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Species-specific differences in hepatic mutant frequency and mutational spectrum among lambda/*lacI* transgenic rats and mice following exposure to aflatoxin B_1

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***In vivo* mutations were studied in lambda/*lacI* (Big Blue®) transgenic C57BL/6 mice and F344 rats following exposure to either AFB_1 (aflatoxin B_1) or DMSO vehicle. Fourteen days after exposure, livers were removed for DNA extraction and subsequent mutational analysis of the *lacI* gene. Mice injected with a single i.p. dose of AFB_1 at 2.5 mg/kg did not show a significant increase in liver mutant frequency relative to vehicle-treated controls. DNA sequence analysis of *lacI* mutations collected from the AFB_1-treated mice showed a pattern of mutation similar to that of the previously observed spontaneous mouse liver mutational spectrum. In contrast, rats subjected to one-tenth the mouse AFB_1 dosage responded with an approximate 20-fold induction in liver mutant frequency over background. Sequencing of *lacI* mutations also revealed spectral differences between vehicle- and AFB_1-treated rats. A large increase in G:C→T:A transversions was observed among *lacI* mutations isolated from the AFB_1-treated rats. This work is among the first multi-species *in vivo* mutagenicity studies using transgenic rodents harboring the same shuttle vector. Such multi-species *in vivo* assays may prove to be valuable in the areas of mechanistic analysis and risk assessment.**

Introduction

The study of genetic damage sustained *in vivo* in mammals has been greatly facilitated in recent years by the development of a lambda/*lacI* shuttle vector that is readily recovered from transgenic C57BL/6 mice (1). These mice, along with their $B6C3F_1$ hybrids, permit the study of mutation fixation in diverse tissues, and allow the observation of treatment-induced changes in mutational spectra within these tissues (2–4). While transgenic mice provide a way to study the effects of mutagens in several tissues, the recent development of an analogous transgenic F344 (Fischer 344) rat harboring the same lambda/*lacI* shuttle vector makes it possible to perform multiple-species mutation testing *in vivo* in rodents (5).

Aflatoxin B_1 (AFB_1*) is a classic example of a substance for which toxic and carcinogenic potential varies widely between species (6). This well-characterized mycotoxin is produced by the food spoilage fungus *Aspergillus flavus* (7) and demonstrates species-specific carcinogenicity and mutagenicity through differences in metabolic activation and detoxification (recent reviews include 8–10). AFB_1 is an effective liver carcinogen in some species, including rats and humans (7,11–15). Numerous comparative studies using rodents have shown that rats are highly susceptible to the hepatocarcinogenic effects of AFB_1, while mice are relatively resistant (16). Rat hepatocytes have also been shown to be ~10-fold more effective than mouse hepatocytes at generating mutagenic metabolites of AFB_1 (17). The observation that AFB_1-induced mutagenicity and carcinogenicity is more pronounced in rats over mice is thought to be due mainly to the higher levels of glutathione-S-transferase activity in mice (9,18).

To examine whether species-specific differences can be observed using an *in vivo* transgenic mutation assay, mutant frequencies were measured in the livers of lambda/*lacI* F344 rats and C57BL/6 mice following vehicle or AFB_1 exposure. As expected based on earlier studies, rats demonstrated high susceptibility to AFB_1-induced *lacI* mutation within the liver, whereas mice were refractory to the mutagenic effects of this mycotoxin. DNA sequence analysis of *lacI* mutations recovered from AFB_1-treated lambda/*lacI* mouse and rat liver also revealed species-dependent differences. Rats treated with AFB_1 demonstrated a large increase in G:C→T:A transversions compared with the vehicle-treated controls. In AFB_1-treated mice, however, the pattern of mutation in AFB_1-treated animals was very similar to the spontaneous mutational spectrum previously observed in liver.

Materials and methods

Husbandry

Hemizygous Fischer 344 rats from the transgenic lineage QX (Big Blue®, Stratagene, La Jolla, CA), 13–19 weeks of age, were bred at Stratagene in La Jolla, CA. Hemizygous C57BL/6 mice from the transgenic lineage A1 (Big Blue), 13 weeks of age, were bred at Taconic (Germantown, NY) for Stratagene. All animals were housed at 20°C and maintained on a 12 h light cycle (5 a.m. to 5 p.m.). Diet consisted of 4% fat rodent blocks (Harlan Teklad, Madison, WI) and water, both administered *ad libitum*. All husbandry procedures were established according to the standards set within the NIH Guide for the Care and Use of Laboratory Animals.

Aflatoxin B_1 administration

AFB_1, CAS (Chemical Abstracts Registry) no. 1162-65-8, catalog no. A-6636 and DMSO (dimethyl sulfoxide), CAS no. 67-68-5, catalog no. D-8779 were obtained from Sigma Chemical Company (St Louis, MO). Each experimental group consisted of six animals. AFB_1 was dissolved in DMSO and administered in a single i.p. injection at 2.5 mg/kg in both mice and rats. After observing acute toxicity at this dosage in the AFB_1-treated rats, an additional group of male rats was administered a single dose of AFB_1 at 0.25 mg/kg. The dosing volume for both species remained constant at 1 ml/kg. The control groups for both species were given a single i.p. injection of DMSO at 1 ml/kg. An expression period of 14 days was carried out before animals were killed for tissue harvest. After quick removal, the livers were flash-frozen in liquid nitrogen and stored at –80°C until DNA isolation. Compound dosing and tissue harvesting were performed at Microbiological Associates, Inc. (Rockville, MD).

Genomic DNA isolation

For each individual, ~100 mg of liver was homogenized in 3 ml of dounce buffer (6.5 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4;

*Abbreviations: CAS, Chemical Abstracts Registry; AFB_1, aflatoxin B_1; DMSO, dimethyl sulfoxide; F344, Fischer 344 rat strain; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetra-acetic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; pfu, plaque forming units; PCR, polymerase chain reaction; CpG site, 5′-CpG-3′ dinucleotide sequence; TD_{50}, dose that produces a 50% incidence in tumors; AP, apurinic; AFB_1-FAPY, AFB_1-formamidopyrimidine.

