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Sequence context effects in DNA replication blocks induced by aflatoxin B_1

(mutagenesis and carcinogenesis/intrastrand base pairing/DNA palindromes/DNA–ligand interactions/DNA polymerase I)

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ABSTRACT **The genotoxic effects of the potent mutagenic carcinogen aflatoxin B_1 (AFB_1) are believed to be mediated by its reaction with the N-7 atom of guanine residues in DNA. We have analyzed the effect of AFB_1-induced chemical modification on the template function of single-stranded DNA *in vitro*. The experimental strategy involves the elongation of a primer on a modified template by *Escherichia coli* DNA polymerase I (large fragment) and analysis of the products by high-resolution gel electrophoresis. Our data show that (*i*) AFB_1 induces specific replication blocks one nucleotide 3′ to the sites of occurrence of guanine residues on template DNA; (*ii*) AFB_1-induced replication blocks occur predominantly at sequences capable of participation in intrastrand base pairing; (*iii*) within the intrastrand base-paired regions there are strong sequence context effects, in accordance with the previously described [Muench, K. F., Misra, R. P. & Humayun, M. Z. (1983) *Proc. Natl. Acad. Sci. USA* 80, 6–10] specificity "rules" that apply to the reaction of AFB_1 with guanine residues in double-stranded DNA; (*iv*) there is evidence that the (7-guanyl)-AFB_1 adducts as well as secondary derivatives such as the formamidopyrimidine-AFB_1 act as replication blocks. In summary, these data suggest that previously observed inhibition of DNA replication and transcription by AFB_1 is directly attributable to (7-guanyl)-AFB_1 adducts or their secondary reaction products.**

Aflatoxin B_1 (AFB_1) is among the small number of chemical carcinogens implicated in human cancer by highly suggestive epidemiological evidence (1, 2). AFB_1 is a metabolic product of certain widely distributed species of the fungal genus *Aspergillus*, and, as a significant contaminant of the food chain, it is a source of world-wide concern. Experimentally, AFB_1 is a powerful toxin, a potent mutagen, and an extremely effective hepatocarcinogen. The genotoxic effects of AFB_1 have been attributed to its ability to chemically modify cellular macromolecules, in particular, DNA. AFB_1 requires activation for significant reaction with DNA, and evidence suggests that the active species is the 8,9-epoxide.

Reaction of AFB_1 with DNA shows several interesting features. The overwhelmingly favored DNA target appears to be the N-7 position of guanine residues. Also, the reaction of AFB_1 is highly sequence-specific in double-stranded (ds) DNA (3), and AFB_1 appears to have a clear preference for ds DNA as compared to single-stranded (ss) DNA, reacting with the latter only minimally and in an essentially random manner (4).

In vitro and *in vivo* evidence (5, 6) suggests a relatively long half-life for the primary (7-guanyl)-AFB_1 (Gua-AFB_1) adduct and a much longer half-life for the secondary derivatives, such as the guanine imidazole ring-opened form, formamidopyrimidine-AFB_1 (FAPyr-AFB_1). In addition, a fraction of the AFB_1-modified guanine residues are lost from the DNA, generating apurinic (AP) sites. While it is generally assumed that the genotoxic effects of AFB_1 (including the toxic, mutagenic, and carcinogenic effects) are mediated by its reaction with guanine residues in DNA, these assumptions are not yet rigorously proven (1). Although chemical modification of the N-7 atom of guanine residues should not, in principle, directly affect base pairing, AFB_1 modification has been shown to impair the template function of DNA *in vitro* and *in vivo* (1, 7–9). Direct evidence linking guanine modification with impaired template function has not been reported, although it is suspected that the guanine adduct, because of its large size, may prove to be a steric hindrance during replication and transcription.

