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Genetics

Aflatoxin B_1 induces the transversion of G → T in codon 249 of the p53 tumor suppressor gene in human hepatocytes

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ABSTRACT **Approximately half of hepatocellular carcinoma (HCC) from regions in the world with high contamination of food with the mycotoxin aflatoxin B_1 (AFB_1) contain a mutation in codon 249 of the p53 tumor suppressor gene. The mutation almost exclusively consists of a G → T transversion in the third position of this codon, resulting in the insertion of serine at position 249 in the mutant protein. To gain insight into the mechanism of formation of this striking mutational hot spot in hepatocarcinogenesis, we studied the mutagenesis of codons 247–250 of p53 by rat liver microsome-activated AFB_1 in human HCC cells HepG2 by restriction fragment length polymorphism/polymerase chain reaction genotypic analysis. AFB_1 preferentially induced the transversion of G → T in the third position of codon 249. However, AFB_1 also induced G → T and C → A transversions into adjacent codons, albeit at lower frequencies. Since the latter mutations are not observed in HCC it follows that both mutability on the DNA level and altered function of the mutant serine 249 p53 protein are responsible for the observed mutational hot spot in p53 in HCC from AFB_1-contaminated areas. Our results are in agreement with an etiological role of AFB_1 in hepatocarcinogenesis in regions of the world with AFB_1-contaminated food.**

Hepatocellular carcinoma (HCC) represents a major cause of mortality in certain areas of the world. Contamination of food with the mycotoxin aflatoxin B_1 (AFB_1) has been implicated as an etiological factor in certain regions of eastern Asia and subSaharan Africa (1, 2). Indeed, recent studies suggest that hepatitis B virus and AFB_1 may exert a synergistic effect (3). Approximately 50% of HCC in high AFB_1 regions (4, 5), but only 20% in low AFB_1 regions, harbors mutations in the p53 tumor suppressor gene, and the spectrum of mutations is quite different (6, 7). More than half of the tumors from high AFB_1 regions contain G → T transversions in the third position of codon 249 (AGG), resulting in the replacement of arginine by serine (4–8). In contrast, mutations are distributed throughout the highly conserved domains IV and V of p53 in HCC from low AFB_1 regions and no prevalence of G → T transversions is observed (9, 10). These results indicate that the substitution of arginine 249 by serine in the p53 protein is not required for hepatocarcinogenesis in man. Though G → T transversions are in agreement with the mutational specificity of AFB_1 (11, 12) and other carcinogens forming bulky DNA adducts (13, 14), there is no convincing explanation for the prevalence of mutations in codon 249, which almost never harbors mutations in other forms of human cancer. Many factors determine the mutability of a particular gene sequence, in addition to the chemical properties of the ultimate mutagen, and it is difficult to predict on the basis of model experiments. Among them, sequence context, local DNA conformation, chromatin structure, transcriptional activity, and repairability are recognized to play a role. Therefore, we have directly evaluated the mutability to AFB_1 of codons 247–250 in human hepatocarcinoma cells HepG2 by genotypic analysis using *Msp* I and *Hae* III restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) (15–17). Our results indicate that AFB_1 induces the transversion of G → T in the third position of codon 249, with the highest frequency producing the same mutation that is found almost exclusively in HCC from high AFB_1 regions in east Asia and Africa. Our results support the notion that AFB_1 represents a causative carcinogen in hepatocarcinogenesis in these regions of the world.

MATERIALS AND METHODS

HepG2 human hepatocarcinoma cells were grown in minimum essential medium supplemented with 10% fetal calf serum to ≈60% confluency before treatment for 30 min with AFB_1 (0.5 μg/ml) in the presence of rat liver microsomes as described (18–20). The medium was replaced and cell growth continued for 0 hr or for 96 hr, respectively. Despite AFB_1 treatment, the cell number doubled within 96 hr. Control cultures were sham-treated only with rat liver microsomes.