10 mM Na_2EDTA, pH 8.0) supplemented with 20 μl/ml RNAce-It™ ribonuclease cocktail (Stratagene, La Jolla) using a Wheaton 7 ml dounce with pestle size B. The homogenate was then gently combined with 3 ml of 2X protease solution (2 mg/ml proteinase K; 2% SDS; 100 mM Na_2EDTA, pH 7.5) and allowed to digest at 50°C for 3 h, followed by two extractions with phenol:chloroform (pH 8.0), one chloroform extraction, and ethanol precipitation. All extractions were performed gently to encourage the isolation of high molecular weight DNA. After removing the residual ethanol, all DNA precipitates were allowed to dissolve in 200–500 μl of TE solution (10 mM Tris–HCl; 1 mM Na_2EDTA, pH 8.0) at 4°C for at least 2 days. All DNA samples were coded and randomized in order to perform a blind study.

Big blue color-screening assay

All assay reagents were obtained from Stratagene (La Jolla, CA). Mutant frequency was evaluated for each DNA sample in a blind study using a block analysis of vehicle- and AFB_1-treated individuals. Mutant frequencies were measured according to the standardized Big Blue color-screening assay protocol (Stratagene, La Jolla, 19,20). Briefly, shuttle vector was recovered from the genomic DNA using Transpack® *in vitro* packaging extract. In each packaging reaction, 8 μl of genomic DNA was combined with 10 μl of Transpack and incubated at 30°C for 3 h, with the addition of another 10 μl of Transpack midway through the reaction period. Approximately 1 ml of SM medium was added to each packaging reaction. If multiple packaging reactions were performed for a particular DNA sample, each sample's reactions were pooled together. After addition of chloroform (50 μl per ml) to each packaged phage solution, the phage suspensions were gently vortexed and stored at 4°C. The initial titer of each phage solution was estimated by infecting 200 μl of *Escherichia coli* SCS-8 host bacteria [*recA1, endA1, mcrA,* Δ(*mcrBC-hsdRMS-mrr*), Δ(*argF-lac*)*U169,* ϕ80*dlacZ*Δ*M15,* Tn*10*(tet^r)] at 0.5 OD_{600nm} with 1 μl of packaged phage and plating on 100 mm NZY agar plates, in duplicate. The initial titers were used to ensure that phage were plated onto 25 cm^2 Big Blue assay trays at plating densities of ~15 000–20 000 pfu (plaque forming units) per tray. For mutant screening, packaged phage were first adsorbed to *E.coli* SCS-8 host cells at 0.5 OD_{600nm} for 15 min at 37°C. After removing a small aliquot for dilution titering (see below), the host-adsorbed phage were plated onto NZY assay trays using 35 ml top agarose containing 1.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After an appropriate venting period required to remove excess moisture introduced by the top agarose, all assay and titer trays were incubated at 37°C for 16 h. The total number of pfu plated was determined by counting two dilution titer trays per sample. Mutations in the *lacI* gene were scored over white fluorescent light boxes using a red Big Blue plaque reading enhancer screen. Mutant frequency was determined as the ratio of the number of blue plaques to the total number of pfu plated. Statistical tests of differences in mutant frequency between animal groups were performed with a two-tailed Student's *t*-test using the analysis tools in Microsoft® Excel software.

Recovery and analysis of lacI mutants

Mutant plaques were recovered according to the standardized Big Blue color-screening assay protocol (19,20), and were purified and sequenced as described by de Boer *et al.* (21). Briefly, mutant plaques were confirmed and purified by infecting *E.coli* SCS-8 cells with the mutant phage and replating on medium containing X-gal. The *lacI* gene and flanking regions were amplified by PCR (polymerase chain reaction), and the products were purified on Wizard™ PCR Prep DNA purification columns (Promega, Madison, WI). Thermal cycle sequencing of the *lacI* gene was performed using A.L.F. automated DNA sequencers (Pharmacia, Piscataway, NJ). DNA sequence data were managed and analyzed using custom software (22) To ensure that independent mutational events were scored, the data were corrected for possible clonal expansions (21, de Boer *et al.*, in preparation) by counting only one mutation for those which were recovered more than once from a single animal. Statistical tests of differences between mutational spectra were done using the Monte Carlo method (with 2500 iterations) of Adams and Skopek (23), using a computer program provided by the authors.

Results

Four groups of six animals each were subjected to a single i.p. injection of either AFB_1 at 2.5 mg/kg or DMSO vehicle. While all six of the AFB_1-treated mice survived through day 14, five out of the six AFB_1-treated rats died by the second day. (The liver of the surviving rat was harvested on the 14th day, post-injection, and stored at –80°C but not subjected to either mutant frequency or spectral analysis.) Approximately 1 month later, another group of six rats was dosed with AFB_1 at 0.25 mg/kg, resulting in 100% survival through day 14. The vehicle-treated rats from the first set of injections were used as the control group for rats in this study. Consequently, the vehicle-treated rats were ~6 weeks older than their AFB_1-treated counterparts.

The mutant frequency data collected using a block analysis treatment of vehicle- and AFB_1-treated samples are summarized in Table I. Transgenic C57BL/6 mice subjected to a single dose of AFB_1 at 2.5 mg/kg showed no induction in mutant frequency over the vehicle-treated mouse group ($P = 0.25$). Transgenic F344 rats subjected to one-tenth the mouse dosage, however, were highly susceptible to the mutagenic effects of AFB_1. At a mean mutant frequency of 49×10^{-5} for the AFB_1-treated group, induction in rat liver was nearly 20-fold over background ($P = 0.001$).