We have examined the effect of AFB_1 modification on the template function of DNA at the sequence level. Although several approaches are available, we have opted for the powerful technique pioneered by Strauss and co-workers (10–12), in which a defined primer is permitted to elongate on a chemically modified template and the products are analyzed on DNA sequence gels. We report below that chemical modification of ss DNA by AFB_1 creates replication stops one nucleotide 3′ to the sites of occurrence of template guanine residues, which constitute the primary target base for AFB_1, and that there are striking sequence context effects in the pattern of such replication blocks.

MATERIALS AND METHODS

AFB_1 (Calbiochem) and [^{3}H]AFB_1 (12 Ci/mmol; Moravek Biochemicals, Brea, CA; 1 Ci = 37 GBq) were used without further purification. ^{3}H-labeled AFB_1 8,9-dichloride (AFB_1Cl_2; 155 mCi/mmol) was synthesized according to Swenson *et al.* (13). Primers for DNA synthesis (indicated in Fig. 2) either were purchased from New England Biolabs or were synthesized in the laboratory of O. P. Bhanot. Various M13 phage cloning vector ss DNAs were prepared by standard techniques in *Escherichia coli* JM101 (14). *E. coli* DNA polymerase I large "Klenow" fragment deficient in 5′-to-3′ exonuclease activity ($polI_K$) was purchased from New England Biolabs.

Template DNAs were modified by [^{3}H]AFB_1Cl_2 (with precautions to minimize exposure to ambient light) according to the following protocol. [^{3}H]AFB_1Cl_2 (0.06–6 nmol) in 200 μl of CH_2Cl_2 was dried down in a 10 × 75 mm glass test tube under reduced pressure. Then 200 μl of a sodium phosphate

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Abbreviations: AFB_1, aflatoxin B_1; AFB_1Cl_2, aflatoxin B_1 8,9-dichloride; AP, apurinic; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA; Gua-AFB_1, (7-guanyl)-AFB_1; FAPyr-AFB_1, formamidopyrimidine-AFB_1; $polI_K$, *Escherichia coli* DNA polymerase I large "Klenow" fragment.

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buffer (20 mM, pH 6.8) containing 4–10 pmol of template DNA was warmed to 37°C and added to the reaction tube (containing dried [^{3}H]AFB$_1$Cl$_2$), which was briefly swirled on a Vortex mixer and shaken vigorously for 10 min in a 37°C waterbath. The reaction was terminated by adding 10 μl of 2-mercaptoethanol and the DNA was recovered by three rounds of precipitation with ethanol (15) and dialysis at 4°C for 2 hr against one of the following buffers: Hepes (20 mM, pH 6.8, 7.0, or 7.5) or 10 mM Tris·HCl/10 mM NaCl (pH 7.5). Mock reactions were identical except for the omission of [^{3}H]AFB$_1$Cl$_2$ or its substitution by (unactivated) [^{3}H]-AFB$_1$ of matching specific activity. Levels of modification (adducts per template molecule), calculated by measuring ^{3}H, increased linearly with [^{3}H]AFB$_1$Cl$_2$ dose and ranged from approximately 0.65 hit per molecule (AFB$_1$Cl$_2$ = 0.06 nmol) to 23 hits per molecule (AFB$_1$Cl$_2$ = 6 nmol) at a DNA (M13mp8) concentration of 10 pmol/200 μl. (AFB$_1$, a suspected human carcinogen, should be handled with appropriate precautions.)

