In Vitro Mutational Spectrum of Aflatoxin B\(_1\) in the Human Hypoxanthine Guanine Phosphoribosyltransferase Gene\(^1\)

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**ABSTRACT**

The *in vitro* mutational spectrum of aflatoxin B\(_1\) (AFB\(_1\)) in exon 3 of the human hypoxanthine guanine phosphoribosyltransferase gene in B-lymphoblasts was examined by a combination of polymerase chain reaction and denaturing gradient gel electrophoresis. The cell line used in this study contained an expression vector that produced high levels of human cytochrome P450 CYP1A1. CYP1A1 metabolizes AFB\(_1\) to form an epoxide intermediate which can react with DNA.

About 1200 independent mutants were induced at the hypoxanthine guanine phosphoribosyltransferase locus by AFB\(_1\) and were selected en masse by addition of 6-thioguanine to the bulk culture. Two independent cultures were treated with AFB\(_1\). Polymerase chain reaction was used to amplify exon 3 from the complex mutant population, and denaturing gradient gel electrophoresis was used to separate wild-type DNA sequences from mutant sequences. Mutational hotspots were visible as discrete bands on the denaturing gradient gel. Scanning densitometry was used to determine the fraction of the complex population that was represented in each non-wild-type band. The bands containing the mutations were excised from the denaturing gradient gel and sequenced. In this way, the nature and frequency of mutational hotspots in a population of >1000 mutants were determined.

AFB\(_1\) produced one strong mutational hotspot in exon 3. Between 10 and 17% of the AFB\(_1\)-induced mutants contained a single GC→TA base substitution at base pair 209. This hotspot occurred in a GGGGGG sequence (the mutated base is underlined). This mutation was observed reproducibly in two independently treated cultures. Several other mutations were observed in only one culture but at a lower frequency. Our results are the first report of the mutational spectrum of AFB\(_1\) in a native human gene.

**INTRODUCTION**

AFB\(_1\)^1 is produced by *Aspergillus flavus*, a mold commonly found as a contaminant of food crops. Aflatoxin ingestion and hepatitis B virus infection have been identified as major risk factors for the development of human hepatocellular carcinoma in portions of China and Africa (1, 2). AFB\(_1\) is carcinogenic in many animal species and the mechanism of DNA mutation is fairly well understood (for review, see Ref. 3).

Aflatoxin appears to be capable of inducing specific mutations in exposed humans. Mutations of the p53 oncogene are frequently found in human hepatocellular carcinomas, and a specific GC→TA transversion at codon 249 was observed in a high fraction of tumors from regions of high aflatoxin ingestion (4-6). This particular p53 mutation is rarely found in hepatocellular carcinomas from people living in regions of low aflatoxin exposure. The observed p53 mutation is consistent with the observed nature of AFB\(_1\)-induced mutation *in vitro*.

The mutational spectra of AFB\(_1\) have been examined in bacteria (7, 8) and in shuttle vector systems in human cells (9, 10). In all studies, the great majority of single-base substitutions occurred at GC base pairs; however, the specific nature of the AFB\(_1\)-induced mutations varied among the studies. In general, AFB\(_1\) induced mainly GC→TA transversions; however, a substantial fraction of GC→AT and GC→CG substitutions were also produced. The fraction of GC→TA transversions varied from study to study.

In this paper, we present the *in vitro* mutational spectra of AFB\(_1\) in exon 3 of the endogenous human HPRT gene. The general method to analyze the mutational spectrum of a complex population of several thousand mutants was presented by Cariello et al. (11). Briefly, mutations were induced by treating a large population of human B-lymphoblastoid cells with AFB\(_1\). Mutants at the HPRT locus were selected by adding the purine analogue TG directly to the bulk culture. After the TG\(^2\) mutants induced by treatment overgrew the culture as an exponentially growing population, DNA was isolated and examined.