Table II summarizes the *lacI* sequence data for liver mutations induced by AFB_1 in lambda/*lacI* mice and rats (a list of all sequenced mutants is included in the Appendix). The vehicle-treated rat liver *lacI* data from this study are also presented, as well as historical data from untreated C57BL/6 mouse liver (*lacI* database; 22). All mutation data were corrected for possible clonal expansions (21, de Boer *et al.*, in preparation), resulting in the elimination of seven out of 84 mutants from the historical dataset for untreated mice, five out of 47 mutants from AFB_1-treated mice, five out of 49 mutants from vehicle-treated control rats, and three out of 48 mutants from AFB_1-treated rats.

In 45 (corrected) mutants from AFB_1-treated rats, 78% of the mutations were G:C→T:A transversions [71% of these transversions occurring at CpG sites (5′-CpG-3′ dinucleotide sequences)], followed by 11% G:C→C:G transversions (80% at CpG sites) and only 4% G:C→A:T transitions (50% at CpG sites). In 44 (corrected) mutants from vehicle-treated rat liver, we observed 27% transversion (11% as G:C→T:A) and 48% G:C→A:T transition mutations (76% at CpG sites). In the mouse, sequence analysis of 42 (corrected) mutants from AFB_1-treated animals revealed 19% G:C→T:A transversions (38% at CpG sites) and 57% G:C→A:T transitions (71% at CpG sites). This spectrum is similar to the C57BL/6 spontaneous liver mutational spectrum.

Discussion

The transgenic A1 mouse and QX rat lineages used in this study are currently commercially available as the Big Blue mouse and rat, respectively. They each harbor the lambda-based shuttle vector transgene (λLIZ), which employs the *lacI* gene from *E.coli* as a mutational target (1–5). The copy number of the lambda/*lacI* shuttle vector in the hemizygous Big Blue mouse is ~40 per cell, while that of the hemizygous Big Blue rat is approximately 15–20 per cell. Although homozygous versions of Big Blue animals are available, all animals used in this study were hemizygous.

The existence of a multi-species mutation assay using transgenic rodents that share the same target gene/shuttle vector should afford the opportunity to perform tissue-specific, inter-species mutagenesis testing in mammals. The study described here was designed to test the ability of a transgenic-based assay to show an *in vivo* species-specific differential response to mutagen exposure.

AFB_1 was chosen for this study because its biological effects are relatively well-characterized (24). The observation that AFB_1 was highly mutagenic in the livers of transgenic F344

Table I. *lacI* mutation data from the livers of male lambda/*lacI* mice and rats exposed to AFB_1

Species	Age at tissue harvest (weeks)	Treatment[a]	Expression period[b]	Animal ID	Mutant pfu[c]	Total pfu[d]	Mutant frequency[e]	Mean ± SD[f]
C57 mouse	13	vehicle-treated	14	26	8	311 376	2.6	3.1 ± 0.88 (28%)
				27	7	323 722	2.2	
				28	21	668 417	3.1	
				29	16	340 720	4.7	
				30	8	279 856	2.9	
				31	14	407 728	3.4	
C57 mouse	13	2.5 mg/kg AFB_1	14	32	11	331 038	3.3	4.1 ± 1.6 (39%)
				33	21	342 696	6.1	
				34	10	330 634	3.0	
				35	9	326 598	2.8	
				36	30	495 288	6.1	
				37	10	331 049	3.0	
F344 rat	19	vehicle-treated	14	76	7	272 840	2.6	2.7 ± 0.69 (26%)
				77	12	401 720	3.0	
				78	11	372 020	3.0	
				79	5	326 918	1.5	
				80	11	304 980	3.6	
				81	8	313 901	2.5	
F344 rat	13	0.25 mg/kg AFB_1	14	166	100	283 506	35.3	49 ± 17 (35%)
				167	195	304 594	64.0	
				168	234	363 240	64.4	
				169	144	296 272	48.6	
				170	201	356 520	56.4	
				171	72	317 232	22.7	

[a]Single dose, i.p. injection, DMSO solvent, 1 ml/kg dosing volume.
[b]Number of days between injection and euthanasia/tissue collection.
[c]Number of blue pfu.
[d]Total number of pfu screened.
[e]Mutant frequency is expressed as the number of blue (*lacI* mutated) plaques per 100 000 pfu screened.
[f]Numbers in parentheses are standard deviations expressed as a percentage of the mean.

Table II. Spectra of spontaneous and AFB_1-induced mutations in the *lacI* gene in male C57BL/6 mouse and F344 rat liver[a]

	C57BL/6 Mouse Spontaneous[b]			C57BL/6 Mouse 2.5 mg/kg AFB_1			F344 Rat vehicle-treated			F344 Rat 0.25 mg/kg AFB_1		
	No.	%	% @ CpG	No.	%	% @ CpG	No.	%	% @ CpG	No.	%	% @ CpG
Transitions												
G:C→A:T	37	48.0	75.7	24	57.1	70.8	21	47.7	76	2	4.4	50
A:T→G:C	3	3.9		0	0		1	2.3		0	0	
Transversions												
G:C→T:A	17	22.1	23.5	8	19.0	37.5	5	11.4	100	35	77.8	71
G:C→C:G	3	3.9	33.3	1	2.4	100	4	9.1	50	5	11.1	80
A:T→T:A	2	2.6		1	2.4		2	4.5		1	2.2	
A:T→C:G	4	5.2		1	2.4		1	2.3		0	0	
Other mutations												
+1 Frameshifts	3	3.9		0	0		0	0		0	0	
−1 Frameshifts	4	5.2		1	2.4		2	4.5		0	0	
Deletions	1	1.3		2	4.8		3	6.8		0	0	
Insertions	0	0		1	2.4		2	4.5		0	0	
Complex changes	1	1.3		1	2.4		3	6.8		1	2.2	
Double mutants	2	2.6		2	4.8		0	0		1	2.2	
Total others	11	14.3		7	16.7		10	22.8		2	4.4	
Totals	77	100		42	100.1[c]		44	99.9		45	99.9	