Preparation of DNA Enriched in p53 Sequences with Mutations in Codons 247–250. The DNA from controls and AFB_1-treated cells was restricted with *Bam*HI and a 6- to 9.5-kb fragment population was purified by agarose gel electrophoresis (15, 16). The recovery of the p53 sequences was determined by Southern blotting with a 1.8-kb cDNA probe (21) consisting of the entire gene. DNA preparations containing 3.5×10^7 copies of p53 were exhaustively digested with *Msp* I and *Hpa* II. To these preparations 10 copies of mutant standard (MS; see below and Fig. 1) were added. The samples were enriched in sequences with mutated *Msp* I/*Hpa* II recognition sequence 14067–14070, which spans the third position of codon 247 and the entire codon 248 (see Fig. 1), by gel isolation of a 380- to 500-bp fragment population. These DNA preparations contain MS and the predicted mutated 468-bp p53 fragment, which extends from the flanking 5′ *Msp* I site (13768) to the flanking 3′ *Msp* I site (14235).

For analysis of mutations in *Hae* III site 14072–14075 (codons 249 and 250), DNA containing 3.5×10^7 copies of p53 was digested with *Hae* III, and five copies of MS (see below and Fig. 1) were added before gel isolation of a 100- to 200-bp fragment population containing a mutated 159-bp p53 segment, which extends from flanking 5′ *Hae* III site (13981) to the flanking 3′ *Hae* III site (14139) plus MS.

High-Fidelity Amplification of a p53 Exon VII Fragment Containing Codons 247–250. From the enriched DNA preparations described above a 116-bp fragment spanning residues 13999–14114 was amplified with *Pyrococcus furiosus* DNA polymerase (22) for 40 cycles using sense primer no. 1 and antisense primer no. 2 (see Fig. 1) under conditions specified by the supplier of the polymerase (Stratagene). A second set of nested primers, nos. 3 and 4, with *Eco*RI tails

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Abbreviations: HCC, hepatocellular carcinoma; AFB_1, aflatoxin B_1; RFLP/PCR, restriction fragment length polymorphism/polymerase chain reaction; MS, mutant standard.

was used in an additional 10 amplification cycles with *Taq* polymerase (Roche, Gipf-Oberfrick, Switzerland) under the conditions defined by Eckert and Kunkel (23), producing a 101-bp exon VII fragment extending from residue 13999 to residue 14099. The temperature cycles consisted of 30 sec at 96°C and 1 min at 45°C for amplification with either polymerase.

The RFLP/PCR products were purified and cloned into λgt10, and the phage were plated on *Escherichia coli* C600 Hfl. Colony/plaque screens were hybridized with ^{32}P-labeled oligonucleotide probes specific for each single base-pair mutation, wild-type and MS. Selective washing temperatures were determined with λ constructs containing authentic mutant, wild-type, or MS inserts (see below). In each experiment authentic mutant constructs were included to ascertain selective hybridization conditions.

Preparation of Authentic Single Base-Pair Changes in Codons 247–250 of p53 and Construction of MS. The principal steps of the PCR protocol for the preparation of authentic single base-pair changes in a particular restriction site and of MS have been described (24).

The MS sequence containing the multiple base-pair changes indicated in Fig. 1 was cloned into the *Eco*RI sites of the pSP65 vector and colony purified on *E. coli* $HB_1$01. For experiments measuring mutations in *Msp* I site 14067–14070, a 462-bp fragment containing the MS sequence and flanking sequences from the vector was removed by restriction with *Nae* I and *Hin*dIII; for experiments on *Hae* III site 14072–14075, the 117-bp MS insert was removed by restriction with *Eco*RI. Two different MS fragments of the indicated lengths are required to allow their coisolation by gel electrophoresis with the 380- to 500-bp fragment population following *Msp* I digestion of cellular DNA or the 100- to 200-bp fragment population following *Hae* III digestion, respectively (see above).