The pattern of mutations in exon 3 was examined by a combination of PCR and DGGE. PCR (12) was used to amplify exon 3 of the HPRT gene from the DNA of the complex mutant populations. DGGE separates DNA molecules on the basis of sequence (13, 14). When duplex DNA has migrated to a certain concentration of denaturant, the lowest temperature melting domain becomes single stranded, causing a severe reduction in electrophoretic mobility. The melting temperature of a domain is often altered by a single-nucleotide substitution, such that the transition to the partially melted state occurs at a different concentration of denaturant. In this way, mutant DNA sequences can be physically separated from wild-type DNA sequences. When DNA from the complex population of AFB\(_1\)-induced mutants was examined by DGGE, non-wild-type bands (mutant DNA sequences) were visible. The mutant DNA sequences were excised from the denaturing gradient gel and sequenced. Using PCR, DGGE, and direct DNA sequencing, we determined the mutational hotspots in the AFB\(_1\)-treated populations.

**MATERIALS AND METHODS**

**Mutagenesis.** The human B-lymphoblastoid cell line h1A2v2 was used (Gentest Corp., Woburn, MA). This cell line contains an expression vector that produces high levels of human cytochrome P450 CYP1A1. Cells were grown in a 225-ml volume in a 275-cm\(^2\) tissue culture flask in a 5% CO\(_2\) atmosphere using RPMI 1640 medium with 10% bovine calf serum. 1-Histidinol (2 mM) was added to ensure that the extrachromosomal plasmid containing the expression vector was maintained in the cells.

Cells were treated with hypoxanthine, aminopterin, and thymidine for 48 h according to the instructions of the supplier to reduce the spontaneous TG\(^\prime\) mutant fraction. Two independent cultures of 10\(^6\) cells at a density of 4.5×10\(^5\) cells/ml were treated for 25 h with 4 ng/ml AFB\(_1\) (Sigma, St. Louis, MO). One untreated culture of about 10\(^6\) cells was also established. After treatment, cells were centrifuged and resuspended in fresh media. Survival was determined by plating the cells in microtiter dishes at 2 cells/well.

The cultures were diluted daily (minimum of 10\(^5\) cells passed) for 7 days to allow for phenotypic expression of HPRT mutants. The mutant frequency in the untreated and the two treated cultures was determined by plating a small aliquot of the bulk culture in microtiter dishes in the presence and absence of...
TG as described by Furth et al. (15). TG (0.6 μg/ml) was then added to the bulk cultures.

After 15 days, viable cells in the bulk cultures could be seen under the microscope, but the Coulter counter cell count was not increasing. About 30% of each culture was centrifuged and resuspended in fresh media; these cells then began to grow as determined by Coulter counter cell count. Cells that were maintained in the original media never resumed normal growth. Resuspending the cells in fresh media was not necessary when using the TK6 lymphoblast cell line (11).

Seven days after the cells were resuspended in fresh media, the cells had a normal doubling time. At this point, the culture contained a complex population of TG' mutants. DNA was then isolated for analysis.

**PCR and Denaturing Gradient Gel Electrophoresis of HPRT Exon 3.** PCR and DGGE were performed as described by Cartello et al. (11) with modifications as noted below. Primers complementary to the intron sequence immediately flanking exon 3 were used to PCR amplify exon 3 from 750 ng of genomic DNA. PCR produced a 224-base pair fragment that contained 184 bases of coding sequence and 40 bases of intron sequence. PCR was performed with Vent DNA polymerase (New England Biolabs, Beverly, MA). Vent polymerase was used because it has a higher fidelity than Taq polymerase (16).

The PCR conditions were: 100 μl total volume, 500 nM primers, 1X Vent buffer, 200 μM deoxyribonucleoside triphosphates, 5 units Vent. Fifty cycles were performed using the following conditions: 1 min 94°C, 1 min 45°C, and 1 min 72°C.

To examine mutations in the 100-base pair, naturally occurring, low-temperature melting domain of exon 3 (Fig. 1), the 224-base pair PCR fragment was 5'-end labeled with [γ-32P]ATP (New England Nuclear, Boston, MA) and T4 kinase (BRL, Gaithersburg, MD) to a specific activity of about 5 × 10^6 cpm/pmol. The DNA was purified by PAGE, and the DNA was recovered by electroelution.