[a]These data were automatically corrected for clonal expansions, using the *lacI* database software (22).
[b]The C57BL/6 mouse spontaneous (untreated) liver mutational spectrum is virtually indistinguishable ($P = 0.72 \pm 0.02$) from that observed in a larger dataset (205 mutants) of $B_6C_3F_1$ mouse spontaneous liver mutants (*lacI* database; 22). There were no statistically significant differences between the mutational spectra from untreated and AFB_1-treated C57BL/6 mice ($P = 0.74 \pm 0.02$), or between untreated C57BL/6 mice and vehicle-treated F344 rats ($P = 0.14 \pm 0.01$). The vehicle-treated and AFB_1-treated F344 rat mutational spectra were significantly different ($P < 0.001$).
[c]Totals may not be 100%, due to rounding errors.

rats and not in those of transgenic C57BL/6 mice is consistent with what is presently known about the metabolism of the toxin in rodents. AFB_1 is converted by cytochrome P450 pathways into the highly reactive AFB_1–8,9-epoxide, which reacts with DNA mainly forming an adduct at the N7-position of guanine (9,12,25–27). A major pathway for the detoxification of the epoxide is through conjugation with glutathione (18,28–30). The balance between activation and detoxification is believed to influence the reaction of AFB_1 with genomic DNA in different species (31). The fact that rats are more efficient than mice in bioactivation of AFB_1 into the mutagenic AFB_1–8,9-epoxide, and less efficient in the conjugation of this active form is reflected by the high sensitivity of rats to the mutagenic effects of AFB_1 while mice that were given ten times the rat dose showed no increase in mutant frequency in this study.

To a lesser extent than species-specific factors, the effects of sex and strain are likely to have had an influence on the results of this study. Sex- and strain-specific differences in carcinogenicity induced by AFB_1 have previously been reported (32). AFB_1-induced TD_{50} values for rat strains range from 1.3 μg/kg per day for male Fischer rats to 12.5 μg/kg per day for female Porton rats (TD_{50} defined as the dose that produces a 50% incidence in tumors). Those for mouse strains range from >70 μg/kg per day for male C3H and C57BL mice to >5300 μg/kg per day for male Swiss mice. This study was performed on male F344 rats and C57BL/6 mice, which are among the more AFB_1-sensitive inbred representatives reported for each species.

Another factor that could conceivably contribute to the differential response between AFB_1-treated rats and mice observed in this study is the effect of transgene integration site. It is possible that the QX rat lineage harbors its 15–20 copies of *lacI* in a region of the genome more accessible to mutagen exposure. Previous studies measuring spontaneous mutant frequencies among different transgenic mouse and rat lineages have supported the assumption that transgene insertion site in the QX rat and A1 mouse lineages has little effect on the outcome of the mutagenicity assay (5). Nevertheless, there is evidence that factors such as DNA conformation and chromatin structure can influence intragenomic localization of carcinogen-DNA binding and repair (32). Thus any mutagenicity assay utilizing a transgenic target is potentially influenced by the integration site of a particular transgenic lineage. A study wherein the response to a known mouse-specific mutagen is measured in both the A1 and QX lineages may help to further address this issue with respect to the lambda/*lacI* rodent assay that was used in our experiments.

It is unlikely that the 6-weeks difference in age between the AFB_1-treated rats and the older vehicle-treated control rats in this study had a significant effect on the increase in mutant frequency measured in the livers of AFB_1-exposed rats. Previous work in lambda/*lacI* mice has shown that spontaneous mutant frequency increases approximately four-fold during the first 24 months of life (33) suggesting that age might have a similar effect on mutant frequency in the rat. Regardless, 6 weeks is a relatively small percentage of the average rat lifespan of 2.5–3 years (34) and is probably not large enough to result in a significant increase in mutant frequency in the control rats relative to the nearly 20-fold induction by AFB_1. Age-related sensitivity to AFB_1 is also not suspected to be a significant factor in this study. Sensitivity to AFB_1 toxicity in F344 rats increases during the first weeks of post-natal development and reaches a plateau at ~65 days of age (35). The AFB_1-treated and vehicle-treated rats used in this study were ~80 and 117 days old, respectively, at the time of i.p. injection.

The spectrum of mutations in the *lacI* gene in the liver of AFB_1-treated lambda/*lacI* mice and rats differed considerably (Table II), probably due to species differences in the metabolic activation and detoxification of AFB_1 (17). In the rat, the mutational spectrum induced by AFB_1 was clearly different ($P < 0.001$) from that observed for vehicle-treated controls, where a large proportion of the mutations are G:C→A:T transitions at CpG sites (this study; de Boer *et al.*, in preparation). After treatment of rats with AFB_1, a dramatic increase was seen in the proportion of G:C→T:A transversions that then accounts for 78% of all mutations compared to only 11% in the vehicle-treated spectrum. This was accompanied by a large decrease in the proportion of G:C→A:T transitions, from 48% to only 4%.

The recovery of a high proportion of AFB_1-induced mutations as G:C→T:A transversions in our study is largely in accordance with the reported mutational specificity of AFB_1 in other systems. For example, in the endogenous *lacI* gene in *E.coli*, metabolically activated AFB_1 predominantly (93%) induced the formation of G:C→T:A transversion mutations (36). In addition, a study of the mutational properties of the primary AFB_1-N7-DNA adduct inserted into bacteriophage M13 DNA by site-directed mutagenesis and replicated in *E.coli*, found that 75% of all mutations were G→T transversions (27), similar to our determination of 78% G:C→T:A transversions in AFB_1-treated rat liver. However, it should be noted that several other studies have found somewhat lower contributions of G:C→T:A transversion mutations, with a higher fraction of other base substitution mutations. For example, in a study in which shuttle vector DNA was treated *in vitro* with AFB_1 and replicated in human cells, 90% of the recovered mutations were base substitutions, but only about one-half of these were G:C→T:A transversions (37). Transfection of *in vitro*-modified phage M13 DNA into DNA repair-deficient *E.coli* cells resulted in approximately equal numbers of G→T transversion and G→A transition mutations (38,39).