For analyzing AFB$_1$-induced replication blocks, we essentially followed the procedure of Strauss and co-workers (10–12) except for some minor changes. A typical experiment was carried out as follows. Template DNA (250 ng) and the appropriate primer (2.5 ng) were annealed at 37°C for 1–2 hr in 8 μl of buffer (50 mM NaCl/10 mM MgCl$_2$/1 mM dithiothreitol/10 mM Tris·HCl, pH 7.5, or Hepes, pH 6.8, 7.0, or 7.5). One microliter of [α-^{32}P]dATP (200 Ci/mmol, Amersham) and 1 μl of polI$_K$ (1 unit) were added to 4 μl of the primed template solution. Elongation reactions were initiated by adding 1.5 μl of a solution containing 0.25 mM each of dGTP, dCTP, and dTTP to 1 μl of the primed template/[α-^{32}P]dATP/polI$_K$ mix. After incubation at 22–24°C for 15 min, 0.5 μl of a "chase" solution (0.25 mM each of dATP, dGTP, dCTP, and dTTP) was added and incubation was continued for an additional 15 min before termination by adding 5 μl of a "stop" solution (0.3% xylene cyanol/0.3% bromphenol blue/0.37% Na$_2$EDTA, pH 7, in formamide; New England Biolabs). Two microliters of this solution was heated at 90°C for 3 min and subjected to electrophoresis on a high-resolution 6% polyacrylamide/8 M urea "thin" gel.

RESULTS

DNA base modifications that can either affect normal Watson–Crick base pairing or present steric hindrance to the progression of a replication fork can be detected and analyzed at the nucleotide sequence level by the following strategy. DNA elongation *in vitro* is permitted from a defined primer on modified templates in the presence of deoxynucleoside triphosphate precursors, Mg^{2+}, and a DNA polymerase. The elongation products are radiolabeled by either a pulsing technique or prior end labeling of the primer and analyzed by high-resolution gel electrophoresis alongside a Sanger sequencing ladder generated from the same primer on unmodified DNA. Replication blocks appear on an autoradiograph as a series of bands not seen in control lanes. These specific bands are presumably due to the stoppage, idling, or slowing-down of the polymerase at or near the site of occurrence of a base modification. This strategy has proven to be useful for analysis of the distribution of replication-blocking DNA modifications at the sequence level as well as for analysis of the behavior of polymerases when presented with such obstacles under defined conditions *in vitro* (10–12).

To react with DNA, AFB$_1$ requires activation via the epoxidation of the 8,9 double bond, although the putative epoxide has not been isolated, presumably due to its reactivity (1). At least three procedures are available for activation of AFB$_1$ *in vitro*. We have previously used (3, 4) activation by rat liver enzyme preparations as well as oxidation by a mild organic oxidant, chloroperbenzoic acid (21). Because of variable nucleic acid and nuclease contamination, the crude liver enzyme procedure cannot be directly applied for the type of work described here. In attempting to use the peracid procedure, and in agreement with a recent report (9), we observed that chloroperbenzoic acid strongly inhibited the template function of DNA such that this activation procedure cannot be used. (We shall describe the peracid-induced inhibition of replication elsewhere.) The third procedure for AFB$_1$ activation (the one used here) involves the synthesis of an electronic model for the 8,9-epoxide—namely, AFB$_1$Cl$_2$ (13), which was previously shown to have essentially the same chemical and biological properties as the epoxidized AFB$_1$. In addition, our unpublished results from alkali-labile site analysis suggest that AFB$_1$Cl$_2$ reacts with DNA with the same sequence specificity as peracid- or enzyme-activated AFB$_1$. Because of these considerations and because of the mutually supportive nature of the present data with previous data obtained by different techniques, we believe that AFB$_1$Cl$_2$ is a valid model for the 8,9-epoxide for the purposes of this study. However, we cannot rule out the possibility of other physicochemical or biological differences between the epoxide and the dichloride.