DNA (0.4 pmol, 20,000 cpm) was placed in 100 μl of 300 mM NaCl, denatured at 95°C for 3 min, and then placed at 65°C for about 16 h. This allows for formation of wild type:mutant heteroduplexes. The DNA was ethanol precipitated and washed before it was resuspended in loading buffer. Samples were loaded onto a 18–36% denaturing gradient gel and run for 16 h at 150 V. The gel was then fixed, dried, and exposed to X-ray film.

To examine mutations in the 80-base pair, naturally occurring, high-temperature melting domain of exon 3 (Fig. 1), it was necessary to alter the melting characteristics of the molecule by addition of a GC-rich section. This was accomplished by performing PCR with a primer that contained a 54-base pair GC-rich section (primers are described in Ref. 11). The GC-rich primer was 5'-end labeled with [γ-32P]ATP using T4 kinase.

About 10^5 copies of the PCR-amplified 224-base pair exon fragment were used as template for a second round of PCR with the GC-rich end-labeled primer. An additional 8 mM MgSO4 was added to the PCR buffer; this increased the efficiency of amplification when using the GC-rich primer. One cycle consisted of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C. The DNA was amplified about 100-fold with Vent polymerase, and the specific activity of the DNA produced was about 1 × 10^6 cpm/pmol.

The DNA was purified by PAGE, and the 179-base pair fragment containing the original, naturally occurring, high-temperature melting domain of exon 3 (Fig. 1) was recovered by electroelution. To form heteroduplex molecules, 1.5 pmol of DNA (15,000 cpm) was heat denatured in 100 μl of 50 mM NaCl and allowed to anneal in solution at 65°C for about 16 h. The DNA was ethanol precipitated, washed, resuspended in loading buffer, and loaded onto a 38–53% denaturing gradient gel. The gel was run for 16 h at 150 V, fixed, dried, and exposed to X-ray film.

**Sequencing the Mutant DNA from the AFB1-treated Populations.** Once the mutant bands were identified using autoradiography (Fig. 2), they were excised from the gel for sequencing. To facilitate excision of the mutant DNA bands, about 500 ng of the 179-base pair fragment (heteroduplex DNA from the complex populations) was loaded on a denaturing gradient gel using 1-cm-wide combs. After electroelution, this gel was not dried but, rather, stained with ethidium bromide and exposed briefly to UV light while the mutant bands were cut from the gel.

The gel slices containing the mutant bands were placed in 100 μl of water, crushed, and placed at 55°C for 8 h to allow the DNA to diffuse out of the gel slices. The solution (2 μl) was further PCR amplified with Taq polymerase and an end-labeled primer. The DNA was purified by PAGE and recovered by electroelution. Homoduplex DNA is present after amplification. Heteroduplex DNA was formed by denaturing 0.25 pmol of DNA at 95°C for 3 min in 50 mM NaCl and then placing the DNA at 65°C for 16 h. Both homoduplex and heteroduplex DNA were then loaded onto another denaturing gradient gel.

Mutant homoduplexes were recovered from the second denaturing gradient gel. DNA was recovered from the gel slice and was used to generate single-stranded DNA for sequencing using asymmetric PCR. Dideoxy sequencing was performed using fluorescent primers and an ABI 370 automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA).

**RESULTS**

**Toxicity and Mutation Frequency.** AFB1 was mutagenic at the HPRT locus in the human lymphoblastoid cell line expressing human cytochrome P450 CYP1A1. A 25-h exposure to 4 ng/ml AFB1 produced 20–30% survival and increased the mutant fraction 9- to 15-fold above background (Table 1). The TG' mutant fraction was 4.1 × 10^-6 for the untreated control and 62.3 and 38.8 × 10^-6 for the AFB1-treated cultures A and B, respectively. Thus, about 90% of the TG' mutants were induced by AFB1 and about 10% of the mutants were of spontaneous origin.