The variation observed in AFB_1-induced mutational spectra in various studies likely reflects unique characteristics of the test systems, including whether the DNA was reacted with activated AFB_1 *in vitro* or *in vivo*. Conceivably, differences in mutational spectra might reflect differences in the relative formation and repair of the three principal AFB_1-DNA adducts: AFB_1-N7-guanine, the AP (apurinic) site formed by depurination of this principal N7-adduct, and the AFB_1-FAPY (AFB_1-formamidopyrimidine) adduct formed by scission of the imidazole ring of AFB_1-N7-guanine (27,40). Bailey *et al.* (27) have shown that the AFB_1-N7-guanine adduct and not the AP site best explains mutations in AFB_1-treated cells. However, in studies where formation of the AP adduct is enhanced, changes in the AFB_1 mutational spectrum might be expected depending on repair of the AP site or the preference for nucleotide insertion opposite unrepaired AP sites during DNA replication (41,42). It should be noted that the AFB_1-FAPY adduct is believed to block DNA replication, and is thus primarily a lethal lesion unless repaired (40).

In our study, AFB_1-induced G→T transversion events (i.e. G:C→T:A) in rat liver occurred at guanine residues that were flanked 97% of the time (34 of 35 events) on the 5′-side by a guanine or a cytosine, and 57% of the time (20 of 35 events) on the 3′-side by a cytosine (Table III). Forty percent (14 of

Table III. Sequence analysis of G:C→T:A transversion mutations observed in the *lacI* gene from liver tissue of AFB1-treated male lambda/*lacI* transgenic mice and rats (corrected for clonal expansions)

Species	5′-Sequence	Mutated nucleotide[a]	3′-Sequence	Mutation position[b]	Strand	CpG site[c]	Occurrences
C57BL/6	CACCG	G	CATAC	66	non-coding	−	1
(Mouse)	TGGCG	G	AGCTG	158	coding	−	1
	ACGCG	G	TTGGG	178	non-coding	−	1
	TGAAT	G	AGGGC	731	coding	−	1
	GGCGC	G	CATTG	791	non-coding	+	3
	CATGA	G	CTGTC	865	non-coding	−	1
F344	CACCG	G	CATAC	66	non-coding	−	1
(Rat)	TGCCG	G	TGTCT	69	coding	−	1
	GATAA	G	AGACA	75	non-coding	−	1
	CACGC	G	GGAAA	92	non-coding	+*	2
	TTCCC	G	CGTGG	93	coding	+*	3
	GCCAC	G	TTTCT	116	coding	+	1
	CGCGG	G	AAAAA	134	coding	−	1
	TGGCG	G	AGCTG	158	coding	−	1
	CACGC	G	GTTGG	179	non-coding	+*	1
	TGCCC	G	CCAGT	198	non-coding	+*	2
	GATTG	G	CGTTG	222	coding	−	1
	TCGCC	G	CGACA	270	non-coding	+*	1
	TAATC	G	CCGCG	273	non-coding	+	1
	CGCTG	G	CACCC	303	non-coding	−	1
	GCACC	G	CCGCT	357	non-coding	+*	2
	GACGC	G	TTGCG	380	non-coding	+	1
	ACAGC	G	CGATT	576	non-coding	+	1
	GCGCC	G	AGACA	606	non-coding	+	3
	ACATG	G	CACTC	693	non-coding	−	2
	GCATC	G	CAGTG	750	non-coding	+	1
	TTACC	G	AGTCC	803	coding	+	1
	TCGTC	G	TATCC	847	non-coding	+	1
	ATACC	G	AAGAC	857	coding	+	1
	AGCAG	G	CGAAA	910	non-coding	−	1
	TCCAC	G	CTGGT	928	non-coding	+	3

[a]Sequenced C→A transversion mutations were interpreted as originating from G→T mutations in the non-coding strand, and are presented as such in the table.

[b]Nucleotide positions are numbered as described by Farabaugh (51).

[c]A (+) sign indicates the mutation occurred at a CpG site; a (−) sign indicates that the mutation did not occur at a CpG site. An asterisk (*) indicates that a G:C→T:A transversion occurred at a CpG site within the sequence 5′-GCGG-3′ or its complement.

35) of these mutations occurred at the sequence 5′-C<u>G</u>C-3′ (underlining denotes the mutated nucleotide throughout this discussion). Noting that it is difficult to identify simple rules that predict AFB_1 reactivity toward a particular guanine (43), our data agree reasonably well with the specificity of AFB_1 adduct formation in DNA described by Benasutti *et al.* (44), who determined that guanines and cytosines on the 5′-side and guanines and thymines on the 3′-side are the most influential in determining guanine reactivity with AFB_1. The differences between their data and our data might reflect the *in vitro* versus the *in vivo* nature of the experiments, respectively. Our data also agree well with that of Misra *et al.* (45), who found that guanines flanked by A:T sequences were poor targets for AFB_1-induced lesions.

Seventy-one percent (25 of 35) of the G:C→T:A transversion mutations recovered from AFB_1-treated rats occurred at CpG sites. This is substantially higher than the ~30% that is expected statistically (21). Inspection of the flanking DNA sequences revealed that 44% of G→T transversions at CpG sites (11 of 25) occurred at the sequences 5′-GC<u>G</u>G-3′ or 5′-CC<u>G</u>C-3′ (Table III).