RESULTS

We have studied the mutagenesis of residues 14067–14075 of the p53 gene by rat liver microsome-activated AFB_1 in human hepatocarcinoma cells HepG2. This gene fragment extends from the third position of codon 247 to the middle position of codon 250. It contains codon 248 (CGG), which represents a mutational hot spot in ≈10% of human tumors with p53 mutations (25, 26), and codon 249 (AGG), which is mutated in ≈50% of HCC from areas in the world with high levels of AFB_1 in food (4–8). Genotypic analysis was carried out by *Msp* I and *Hae* III RFLP/PCR (15, 16). Sequence information, the locations of the selected *Msp* I site (residues 14067–14070) and *Hae* III site (residues 14072–14075), the chosen amplimers, and the structure of the MS are given in Fig. 1.

The profile of experimentally induced mutations in the sequence of interest has to be known to distinguish them from preexisting, spontaneous mutations contained in cellular DNA. Therefore, we first carried out a detailed analysis of the experimentally induced base-pair changes. We amplified a 101-bp fragment (residues 13999–14099) under the high-fidelity conditions outlined for cellular DNA using as template 5×10^3 copies of a cloned exon VII fragment of p53 and analyzed the *Msp* I site 14067–14070 and the *Hae* III site 14072–14075 for their content in base-pair changes by RFLP/PCR. The predominant polymerase errors were C → T (third base of codon 247, 8.9%), C → A (first base of codon 248, 7.6%), G → A (third base of codon 248, 6.8%), G → T (third base of codon 249, 6.9%), C → T (second base of codon 250, 25%), and C → A (second base of codon 250, 8.6%). The percentage values in parentheses represent relative frequencies of mutant λ plaques that are not directly comparable to those obtained from cellular DNA from untreated cells because, in the latter case, the number of residual wild-type sequences in the amplification reaction is not known.

Fig. 2 gives the relative frequencies in DNA from AFB_1-treated HepG2 cells and controls of all possible base-pair changes in *Msp* I site 14067–14070. This site spans the third residue of codon 247, (A) (A)C, and the entire codon 248, CGG. The DNA that was exhaustively restricted with *Msp* I contained 3.5×10^7 initial p53 copies from untreated HepG2 controls and AFB_1-treated cells alike. It is evident that the level of background mutations was low in untreated controls. In particular, there was no evidence for spontaneous deamination at the CpG dinucleotide of codon 248, since the levels of C → T transitions at C 14068 and of G → A transitions at G 14069 were very low. Since spontaneous deamination is expected to occur preferentially at 5-methylcytosine rather than cytosine we analyzed the methylation status of the CpG sequence of codon 248 in HepG2 cells. Comparative Southern blotting following *Msp* I and *Hpa* II restriction with a 101-bp exon VII probe extending from residue 13999 to residue 14099 indicated a high level of 5-methylcytosine in