The PCR/DGGE method described in this paper is used to examine a population of mutants rather than to isolate and sequence individual clones. AFB1 mutagenesis was performed using a large number of cells (10^9) to induce about 1200 mutants in a treated culture. Two cultures were independently treated with AFB1, and an untreated culture served as the control. The data are summarized in Table 1.
Table 1 Survival, mutant fraction, and the number of mutants in untreated and aflatoxin B1-treated cultures

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DGGE Melting Characteristics of HPRT Exon 3. Exon 3 contains the largest contiguous section of the HPRT-coding frame and thus offers a sizable mutational target; 28% of the HPRT-coding region is contained in exon 3. The behavior of a DNA fragment on a denaturing gradient gel can be predicted from the base pair sequence (13, 14, 17). The calculated melting map of HPRT exon 3 is given in Fig. 1. Exon 3 has a naturally occurring, low-temperature melting domain of 104 base pairs (bases 215-318) and a high-temperature melting domain of 80 base pairs (bases 135-214). Since DGGE only detects mutations in the low-temperature melting domain(s) of a molecule, we could examine 104 base pairs of exon 3 using the natural DNA sequence.

It is possible to alter the melting characteristics of a DNA fragment by addition of a GC-rich sequence, termed a GC-clamp; the GC-clamp can be added by performing PCR with a GC-rich oligonucleotide (11, 18). Addition of a 54-base pair GC-clamp to exon 3 creates a new high-temperature melting domain (Fig. 1), and mutations from base pairs 135–214 can now be detected by DGGE. Thus, mutations at all base pair locations in exon 3 could be detected.

DGGE Analysis of HPRT Exon 3. DNA was amplified from the AFB1-treated, complex mutant populations using PCR, and the DNA was then denatured and reannealed in solution to form mismatches. Since we are examining only exon 3 which contains about 28% of the HPRT-coding region, most of the DNA in the complex mutant population will be wild type for exon 3. Thus, in solution hybridization, the wild-type DNA serves as the “driver” to ensure that wild type:mutant heteroduplexes form.

DGGE is extremely sensitive to mismatch formation, and all single-base mismatches in the low-temperature melting domain of a molecule can be detected (17). The sensitivity of the present PCR/DGGE approach is such that a particular mutant making up about 1% of the population can be detected (11). To achieve this level of sensitivity, it was necessary to amplify the DNA with Vent DNA polymerase, since the lower fidelity of Taq polymerase introduces a high level of undesirable polymerase-induced errors (16).

DGGE analysis of the naturally occurring low-temperature melting domain of exon 3 revealed no common mutant bands in the AFB1-treated cultures (data not shown). Thus, AFB1 does not appear to be producing mutational hotspots from bases 215–318.

However, multiple common bands appeared in both treated cultures when the high-temperature melting domain of exon 3 (bases 135–214) was examined by DGGE. Fig. 2 shows the control (untreated) TG' population, two AFB1-treated TG' populations, and wild-type DNA containing a 10% fraction of a known exon 3 mutant; Untreated Lane, untreated control culture; Lanes AFT A and AFT B, aflatoxin B1-treated cultures A and B, respectively.

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The novel bands in the AFB1-treated samples were excised from the denaturing gradient gel and reamplified by PCR. Since the band cut from the gel often contained a mutant DNA strand and a wild-type DNA strand, PCR amplification produced both a wild-type and mutant species. To separate the mutant from the wild-type species, the amplified DNA was loaded onto a second denaturing gradient gel as a homoduplex (perfectly base paired). Heteroduplex DNA was also loaded for reference. In all cases, the mutant homoduplexes resolved from the wild-type homoduplex, and the mutant homoduplexes were excised from the gel and sequenced.

It was not possible to sequence the bands designated as A1, A3, and A4 because the intensity of these bands proved to be too weak to yield an unambiguous interpretation. Sequence information was obtained from all other mutant bands shown with an arrow in Fig. 3.

The same mutation produced bands B1, B2, and B5. Band B1 contained the mutant:mutant homoduplex, while the mutant bands B2 and B5 contained the wild type:mutant and mutant:wild type heteroduplexes. The mutation that produced these bands was a GC→TA transversion at base 209. Taking into account all of the bands corresponding to this mutation, we calculated that this mutant made up about 17% of the TG' population. This mutation appeared to represent an aflatoxin-induced mutational hotspot. The information about specific mutations is summarized in Table 2.