Since 5′-GCGG-3′ and 5′-CCGC-3′ are complementary, it appeared that CpG sites flanked by G:C basepairs (specifically, 5′-GCGG-3′/5′-CCGC-3′ sequences) might be hotspots for AFB_1-induced mutation. While there are 95 CpG sites per strand in *lacI*, only 39 are known to generate a mutant phenotype when mutated by a G:C→T:A transversion (*lacI* database, 22). Of these 39 CpG sites, five occur at 5′-GCGG-3′/5′-CCGC-3′ sequences and 34 occur at other CpG sites. If the 25 recovered G:C→T:A mutations at CpG sites (from AFB_1-treated rats) were distributed randomly among the 39 recoverable mutable sites, we would expect to recover 3.2 mutations at 5′-GCGG-3′/5′-CCGC-3′ sequences and 21.8 mutations at other CpG sites. However, we observed 11 such mutations at 5′-GCGG-3′/5′-CCGC-3′ sequences, and 14 mutations at other CpG sites. Therefore, AFB_1-induced G→T mutations at 5′-GC<u>G</u>G-3′ or 5′-CC<u>G</u>C-3′ occurred significantly more frequently ($P < 0.0076$, Fisher exact test) than at other CpG sites, suggesting that these sequences are indeed hotspots for mutation induced by AFB_1. It may be noted that 100% of the recoverable G→T mutations at 5′-GC<u>G</u>G-3′ or 5′-CC<u>G</u>C-3′ were observed (with 11/5 or 2.2-fold 'saturation'), whereas only 41% (14 of 34) of recoverable G→T mutations at other CpG sites were observed.

The five 5′-GCGG-3′/5′-CCGC-3′ regions in *lacI* containing CpG sites at which G→T transversions are recoverable were also examined to determine if a strand bias existed for such mutations induced by AFB_1 in rat liver. Eight of the 11 recovered G→T transversions at 5′-GC<u>G</u>G-3′ or 5′-CC<u>G</u>C-3′ occurred in the non-coding strand (Table III). However, such

transversions are known to generate a mutant phenotype at all of the five above mentioned regions when occurring in the non-coding strand but only at two of these five regions when occurring in the coding strand. The ratio of observed coding:non-coding strand mutations (3:8, Table III) is very close to the ratio of recoverable coding:non-coding strand mutations (2:5), suggesting that there is no strand bias for AFB_1-induced G→T mutation at 5′-GCGG-3′/5′-CCGC-3′ sequences in the *lacI* gene.

Activated AFB_1 is known to react almost exclusively with guanines, at the N7-position (25). It has previously been suggested that AFB_1 may preferentially induce mutations at certain sites, including 5′-GpG-3′, 5′-CpC-3′ and 5′-CpG-3′ sequences (45). In a bacterial forward mutagenesis assay using a *lacZ* segment in M13, the sequence 5′-TGGCG-3′ was found to be an AFB_1 hotspot, with the underlined guanine having the most mutations (38). Other AFB_1-induced hotspots have been reported. For example, in the human *hprt* gene, activated AFB_1 produced a G:C→T:A transversion at basepair 209 in exon 3 in 10–17% of all mutants (46). This hotspot occurs in a GGGGGG sequence. These observations, and our data (5′-GCGG-3′/5′-CCGC-3′ hotspot), correlate reasonably well with data from a study that indicated that 5′-(G/C)G(G/T)-3′ sequences in DNA react preferentially with activated AFB_1 (44).

The induction of G:C→T:A transversions by AFB_1, particularly at 5′-GpG-3′ dinucleotide sequences, is of particular relevance in regard to activation of oncogenes. In AFB_1-induced hepatocarcinomas in rats and trout, mutations at codons 12 and 13 of the *ras* gene, a 5′-GpG-3′ target, were primarily (83%) G:C→T:A transversions (47,48). In addition, G:C→T:A transversions are frequently found (77%) at 5′-AGG-3′ in codon 249 of the *p53* gene in human hepatocellular carcinomas from humans exposed to dietary aflatoxins (49,50).

In contrast to the dramatic changes in the pattern of mutation observed in the rat, the liver mutational spectrum of AFB_1-treated mice was very similar to the pattern of spontaneous mutations, except that G:C→T:A transversions occurred at CpG sites almost twice as frequently in AFB_1-treated mice as in untreated mice (Table II). In addition, there was a modest increase in the proportion of G:C→A:T transitions (from 48 to 57%) in treated mice. It was determined that there was no significant difference ($P = 0.74 \pm 0.02$) in the mutational spectra between AFB_1-treated and untreated mice.

An intriguing observation in the mouse AFB_1 data was the recovery of a G:C→T:A transversion at position 791, which was found once in each of three animals. Mutation at this position has previously been observed only twice among more than 5800 sequenced *lacI* mutations (unpublished: *lacI* database, de Boer *et al.*, 1995). The rarity of the mutation, and the sequence at which this mutation occurs (5′-TGCGC-3′) tends to support the idea that this mutation was induced by AFB_1. Position 791 may thus be an AFB_1 hotspot in the mouse liver environment, although this event was not recovered in the rat collection.

In this study, the lambda/*lacI* transgenic mutagenesis assay was used to demonstrate a species-specific *in vivo* response to the mycotoxin AFB_1. The results described here are in general concordance with what is known about AFB_1 and its mechanism of action on rodent liver DNA *in vivo*. The sensitivity of the F344 rat to the mutagenic effects of AFB_1 in the liver was evidenced by an increase in *lacI* mutant frequency and a shift in mutational spectrum to predominantly G:C→T:A transversions. The C57BL/6 mouse was relatively resistant to AFB_1-induced liver mutations. This work illustrates the utility of having the same shuttle vector system in both mice and rats for comparative mutation testing. The lambda/*lacI* assay confers the potential to measure species-specific differences in mutant frequency and mutational spectra in essentially any tissue. Moreover, the lambda/*lacI* transgenic assay uses the same model species that have been established for the 2-year rodent bioassay. Thus, carcinogenicity studies can be augmented by transgenic mutational studies, providing detailed sequence-level analysis of *in vivo* mutational events. The information that is now achievable from the two-species lambda/*lacI* transgenic assay may have beneficial applications in many fields, including mechanistic analysis and risk assessment.