Fig. 1 *a* and *b* shows that AFB$_1$ modification of template DNA specifically creates replication blocks for *E. coli* polI$_K$. The panels represent elongation by two different primers within the *lacZ* α-complementing gene segment contained in various M13mp vector phage as indicated in Fig. 2. In Fig. 1*a*, some AFB$_1$-specific replication blocks found (lanes 8–11) are identified by base numbers. In general, AFB$_1$-specific replication blocks are observed one nucleotide 3′ to certain template guanine residues. [Although this feature—i.e., a block one nucleotide before rather than opposite certain template guanine residues—is not clearly ascertainable at all guanine residues in the data in Fig. 1 *a–d*, we have confirmed it by running replication-block lanes interspersed with sequence marker ladders (data not shown).] Although the replication block bands occur one nucleotide 3′ to the site of occurrence of certain template guanine residues, for the sake of clarity, we identify the bands with the base numbers corresponding to the appropriate template guanine residues—e.g., the numbers 6462–3 identify bands that actually have mobilities corresponding to template residues 6463–4; however, since these bands are attributed to stops occurring one nucleotide 3′ to the relevant template guanine residues at 6462–3, we label the bands as "6462–3." We have followed the same conventions for identifying replication blocks throughout this report. Note the striking variations in the intensities of replication blocks—e.g., blocks at 6462–3 are very strong, whereas at other template guanine residues the blocks are less intense, barely detectable, or undetectable. In Fig. 1*a*, the strongest cluster of blocks is at guanine residues 6444 through 6471. Note that with increasing levels of AFB$_1$ adducts, as expected, there is a progressive diminution in large molecular weight products at the top of the gel. In Fig. 1*b*, the strongest AFB$_1$-specific bands within the well-resolved region correspond to positions 6177–8, whereas the bands at 6267–8 are less intense. The results show that (*i*) AFB$_1$-modified DNA shows a series of replication blocks whose intensity increases with the extent of AFB$_1$ modification; (*ii*) the replication blocks, in comparison to the sequence ladder, have occurred one nucleotide 3′ to template guanine residues; (*iii*) there is a striking nonrandomness in the intensity of replication blocks, such that, at certain template guanine residues, the bands are very intense, while at others they are barely visible or not detectable.

Fig. 2 is a part of the template DNA sequence, showing the positions of the primers used as well as some inverted repeat sequences. An analysis of the data in Fig. 1 *a–d* in comparison with the sequence in Fig. 2 suggests that nearly