<table>
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<th>Band in AFT culture A</th>
<th>Bands in AFT culture B</th>
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<tr>
<td>GC→TA, base pair 209</td>
<td>A1 too weak to sequence</td>
<td>B1 homoduplex</td>
</tr>
<tr>
<td></td>
<td>A2 heteroduplex</td>
<td>A5 heteroduplex</td>
</tr>
<tr>
<td></td>
<td>Mutation is 10% of population</td>
<td>Mutation is 17% of population</td>
</tr>
<tr>
<td>GC→TA, base pair 208</td>
<td>A2 heteroduplex</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>A5 heteroduplex</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>Mutation is 2% of population</td>
<td></td>
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<tr>
<td>-G, base pair 207–212</td>
<td>A6 heteroduplex</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>A7 heteroduplex</td>
<td>Mutation is 3% of population</td>
</tr>
<tr>
<td></td>
<td>A4 too weak to sequence</td>
<td>B4 heteroduplex</td>
</tr>
<tr>
<td></td>
<td>Mutation is 2% of population</td>
<td></td>
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<tr>
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<td>GC→TA, base pair 139</td>
<td>A4 too weak to sequence</td>
<td>B4 heteroduplex</td>
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<tr>
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<td>Mutation is 2% of population</td>
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<td>GC→TA, base pair 197</td>
<td>A3 too weak to sequence</td>
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The primary mutation induced by aflatoxin B1 in vitro in exon 3 of the native human hprt gene is a GC→TA transversion at base 209. This mutation was found in duplicate, independently treated cultures and represented between 10 and 17% of the mutants in each culture. Base 209 is found in a run of six consecutive guanines (GGGGGG, base 209 is underlined), which is the longest mononucleotide run in the HPRT-coding region.

The run of six guanines in human HPRT is a hotspot for other mutagens, including N-methyl-N-nitro-N-nitosoguanidine (11), ICR-191 (11), cisplatin (19), and benzo[a]pyrene (20, 21). These four mutagens, as well as AFB1, form premutagenic lesions at guanines. There is some evidence to show that the chemical reactivity of an internal guanine in guanine runs increases with the length of the mononucleotide run (22). There is also evidence to suggest that DNA lesions are repaired at a slower rate in the GGGGGG sequence than in the rest of the gene (20).

The fact that only GC→TA transversions are detected in the GGGGGG sequence (bases 207–212) is not an artifact of the HPRT selection system. We have recently constructed an HPRT database and software for the analysis of mutations at this locus (23, 24). Examination of the database reveals that all possible single-base substitutions (G→A, G→C, and G→T) can be detected at bases 208, 209, 211, and 212. Mutations at base 210 will not be observed, since single-base substitutions at this location will not change the amino acid. Likewise, a GC→AT transition at base 207 will not cause an amino acid substitution, and this mutation will not be observed in the HPRT system. Clearly, the mutational specificity observed is intrinsic to AFB1 and is not conferred by a bias of the selection system itself.

AFB1 has been shown to induce base pair substitutions primarily at GC base pairs in a variety of test systems; however, the percentage of GC→TA transversions varied from nearly 90% (7) to 55% (9), with other studies showing intermediate values (8, 10). In our study, all of the single-base substitutions detected in HPRT exon 3 were GC→TA transversions.

Mutations found at a frequency of about 2-3% in only one AFB1-treated culture included (a) a -G frameshift in a GGGGGG sequence (bases 207–212), (b) a GC→TA transversion at base 139, (c) a GC→TA transversion at base 197, and (d) a GC→TA transversion at base 208.

When multiple cultures are treated with the same dose of a given mutagen and then examined by the DGGE/PCR technique, it is common to observe mutations at a low frequency in only one treated culture (11, 25–27). Generally the bands present in only one of several identically treated cultures represent <2% of the thioguanine-resistant mutants. It should be noted that the major aflatoxin-induced hotspot at base 209 represents a very significant hotspot (10–17% of the mutants), and mutants that make up such a high fraction of the total...
mutant population have always been observed in identically treated cultures.

The cultures must be diluted daily for about 7 days after mutagenic treatment to allow for phenotypic expression of the HPRT mutants, and these dilutions have been shown to contribute to the variance observed in identically treated cultures (28, 29). The variation caused by daily dilutions becomes most pronounced as the number of mutants is decreased. The mutant corresponding to the strong mutational hotspot found in both cultures, namely, a GC→TA at base pair 209, represented between 120 and 195 cells, while those bands appearing in only a single culture represented between 24 and 36 mutants.