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Appendix. List of mutants

Table AI. AFB_1-treated mouse liver mutants, uncorrected

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31C361	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	35	
31A014	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	36	
31E008	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	34	
31F001	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	34	clonal
31A006	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	36	
31C320	substitution	no	C 66 A	none	Ala 13 Asp	AFB1	35	
31G011	substitution	yes	C 92 T	none	Arg 22 Cys	AFB1	32	
31G006	substitution	yes	G 93 A	none	Arg 22 His	AFB1	32	
31G009	substitution	yes	G 93 A	none	Arg 22 His	AFB1	32	clonal

Table AI. *Continued*

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31F003	substitution	yes	G 93 A	none	Arg 22 His	AFB1	34	
31C270	substitution	yes	G 95 A	none	Val 23 Met	AFB1	33	
31A013	substitution	no	G 158 T	none	Glu 44 amber	AFB1	36	
31G019	substitution	no	C 178 A	none	Asn 50 Lys	AFB1	37	
31A009	substitution	yes	C 179 T	none	Arg 51 Cys	AFB1	36	
31C268	substitution	yes	G 180 A	none	Arg 51 His	AFB1	33	
31C269	substitution	yes	G 180 A	none	Arg 51 His	AFB1	33	clonal
31E001	multiple	no	G 184 T	C 186 T	Val 52 Val	AFB1	34	also Ala(53) → Val
31C264	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	33	
31C267	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	33	clonal
31E003	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	34	
31G005	substitution	no	C 206 T	none	Gln 60 amber	AFB1	32	
31E002	substitution	no	G 222 A	none	Gly 65 Asp	AFB1	34	
31C323	substitution	no	G 222 A	none	Gly 65 Asp	AFB1	35	
31G015	insertion	no	620	none		AFB1	37	+ TGGC @ 620/631
31G016	complex	no	T 246 A	none		AFB1	37	also - 9bp @ 234/242
31C326	substitution	no	C 260 T	none	Gln 78 ochre	AFB1	35	
31C266	substitution	yes	G 269 A	none	Ala 81 Thr	AFB1	33	
31G017	substitution	yes	G 269 A	none	Ala 81 Thr	AFB1	37	
31F002	substitution	yes	C 270 T	none	Ala 81 Val	AFB1	34	
31G002	substitution	yes	C 329 T	none	Arg 101 opal	AFB1	32	
31G020	substitution	yes	C 329 T	none	Arg 101 opal	AFB1	36	
31A008	substitution	no	T 369 G	none	Leu 114 Arg	AFB1	36	
31D005	-1 frameshift	no	429	none		AFB1	35	- T @ 429/430
31G014	substitution	no	C 513 T	none	Ser 162 Phe	AFB1	37	
31C325	deletion	no	637	none		AFB1	35	deletion @ 637/638
31G007	multiple	no	A 689 T	G 690 A		AFB1	32	AGT(Ser) → TAT(Tyr)
31A011	substitution	no	C 719 T	none	Gln 231 ochre	AFB1	36	
31G001	substitution	no	G 731 T	none	Glu 235 amber	AFB1	32	
31G004	substitution	no	G 731 T	none	Glu 235 amber	AFB1	32	clonal
31H001	deletion	no	777	none		AFB1	37	8bp deletion @ 777–784
31G012	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	32	
31C265	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	33	
31C360	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	35	
31C324	substitution	yes	G 794 C	none	Ala 256 Pro	AFB1	35	
31G010	substitution	no	C 865 A	none	Ser 279 Arg	AFB1	32	
31A012	substitution	no	C 944 T	none	Gln 306 ochre	AFB1	36	
31A010	substitution	no	A1004 T	none	Arg 326 opal	AFB1	36	

Table AII. Vehicle-treated control rat liver mutants, uncorrected

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31J002	complex	no	none	none		none	77	T(704)→ A; C(762)→ A
31C365	complex	no	none	none		none	79	AC(1010/1011) → G
31E051	complex	no	none	none		none	81	30–43 replaced with GT
31E050	substitution	yes	G 29 T	none	Val 30 Phe	none	81	
31G134	-1 frameshift	no	61	none		none	80	- G @ 61
31B001	substitution	no	T 64 G	none	Tyr 12 amber	none	78	
31E164	deletion	no	68	none		none	77	GGTGCTCTTA del @ 68/78
31E006	substitution	no	A 81 T	none	Gln 18 Leu	none	77	
31B003	substitution	yes	C 92 T	none	Arg 22 Cys	none	78	
31G130	substitution	no	C 104 T	none	Gln 26 amber	none	80	
31E043	substitution	no	G 140 A	none	Val 38 Met	none	81	
31B007	substitution	no	C 174 G	none	Pro 49 Arg	none	78	
31B004	substitution	yes	G 180 A	none	Arg 51 His	none	78	
31C362	substitution	no	G 185 A	none	Ala 53 Thr	none	79	
31E166	substitution	no	T 195 C	none	Leu 56 Pro	none	77	
31B005	substitution	yes	C 198 A	none	Ala 57 Glu	none	78	
31B006	substitution	yes	C 198 A	none	Ala 57 Glu	none	78	clonal
31H008	substitution	yes	C 198 T	none	Ala 57 Val	none	76	
31A004	substitution	yes	C 198 T	none	Ala 57 Val	none	78	
31D002	substitution	yes	C 198 T	none	Ala 57 Val	none	79	
31E049	substitution	yes	C 198 T	none	Ala 57 Val	none	81	