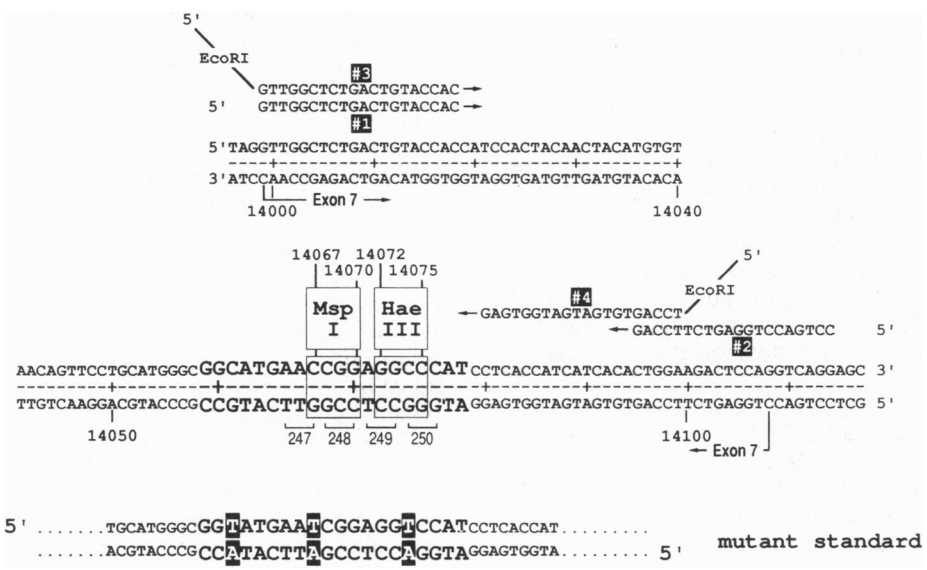

FIG. 1. Genotypic mutation analysis of codons 247–250 (residues 14067–14075) of exon VII of the human p53 gene by *Msp* I and *Hae* III RFLP/PCR. Sequence information is given for the restriction recognition sequences of interest, the MS, and the amplimers.

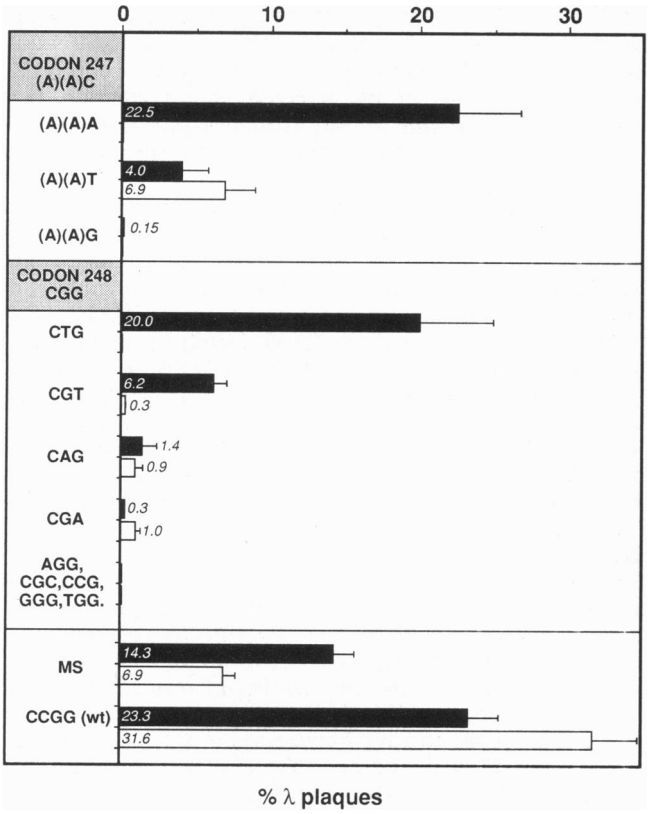

FIG. 2. Relative frequencies of AFB_1-induced base-pair changes in codons 247 and 248 of p53 in HepG2 cells. HepG2 cells were treated with rat liver microsome-activated AFB_1 or sham-treated only with microsomes. Solid bars represent data from AFB_1-treated cells and open bars from untreated controls. After 4 days of continued growth their DNA was analyzed for base-pair changes in the third position of codon 247, (A)(A)C, and codon 248, CGG, corresponding to residues 14067–14070 by *Msp* I RFLP/PCR. The residues in parentheses are not part of the *Msp* I site and were not analyzed. Ten copies of mutant standard were added to 3.5×10^7 initial copies of the p53 gene. The RFLP/PCR products were cloned into λgt10 and plated on *E. coli* C600 Hfl, and colony/plaque screens were analyzed by hybridization with 12 mutant-specific oligonucleotide probes as well as probes for the wild-type (wt) sequence and MS. Analysis included 1768 plaques for DNA from AFB_1-treated cells and 1721 plaques for control DNA on 16 Petri dishes. Error bars indicate standard deviations.

codon 248. The expected 300-bp fragment extending from 5′ *Msp* I/*Hpa* II site 13768 to the 3′ flanking *Msp* I/*Hpa* II site 14067 was only produced following *Msp* I but not *Hpa* II digestion of HepG2 DNA (data not shown).

