and Harris, T. M. (2002). Reaction of aflatoxin B_1 oxidation products with lysine. *Chem. Res. Toxicol.* **15,** 780–792.

Guengerich, F. P., Cai, H., McMahon, M., Hayes, J. D., Sutter, T. R., Groopman, J. D., Deng, Z., and Harris, T. M. (2001). Reduction of aflatoxin B_1 dialdehyde by rat and human aldo-keto reductases. *Chem. Res. Toxicol.* **14,** 727–737.

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974). Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **219,** 7130–7139.

Hayes, J. D., Judah, D. J., and Neal, G. E. (1993). Resistance to aflatoxin B_1 is associated with the expression of a novel aldo-keto reductase which has catalytic activity towards a cytotoxic aldehyde-containing metabolite of the toxin. *Cancer Res.* **53,** 3887–3894.

Hinshelwood, A., McGarvie, G., and Ellis, E. M. (2003). Substrate specificity of mouse aldo-keto reductase AKR7A5. *Chem. Biol. Interact.* **144,** 263–269.

Ireland, L. S., Harrision, D. J., Neal, G. E., and Hayes, J. D. (1998). Molecular cloning, expression and catalytic activity of a hum n AKR7 member of the aldo-keto reductase superfamily: Evidence that the major 2-carboxybenzaldehyde reductase from human liver is a homologue of rat aflatoxin B_1-aldehyde reductase. *Biochem. J.* **332,** 21–34.

Johnson, D. N., Egner, P. A., OBrian, G., Glassbrook, N., Roebuck, B. D., Sutter, T. R., Payne, G. A., Kensler, T. W., and Groopman, J. D. (2008). Quantification of urinary aflatoxin B_1 dialdehyde metabolites formed by aflatoxin aldehyde reductase using isotopic dilution tandem mass spectrometry. *Chem. Res. Toxicol.* **21,** 752–760.

Johnson, W. W., and Guengerich, F. P. (1997). Reaction of aflatoxin B_1 *exo*-epoxide with DNA: Kinetic analysis of covalent binding and DNA-induced hydrolysis. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6121–25.

Johnson, W. W., Harris, T. M., and Guengerich, F. P. (1996). Kinetics and mechanism of hydrolysis of aflatoxin B_1 *exo*-8,9-oxide and rearrangement of the dihyrodiol. *J. Am. Chem. Soc.* **118,** 8213–8220.

Kaden, D. A., Call, K. M., Leong, P.-M., Komives, E. A., and Thilly, W. G. (1987). Killing and mutation of human lymphoblast cells by aflatoxin B_1: Evidence for and inducible repair response. *Cancer Res.* **47,** 1993–2001.

Kensler, T. W., Egner, P. A., Davidson, N. E., Roebuck, B. D., Pikul, A., and Groopman, J. D. (1986). Modulation of aflatoxin metabolism, aflatoxin-N^7-guanine formation, and hepatic tumorigenesis in rats fed ethoxyquin: Role of induction of glutathione S-transferases. *Cancer Res.* **46,** 3924–3931.

Kensler, T. W., Gange, S. J., Egner, P. A., Dolan, P. M., Muñoz, A., Groopman, J. D., Rogers, A. E., and Roebuck, B. D. (1997). Predictive value of molecular dosimetry: Individual *versus* group effects of oltipraz on aflatoxin-albumin adducts and risk of liver cancer. *Cancer Epidemiol. Biomarkers Prev.* **6,** 603–610.

Kensler, T. W., Groopman, J. D., Eaton, D. L., Curphey, T. J., and Roebuck, B. D. (1992). Potent inhibition of aflatoxin-induced hepatic tumorigenesis by the monofunctional enzyme inducer 1,2-dithiole-3-thione. *Carcinogenesis* **13,** 95–100.

Kensler, T. W., Groopman, J. D., Sutter, T. R., Curphey, T. J., and Roebuck, B. D. (1999). Development of cancer chemopreventive agents: Oltipraz as a paradigm. *Chem. Res. Toxicol.* **12,** 113–126.