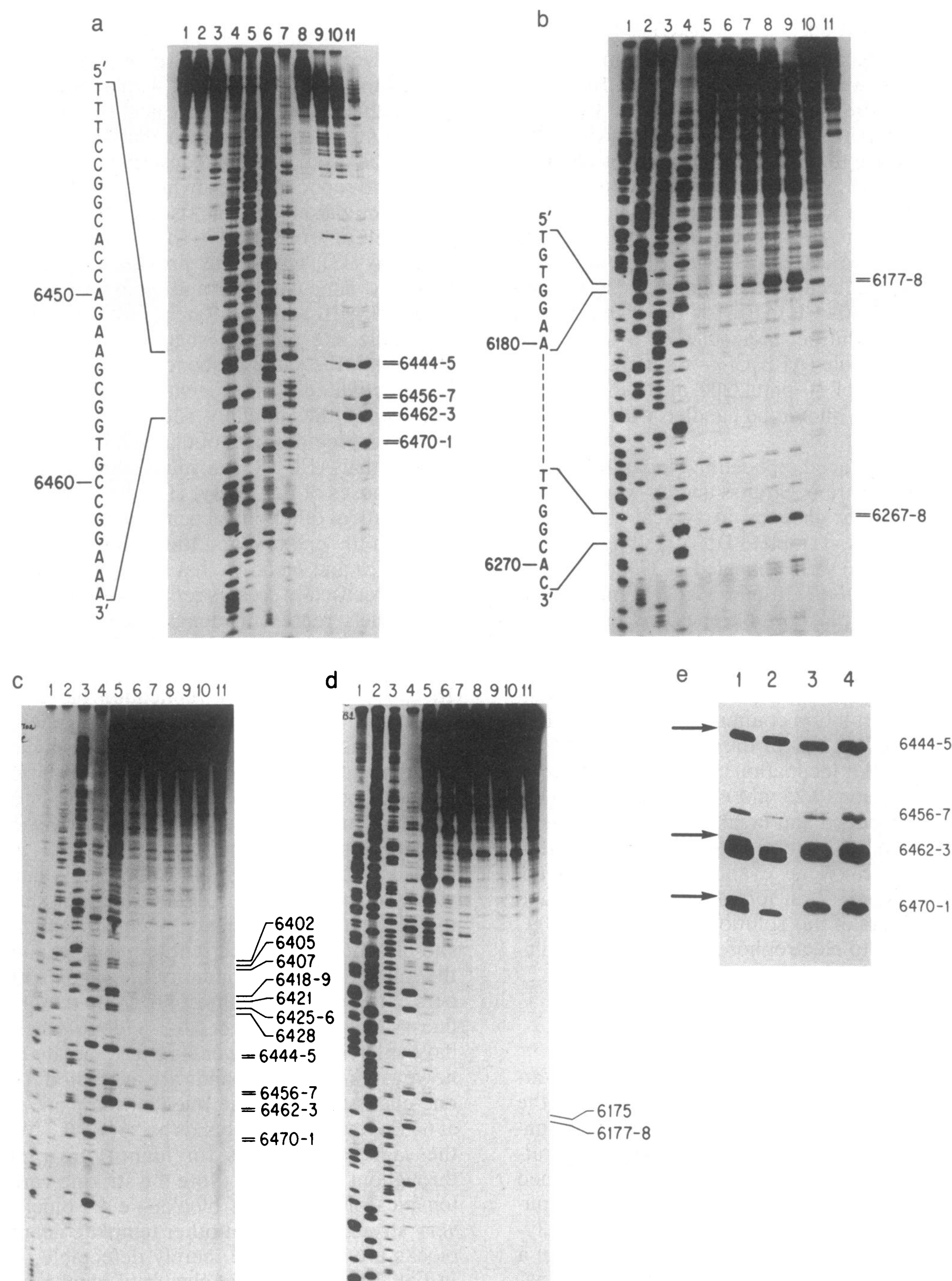

FIG. 1. Autoradiographs of DNA sequence gels on which products obtained by elongation of the appropriate primer on various M13mp8 (BK8) templates were fractionated. The sequences shown are those of the template, not the synthesized strand. (*a*) Primer used was primer 3 (see Fig. 2). Lane 1, untreated template; lane 2, template DNA subjected to mock modification (unactivated AFB_1); lane 3, template DNA subjected to mock modification (no AFB_1); lanes 4–7, standard Sanger (16) sequence channels G, A, T, and C, respectively; lanes 8–11, AFB_1-modified template DNAs with 1, 2, 4, and 20 AFB_1 adducts per DNA molecule. Elongation conditions were 37°C and pH 7.5 (Tris·HCl). Essentially similar results were obtained under the elongation conditions 23°C or 37°C, pH 6.8, 7.0, 7.5 (Hepes), or 7.5 (Tris·HCl). (*b*) Primer used was NEB (Fig. 2). Lanes 1–4, Sanger sequence channels G, A, T, and C; lanes 5–9, template DNAs with 1.5, 4, 6, 12, and 25 adducts per molecule, respectively; lane 10, mock-treated template (unactivated AFB_1); lane 11, untreated template. Elongation conditions were 23°C and pH 6.8 (Hepes). Other conditions, as described for *a*, gave similar results. (*c* and *d*) Primers used were primer 3 (*c*) and NEB (*d*). Lanes 1–4, Sanger sequence lanes G, A, T, and C. Lanes 5–9, template DNAs modified by the standard protocol except for formamide concentrations during modification of 0, 10, 20, 40, and 50% (vol/vol), respectively, resulting in 10.4, 3.8, 1.7, 1.8, and 1.4 AFB_1 adducts per DNA molecule. Lane 10, mock-treated template (no AFB_1); lane 11, untreated template. (*e*) Composite autoradiograph of replication block gels (primer 3). Lane 1, AFB_1-modified DNA (initial adduct level, 20 per molecule) incubated at 37°C for 48 hr in Hepes buffer (pH 6.8; final adduct level, 6 per molecule). These conditions are known to cause depurination as well as loss of AFB_1 diol (5, 6). Lanes 2 and 4, DNA not subjected to postmodification treatment (control). Lane 3, AFB_1-modified DNA (initial adduct level, 20 per molecule) incubated at 37°C for 1 hr in 65 mM glycine/NaOH buffer (pH 10.5) followed by incubation at 37°C for 48 hr in Hepes buffer, pH 6.8 (final adduct level, 16 per molecule). Incubation at pH 10.5 for 30 min at 37°C was previously shown (17) to result in the conversion of the major proportion (greater than 90%) of Gua-AFB_1 adduct to the stable secondary derivatives of the type FAPyr-AFB_1.