Table AII. *Continued*

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31B008	substitution	no	T 209 A	none	Ser 61 Thr	none	78	
31E160	substitution	yes	G 269 A	none	Ala 81 Thr	none	77	
31E044	substitution	yes	G 269 A	none	Ala 81 Thr	none	81	
31E165	substitution	yes	C 329 T	none	Arg 101 opal	none	77	
31A001	substitution	yes	C 329 T	none	Arg 101 opal	none	78	
31G131	substitution	yes	C 329 T	none	Arg 101 opal	none	80	
31G137	substitution	yes	C 329 T	none	Arg 101 opal	none	80	clonal
31G138	substitution	yes	C 329 T	none	Arg 101 opal	none	80	clonal
31H006	substitution	yes	C 380 A	none	Arg 118 Ser	none	76	
31A003	substitution	yes	G 381 A	none	Arg 118 His	none	78	
31G129	substitution	yes	G 381 A	none	Arg 118 His	none	80	
31E046	substitution	yes	G 381 A	none	Arg 118 His	none	81	
31E162	substitution	yes	G 383 T	none	Val 119 Phe	none	77	
31G141	substitution	yes	C 530 T	none	Arg 168 opal	none	80	
31G142	substitution	yes	C 530 T	none	Arg 168 opal	none	80	clonal
31H007	substitution	yes	G 575 C	none	Ala 183 Pro	none	76	
31G139	substitution	yes	G 575 C	none	Ala 183 Pro	none	80	
31E045	deletion	no	620	none		none	81	- TGGC @ 620
31H005	insertion	no	620	none		none	76	+ TGGC @ 620/632
31E158	insertion	no	620	none		none	77	+ CTGG @ 620
31E163	insertion	no	620	none		none	77	+ TGGC @ 620; clonal
31J001	substitution	no	G 687 A	none	Trp 220 amber	none	77	
31G132	substitution	yes	G 803 A	none	Glu 259 Lys	none	80	
31E159	substitution	yes	C 834 A	none	Ser 269 amber	none	77	
31D001	substitution	no	C 953 T	none	Gln 309 amber	none	79	
31E007	substitution	no	G 962 C	none	Ala 312 Pro	none	77	
31A002	deletion	no	96	none		none	78	17 bp del. @ 996–1012
31C363	-1 frameshift	no	1010	none		none	79	- G @ 778

Table AIII. AFB_1-treated rat liver mutants, uncorrected

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31A056	substitution	no	G 31 C	none	Val 1 Val	AFB1	171	Initiation codon
31A041	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	171	
31H125	substitution	yes	G 56 C	none	Ala 10 Pro	AFB1	166	
31A040	substitution	no	T 64 A	none	Tyr 12 ochre	AFB1	171	
31H122	substitution	no	C 66 A	none	Ala 13 Asp	AFB1	166	
31C037	substitution	no	G 69 T	none	Gly 14 Val	AFB1	170	
31H123	substitution	no	C 75 A	none	Ser 16 Tyr	AFB1	166	
31E059	substitution	no	C 80 T	none	Gln 18 amber	AFB1	168	
31H130	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	166	
31C002	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	169	
31C003	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	169	clonal
31G021	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	167	
31E053	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	168	
31E054	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	168	clonal
31C010	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	169	
31A036	substitution	yes	G 116 T	none	Val 30 Phe	AFB1	171	
31C007	substitution	yes	G 132 C	none	Arg 35 Pro	AFB1	169	
31H127	substitution	no	G 134 T	none	Glu 36 ochre	AFB1	166	
31C038	substitution	no	G 158 T	none	Glu 44 amber	AFB1	170	
31G026	substitution	yes	C 179 A	none	Arg 51 Ser	AFB1	167	
31G028	substitution	yes	G 180 C	none	Arg 51 Pro	AFB1	167	
31C034	substitution	yes	C 198 A	none	Ala 57 Glu	AFB1	170	
31A038	substitution	yes	C 198 A	none	Ala 57 Glu	AFB1	171	
31C008	substitution	no	G 222 T	none	Gly 65 Val	AFB1	169	
31E060	multiple	no	C 270 A	C 273 A	Ala 81 Glu	AFB1	168	also Ala(82) → Glu
31C035	substitution	yes	C 270 A	none	Ala 81 Glu	AFB1	170	
31C040	substitution	yes	C 270 A	none	Ala 81 Glu	AFB1	170	clonal
31E052	substitution	yes	C 273 A	none	Ala 82 Glu	AFB1	168	
31H121	substitution	no	C 303 A	none	Ala 92 Asp	AFB1	166	
31G024	substitution	yes	C 357 A	none	Ala 110 Glu	AFB1	167	

Table AIII. *Continued*

Mutant ID no.	Mutation type	CpG site	Mutated base no. 1	Mutated base no. 2	Amino acid change	Mutagen	Animal ID no.	Other changes, comments
31E055	substitution	yes	C 357 A	none	Ala 110 Glu	AFB1	168	
31A039	substitution	yes	C 380 A	none	Arg 118 Ser	AFB1	171	
31A043	substitution	yes	C 576 A	none	Ala 183 Glu	AFB1	171	
31H124	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	166	
31G025	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	167	
31C001	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	169	
31G023	substitution	no	C 693 A	none	Ala 222 Asp	AFB1	167	
31A037	substitution	no	C 693 A	none	Ala 222 Asp	AFB1	171	
31G027	substitution	yes	C 750 A	none	Ala 241 Glu	AFB1	167	
31C041	complex	yes	C 750 A	none		AFB1	170	also + T @ 703/706
31H128	substitution	yes	G 785 C	none	Ala 253 Pro	AFB1	166	
31H126	substitution	yes	G 803 T	none	Glu 259 amber	AFB1	166	
31E057	substitution	yes	C 847 A	none	Tyr 273 ochre	AFB1	168	
31A042	substitution	yes	G 857 T	none	Glu 277 ochre	AFB1	171	
31C006	substitution	no	C 910 A	none	Arg 294 Arg	AFB1	169	
31H129	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	166	
31E056	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	168	
31C036	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	170	