Microsome-activated AFB_1 strongly induced the transversions C 14067 → A in the third position of codon 247 and of G 14069 → T in the middle position of codon 248. The corresponding G 14070 → T transversion at the third position of codon 248 occurred with ≈3-fold lower frequency and the C 14068 → A transversion in the first position of codon 248 was not observed. The percentages of λ plaques that contained MS or wild-type sequences are indicated at the bottom of Fig. 2.

Fig. 3 *A* and *B* give the relative frequencies of base-pair changes in *Hae* III site 14072–14075, which includes the second and third base of codon 249, (A)GG, and the first and second base of codon 250, CC(C). The highest relative frequencies for background mutations in DNA from untreated controls are observed for G 14073 → A (third base of codon 249), G 14073 → T (third base of codon 249), C 14074 → A (first base of codon 250), and C 14075 → A (second base of codon 250). Microsome-activated AFB_1 selectively induced the transversions of G 14073 → T at the third base of codon 249 and C 14074 → A at the first base of codon 250. No significant increases above background are observed for the remaining 10 possible base-pair changes.

Absolute mutation frequencies were calculated from the MS content of the RFLP/PCR products, the initial number of MS copies, and the number of copies of the p53 gene present at the time of the addition of MS to the cellular DNA. The data for the AFB_1-induced G → T and C → A transversions in the 8 bp that have been analyzed are shown in Fig. 4. It is evident that the G 14073 → T transversion involving the third position of codon 249 is induced most strongly with a frequency of 8.4×10^{-7}. It is followed by the C 14074 → A transversion with a frequency of 5.9×10^{-7} at the first position of codon 250.

It was conceivable that AFB_1–DNA adducts that may persist after 96 hr of posttreatment incubation result in misincorporation during the early amplification cycles. This possibility was ruled out by experiments in which DNA was prepared immediately following treatment and analyzed by *Hae* III RFLP/PCR. Since the cells were given no time for repair, these DNA preparations are expected to contain maximal adduct levels. The absolute frequency of the G 14073 → T transversion was reduced 52-fold (to 1.6×10^{-8}) and the frequency of the C 14074 → A transversion was reduced 65-fold (to 0.9×10^{-8}) relative to DNA from cell cultures that were allowed to grow for 96 hr following exposure to AFB_1.

DISCUSSION

Our results indicate that AFB_1 induces the transversion of G → T in the third position of codon 249 in the p53 segment extending from codon 247 to 250 with highest absolute frequency. The transversion of C → A in the adjacent first position of codon 250 represents the second most frequent mutation. The additional base-pair changes observed in codons 248 and 247 occur at 2-fold lower frequencies. All observed base-pair changes are transversions in agreement with the mutagenic specificity of AFB_1 (11, 12) and other carcinogens that form bulky DNA adducts (13, 14, 27–29). The reactions of AFB_1–G adducts in DNA have been studied in detail *in vitro* (18, 30) and in cellular DNA (31–33). Activated AFB_1 forms the chemically unstable 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 adducts, which, in secondary reactions, either lose their substituent and revert to intact guanine, form apurinic sites, or ring open to a stable derivative referred to as AFB_1–triamino-Py or FAPYR (18, 19, 31). It appears that the latter adducts are removed only slowly by cellular repair processes in mammalian cells (30) and, therefore, they represent likely premutagenic lesions. Therefore, the observed G → T and C → A transversions most likely are a consequence of polymerase miscoding at guanine adducts in the coding and noncoding strands, respectively, but they could also originate from miscoding opposite apurinic sites. Only weak strand bias was observed in favor of the transversion of G → T in the third position of codon 249 of the coding strand. In agreement with our findings, no strand bias was observed for AFB_1 mutagenesis in a shuttle vector system in human fibroblasts (12). This might be expected for persistent lesions that are only poorly repaired in either strand.