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6171                                              MET
TTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGA
             HAIRPIN 6187
6221                GATC
CCATGATTACGAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTTGGCA
             HAIRPIN 6239             HAIRPIN 6263
6271
CTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCA
                    NEB PRIMER
6321
ACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG
             PRIMER 1           HAIRPIN 6356
6371
AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGC
6421
GAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTG
                                HAIRPIN 6452
6471
GCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACT
6521
GGCAGATGCACGGTTACGATGCGCCCAT
             PRIMER 3
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FIG. 2. DNA sequence of a part of M13mp8 (BK8), showing the positions of various primers and some inverted repeat sequences potentially capable of intrastrand base pairing to generate "hairpins," identified by the base number at the center of the symmetry. BK8 is a variant of M13mp8 with G-A-T-C inserted between nucleotides 6246 and 6247 such as to eliminate the *BamHI* site and to frameshift the *E. coli lacZ* (α-complementing segment) gene, whose initiation codon is at position 6217.

all of the strong blocks (i.e., modification sites) are within inverted repeat sequences capable of assuming intrastrand secondary structures (hairpins). Within the template sequences examined, there are two notable hairpins, one extending from base 6440 to base 6467 (hairpin 6452, Fig. 2) and another between bases 6172 and 6207 (hairpin 6187, Fig. 2), corresponding to the *E. coli lac* operator. Fig. 3 is a graphic representation of the relevant sequences folded to show potential intrastrand base pairing. Examination of the autoradiographic data in Fig. 1 in comparison to the sequence features shown in Figs. 2 and 3 suggests that almost all strong stops (modification sites) occur within potentially base-paired regions and that the relative intensity of a stop is dependent, to a certain extent, on the predicted stability of the hairpin in terms of parameters (18) hypothesized to affect RNA intrastrand structures, such as the length of the inverted repeat, the number of matches, loop size, and G+C content. More specifically, in Fig. 1*a*, lane 11, the cluster of strong bands extending from 6471 to 6444 falls within hairpin 6452. Similarly, in Fig. 1*b*, lane 9, the strong bands at 6177–8 fall within hairpin 6187. Also note that in Fig. 1*b*, lane 9, the moderately strong bands at 6267–8 occur within the inverted repeat sequence labeled as hairpin 6356 in Fig. 2.

To test whether strong stops within potentially base-paired regions are due to preferential attack of AFB_1 on such structures, we carried out the following three types of experiments. (*i*) Fig. 1 *c* and *d* shows the effect of various concentrations of the denaturant formamide during AFB_1 modification on the pattern of replication blocks. At higher formamide concentrations, presumably due to the melting of the secondary structures, there is a progressive loss of DNA