Several possibilities have been put forth as explanations for the fact that mutations are sometimes seen in only one of several identically treated cultures. These explanations include (a) preferential amplification of a mutant DNA sequence during PCR (11, 25–27), (b) the presence of the variance induced by daily cell culture dilutions as the reason for the presence of a single mutant that contains multiple copies of the HPRT locus caused by gene amplification (27). However, when investigated experimentally, none of the above possibilities were shown to have an effect on culture-to-culture variation. At this point, we favor the variance induced by daily cell culture dilutions as the reason for the variation in independent cultures.

Since some culture-to-culture variation is often seen with the PCR/DGGE method, we accept only those mutations found in multiple independently treated cultures as the mutational spectrum. Applying these criteria to AFB1, the mutational spectrum reduces to a single mutation, a GC→TA transversion at base 209.

However, it should be noted that mutant bands exist in the aflatoxin A culture (A3 and A4) at a position corresponding to mutant bands in the aflatoxin B culture B3 (GC→TA, base pair 197) and B4 (GC→TA, base pair 139). The signal:noise ratio was too low to permit sequencing of the bands from the aflatoxin A culture; therefore, it is possible that GC→TA mutations at bases 197 and 139 exist in both cultures.

We have examined the DGGE patterns of both aflatoxin-treated cultures after an addition 15 generations of growth in tissue culture. The DGGE patterns of these cultures were identical with the patterns prior to additional growth. This suggests that no sizable mutant subpopulation exists with a different doubling time than the bulk culture. Thus, the variability seen from culture to culture does not appear to be caused by differential doubling times for specific mutants.

Sensitive AFB1 adduct-based assays exist that provide a molecular dosimeter of dietary exposure (for review, see Ref. 30). The adduct-based assays essentially measure current AFB1 exposure. We are exploring the feasibility of using a DNA mutation assay as an indicator of aflatoxin exposure. It is possible to isolate and clone human T-cells from peripheral blood which are mutant at the HPRT locus (31, 32). Several groups are using mutations at the HPRT locus as a human biomonitor; cohorts heavily mutated by the same agent are expected to show a similar pattern of mutation. Mutations at the HPRT locus can persist years after the genetic insult (33), thus, it may be possible to use the mutational spectrum at the HPRT locus as a dosimeter of cumulative dose.

In collaboration with Dr. Geng-Sun Qian of the Shanghai Cancer Institute and Dr. John Groopman of Johns Hopkins University, we obtained viable human T-cells from 13 individuals in the People’s Republic of China who have a high dietary aflatoxin intake. The average HPRT mutant fraction of this cohort of 13 people was $14 \times 10^{-6}$ (ranging from $3-24 \times 10^{-6}$). This is higher than the normal value of $7.05 \pm 4.26$ (mean ± SE) for a cohort with a mean age of <40 years (34).

The AFB1-induced mutation found in tissue culture, namely, the GC→TA transversion at base 209, could be useful as a human biomonitor of aflatoxin exposure. The utility of this mutation will depend on the frequency of this mutation in the general population which is not exposed to aflatoxin. Analysis of the HPRT database (release 3) (23, 24) reveals that the GC→TA transversion at base 209 is rare in mutants isolated from the peripheral blood of humans. Present in the database are 269 single-base substitutions from mutants isolated in vivo. Among this collection, two GC→TA transversions at base 209 were found; one mutation was observed in a smoker and the other was seen in a Xeroderma pigmentosum patient.

We are currently determining the mutational spectrum at the HPRT locus from aflatoxin-exposed individuals from China. To date, a specific HPRT mutational spectrum has not been identified in any exposed population, including smokers and individuals occupationally exposed to mutagenic agents. The in vitro mutational spectrum of aflatoxin B1 identified in this paper may prove important in the interpretation of the data from the exposed individuals.

ACKNOWLEDGMENTS

A thoughtful reading of the manuscript by Dr. Adriana Oller is greatly appreciated.

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In Vitro Mutational Spectrum of Aflatoxin B₁ in the Human Hypoxanthine Guanine Phosphoribosyltransferase Gene

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