It has been reported that adducts are formed preferentially at GG dinucleotides in DNA *in vitro* (12, 34). The p53 segment investigated in our work, which extends from codon 247 to codon 250, contains four GG dinucleotides in the coding and noncoding strands. Interestingly, this segment contains an axis of symmetry that lies in the A·T base pair (14071) at the first position of codon 249 (see Fig. 1). It is conceivable that this unique sequence context favors the formation of

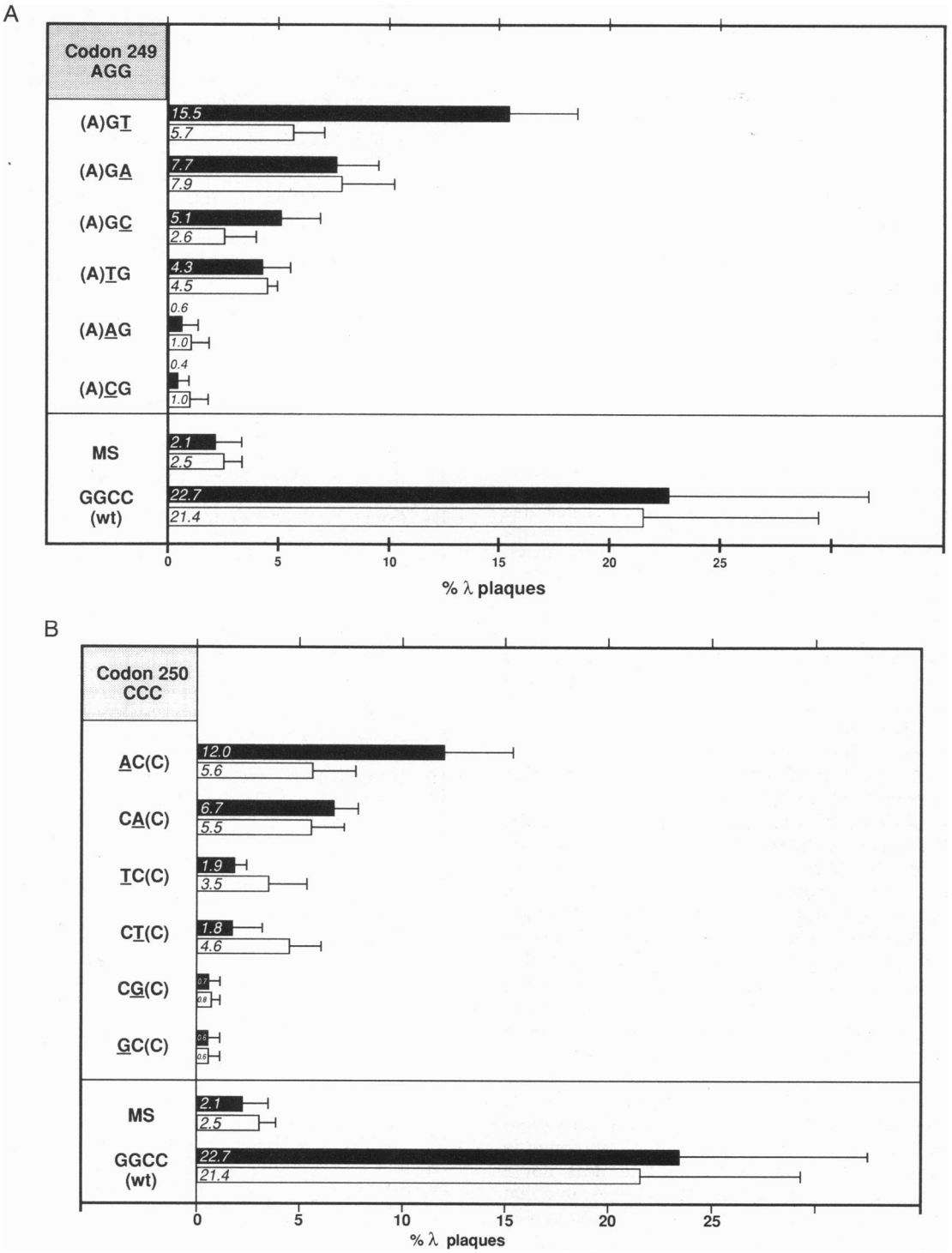

FIG. 3. Relative frequencies of AFB_1-induced base-pair changes in codon 249 (*A*) and codon 250 (*B*) of p53 in HepG2 cells. Experimental conditions were as described in the text and the legend to Fig. 2. Solid bars represent data from AFB_1-treated cells and open bars from untreated controls. Base-pair changes were measured in the second and third positions of codon 249, (A)GG, and the first and second positions of codon 250, CC(C) (corresponding to residues 14072–14075), by *Hae* III-RFLP/PCR. The residues in parentheses are not part of the *Hae* III site and were not analyzed. Five copies of MS were added to 3.5×10^7 initial copies of the p53 gene. Analysis with 12 mutant-specific oligonucleotide probes and probes for the wild-type sequence and MS included 1595 plaques for DNA from AFB_1-treated cells and 1639 plaques for control DNA on 16 Petri dishes. Error bars indicate standard deviations.

AFB_1–G adducts. However, in many instances there is no simple relationship between the initial adduct distribution and the ultimate mutational spectrum (12). Alternatively, local DNA and chromatin conformation may interfere with repair processes.

Approximately 85% of p53 mutations at hot spot codon 248 in human tumors occurs at the CpG dinucleotide and they consist to equal proportions of C → T and G → A transitions (25, 26). It has been speculated that these mutations occur by the spontaneous or induced deamination of 5-methylcytosine at these sequences (35). This does not appear to occur in HepG2 cells. Despite the high level of 5-methylcytosine at codon 248, these mutations were not detected. An indication about the origin of the background mutations in the RFLP/