Knight, L. P., Primiano, T., Groopman, J. D., Kensler, T. W., and Sutter, T. R. (1999). cDNA cloning, expression and activity of a second human aflatoxin B_1-metabolizing member of the aldo-keto reductase superfamily, AKR7A3. *Carcinogenesis* **20,** 1215–1223.

Liu, Y. L., Roebuck, B. D., Yager, J. D., Groopman, J. D., and Kensler, T. M. (1988). Protection by 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) against the hepatotoxicity of aflatoxin B_1 in the rat. *Toxicol. Appl. Pharmacol.* **93,** 442–451.

Maxuitenko, Y. Y., Curphey, T. J., Kensler, T. W., and Roebuck, B. D. (1996). Protection against aflatoxin B_1-induced hepatic toxicity as a short term screen of cancer chemopreventive dithiolethiones. *Fundam. Appl. Toxicol.* **32,** 250–259.

McCoy, L. F., Scholl, P. F., Schleicher, R. L., Groopman, J. D., Powers, C. D., and Pfieffer, C. C. (2005). Analysis of aflatoxin B_1-lysine adduct in serum using isotope dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **19,** 2203–2210.

Newberne, P. M. (1973). Chronic aflatoxicosis. *J. Am. Vet. Med. Assoc.* **163,** 1262–1269.

Roebuck, B. D. (2004). Hyperplasia, partial hepatectomy, and the carcinogenicity of aflatoxin B_1. *J. Cell. Biochem.* **91,** 243–249.

Roebuck, B. D., Curphey, T. J., Li, Y., Baumgartner, K. J., Bodreddigari, S., Yan, J., Gange, S. J., Kensler, T. W., and Sutter, T. R. (2003). Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. *Carcinogenesis* **24,** 1919–28.

Roebuck, B. D., Liu, Y.-L., Rogers, A. E., Groopman, J. D., and Kensler, T. W. (1991). Protection against aflatoxin B_1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (Oltipraz): predictive role for short-term molecular dosimetry. *Cancer Res.* **51,** 5501–5506.

Roebuck, B. D., and Maxuitenko, Y. (1994). Biochemical mechanisms and biological implications of the toxicity of aflatoxins as related to aflatoxin carcinogenesis. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D. L. Eaton and J. D. Groopman, Eds.), pp. 27–43. Academic Press, New York.

Sabbioni, G., Skipper, P. L., Buchi, G., and Tannenbaum, S. R. (1987). Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 in vivo in rats. *Carcinogenesis* **8,** 819–824.

Smela, M. E., Currier, S. S., Bailey, E. A., and Essigmann, J. M. (2001). The chemistry and biology of aflatoxin B_1: from mutational spectrometry to carcinogenesis. *Carcinogenesis* **22,** 535–545.

Wogan, G. N. (1974). Naturally occurring carcinogens. In *The Physiopathology of Cancer. Biology and Biochemistry* (P. Shubik, Ed.), Vol. 1, pp. 64–109. Karger, Basel.

Wogan, G. N., Paglialunga, S., and Newberne, P. M. (1974). Carcinogenic effects of low dietary levels of aflatoxin B1 in rats. *Fd Cosmet. Toxicol.* **121,** 681–685.

Yamanaka, S., Balestra, M., Ferrell, L. D., Fan, J., Arnold, K. S., Taylor, S., Taylor, J. M., and Innerarity, T. L. (1995). Apolipoprotein B mRNA-editing protein induces hepatocellular carcinoma and dysplasia in transgenic animas. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8483–8487.

Yates, M. S., Kwak, M.-K., Egner, P. A., Groopman, J. D., Bodreddigari, S., Sutter, T. R., Baumgartner, K. J., Roebuck, B. D., Liby, K. T., Yore, M. M., *et al.* (2006). Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the synthetic triterpenoid, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oly]imidazole. *Cancer Res.* **66,** 2488–2494.