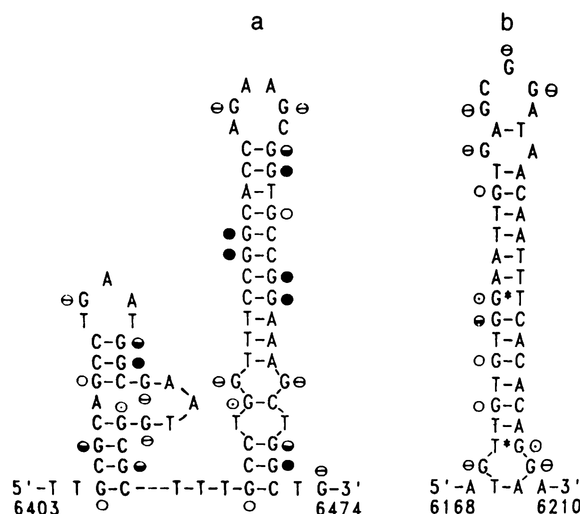

FIG. 3. Diagram of parts of the DNA sequence from Fig. 2 to show possible intrastrand base pairing near hairpin 6452 (*a*) and hairpin 6187 (*b*). Although in Fig. 2 only an 11-nucleotide inverted repeat (hairpin 6452) is shown for clarity, a more extensive folding of the DNA is possible (*a*). If one assumes the indicated base pairing, one can predict the relative reactivities of individual guanine residues on the basis of "rules" for ds DNA (3, 4), as follows: ○, poor; ◒, intermediate; and ●, strong. ⊖ represents random low-level reactivity of non-base-paired guanine residues, while ⊙ denotes a guanine residue that may or may not be base paired and, therefore, whose reactivity cannot be predicted. Asterisks identify possible G·T base pairing.

binding of AFB_1 (see legend for Fig. 1) and a concomitant loss of replication blocks such that above 40% formamide the replication blocks are minimal and essentially random. Note that hairpin 6187 (6171–6207; Fig. 3*b*) is relatively rich in A·T base pairs [$\Delta G°$ of formation = −12.7 kcal/mol (1 kcal = 4.18 kJ)] and is expected to be less resistant to denaturation in comparison to a G+C-rich hairpin. Accordingly, AFB_1 reactivity at formamide concentrations in excess of 20% seems to be virtually eliminated at positions 6177–8 (Fig. 1*d*). Similarly, the "satellite" hairpin (6405–6429) depicted to the left of the "major" hairpin in Fig. 3*a* is expected to be less stable ($\Delta G°$ = −10.7 kcal/mol) due to the "loop-out" in the stem compared to the major hairpin (6439–6466; $\Delta G°$ = −33.5 kcal/mol). Accordingly, reactivity of guanine residues in the cluster 6402–6428 (Fig. 1*c*) seems to have abruptly diminished at a formamide concentration of 10%. On the other hand, reactivity of guanine residues in the major hairpin stem (6439–6466) has persisted at higher formamide concentrations (Fig. 1*c*), presumably due to the higher G+C content, as well as the length of the stem, both of which should confer resistance to denaturation. Also note that the relatively strong reaction at position 6178 (Fig. 1*b*) implies a relatively stable helical structure due to a possible G·T base pair between the guanine residue at 6178 and the thymine residue at 6200, as shown in Fig. 3*b*. (*ii*) We observed that random depurination of template DNA by acid treatment generates numerous relatively weak stops with no clear correlations with intrastrand structures (data not shown). (*iii*) Similarly, modification of the template with the alkylating agent dimethyl sulfate generates numerous replication blocks, with patterns quite distinct from those induced by AFB_1 (data not shown).

The data presented so far also show that within base-paired regions there are striking differences in the intensity of replication blocks at different guanine residues. On the basis of a previous analysis utilizing an entirely different technique, we have deduced a set of rules that govern the reaction of AFB_1 with ds DNA in terms of the local sequence context (3, 4). In Fig. 3, guanine residues have been identi-

fied with symbols representing *predicted* reactivities for ds DNA. It is clear that within the hairpin stems the relative intensities of stops (Fig. 1) are in general agreement with the rules. The requirement for duplex DNA for reactivity is underscored by the stop pattern at the dinucleotide G-G (residues 6456–7), which occurs at the boundary of the hairpin loop (Figs. 2 and 3) and is therefore likely to be relatively more prone to "breathing" in comparison to other residues within the stem. Accordingly, although the qualitative predicted (3, 4) pattern for 5′-G-G-3′ sequences (5′ residue would be intermediate and 3′ residue strong in reactivity) is retained, the overall strength is reduced in comparison to other guanine residues in the stem. Although not specifically pointed out, essentially the same observations regarding context effect rules can be made at other sites.