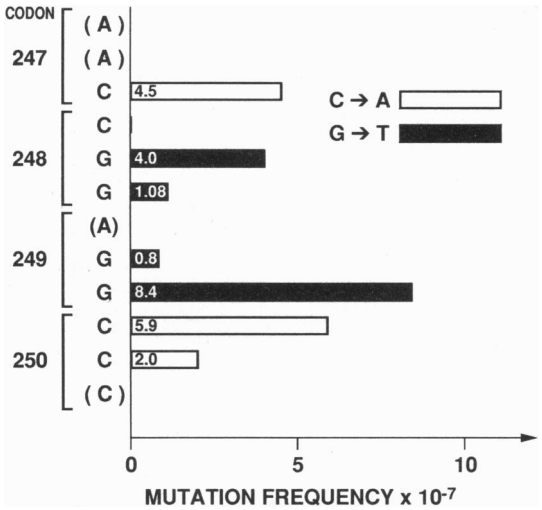

FIG. 4. Absolute frequencies of AFB_1-induced base-pair changes in codons 247–250 of p53 in HepG2 cells: G → T and C → A transversions. Absolute mutation frequencies were calculated from the data given in Figs. 2 and 3 by calibration with the MS contents of the RFLP/PCR products, the initial number of p53 gene copies, and the number of copies of MS added at the outset of the experiment (15–17). The values for background mutations in untreated controls were subtracted.

PCR products from cellular DNA of untreated cells can be obtained by comparison with the data for mutations induced due to polymerase errors in the amplification of the same cloned exon VII segment. The relatively frequent C → A transversions at the first and second positions of codon 250 in cellular DNA are only rarely produced during amplification and may represent spontaneous mutations.

Our results indicate that AFB_1 preferentially induces the same G → T transversion in the third position of codon 249 of p53 as that which is found in HCC from high AFB_1 regions of the world. These findings are in agreement with an etiological role of AFB_1 in HCC from these areas. Although we observed highest mutability of this G·C base pair of codon 249, the difference in mutation frequencies at the neighboring base pair was only moderate. It follows that mutability on the level of DNA that is mostly a consequence of adduct distribution and repair cannot fully explain the almost complete prevalence of this particular G → T transversion in codon 249 in HCC from high AFB_1 regions. Evidently the altered function of the serine 249 mutant p53 protein must play a role in the development of HCC that harbors this mutation. However, the fact that this mutation is only rarely found in HCC from low AFB_1 regions indicates that it is not a prerequisite for hepatocarcinogenesis. It is conceivable that hepatitis B virus and the mutant serine 249 p53 protein play a synergistic role.

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