Other workers (5, 6) have shown that, depending on the conditions of incubation, the primary Gua-AFB_1 adduct is subject to the following secondary reactions: (*i*) loss of AFB_1 diol, resulting in an intact guanine residue; (*ii*) loss of the adducted guanine residue, resulting in an AP site; and (*iii*) guanine imidazole ring opening, resulting in the stable derivative of the type FAPyr-AFB_1. All of the data discussed so far were obtained with freshly modified DNA, and we therefore assume that the major portion of covalently bound $[^3H]AFB_1$ is in the form of the primary adduct. We have attempted to identify the nature of the chemical lesion(s) responsible for the observed replication blocks by subjecting AFB_1-modified DNA to conditions favoring either AP site formation or FAPyr-AFB_1 formation. In Fig. 1*e*, lanes 3 and 4 show that incubation under conditions favoring nearly quantitative conversion of the primary adduct to FAPyr-AFB_1 does not result in significant qualitative or quantitative changes in the pattern of replication blocks. Conditions favoring AP site formation do bring about a qualitative change in the replication block pattern in the form of new bands (arrows) not seen in the control. In the clear example at the bottom of lane 1, the new bands correspond in mobility exactly to the template guanine residues (6470–1), in contrast to the control lane, where the stop bands occur one nucleotide 3′ to these template G residues. On the basis of the previous observation (12) that replication can stop either one nucleotide 3′ or opposite a template AP site, we assume that the new bands correspond to some of the stops caused by AP sites. Since conversion of the primary adduct to FAPyr-AFB_1 should stabilize the AFB_1 moiety and therefore minimize generation of new AP sites, such "shifted-up" bands are not expected, and they are not observed (lanes 3 and 4). These observations suggest that all three types of AFB_1-induced guanine residue damage—namely, the primary adduct, FAPyr-AFB_1, and AP sites—can act as replication blocks.

DISCUSSION

The data presented here suggest that the previous observations regarding the effect of AFB_1 on DNA template function *in vivo* and *in vitro* (1, 7–9) may be attributable to modification of guanine residues. In addition, our results vividly confirm the ds DNA preference shown by AFB_1. Thus, within the ss DNA molecules examined, the preferred sites of modification are seen to be within sequences capable of assuming double-strand configuration through intrastrand base pairing. This explanation for AFB_1 hotspots in ss DNA is supported by the observation that sequence specificity within the potentially ds DNA parallels that previously reported for fully ds DNA (3, 4).

We have previously suggested a precovalent association (presumably in the major groove of B DNA) between AFB_1 and particular DNA sequences in ds DNA as a plausible mechanism for the observed sequence specificity (4). A subsequent report by Nordheim *et al.* (19) on the inhibitory effect of AFB_1 modification on the conversion of B-DNA into Z conformation is consistent with our "lock-and-key fit" model (4) for a specific precovalent association. These authors speculate that B-DNA is locked in its conformation due to possible hydrogen bonding of the AFB_1 adduct within the major groove. In accordance with the lock-and-key model (irrespective of the forces stabilizing such an intermolecular fit), the greater accessibility of the N-7 atom of guanine residues in Z-DNA (20) by itself should not enhance its reactivity with AFB_1, a prediction supported by an experiment cited by Nordheim *et al.* (19), as well as by our unpublished preliminary results.

We have speculated on the potential biological significance of AFB_1 sequence specificity elsewhere (4). Because of its striking target preferences, AFB_1 promises to be a peculiarly well-suited model for studying mutagenesis by bulky carcinogens. The data presented here suggest relatively simple procedures for the experimental determination of the folding of single-stranded polynucleotides and for the targeted delivery of AFB_1 damage to specific loci in sequences inserted into ss DNA cloning vectors.

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