Tijan, R. (1978) *Cell (Cambridge, Mass.) 13*, 165–179.
Tsutsui, K., Tsutsui, K., & Oda, T. (1980) *Eur. J. Biochem. 108*, 497–505.
Warner, J. R. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P., Eds.) p 461, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Warner, J. R. (1979) *J. Cell Biol. 80*, 767.
Weideli, H., Brock, C., & Gehring, W. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3773–3777.
Wensink, P. C., Finnegan, D. J., Donelson, J. E., & Hogenss, D. S. (1974) *Cell (Cambridge, Mass.) 3*, 315–325.
Wiegand, R. C., Godson, G. N., & Radding, C. M. (1975) *J. Biol. Chem. 250*, 8848–8855.
Williams, K. R., & Konigsberg, W. (1981) in *Gene Amplification and Analysis* (Chirikjian, J., & Papas, T., Eds.) Vol. 2, pp 475–508, Elsevier, New York.
Yeoman, L. C., Taylor, C. W., Jordan, J. J., & Busch, H. (1973) *Biochem. Biophys. Res. Commun. 53*, 1067–1076.

Covalent and Noncovalent Interactions of Aflatoxin with Defined Deoxyribonucleic Acid Sequences†

Ravindra P. Misra, Kevin F. Muench, and M. Zafri Humayun*

ABSTRACT: The major stable reaction product of activated aflatoxin B_1 (AFB1) with DNA is the N^7-guanine adduct. By using a simple extension of the Maxam–Gilbert sequencing technique on defined DNA sequences modified by activated AFB1, we have shown [Muench, K. F., Misra, R. M., & Humayun, M. Z. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6–10] that the induction of alkali labile sites is strongly and predictably influenced by the nucleotide sequence context in double-stranded DNA. In this paper, we present data that show the following: (a) sequence-specific effects are abolished in single-stranded DNA while at the same time the overall reactivity of guanine (G) residues is strongly suppressed when compared to double-stranded DNA; (b) in single-stranded DNA capable of forming intrastrand hairpin stem–loop structures, AFB1 reacts strongly with base-paired G residues in a sequence-specified manner but not as efficiently with non-base-paired G residues; (c) certain chemicals related in structure to AFB1, and the intercalating dye ethidium bromide, inhibit the reaction of AFB1 with DNA. These data are consistent with the possibility that a sequence-specific precovalent association between double-stranded DNA and AFB1 is a factor in the observed specificity in covalent reactions. We also present data on another kind of covalent AFB1 binding, namely, the formation of photoadducts. Previous work showed that AFB1, in analogy with related coumaryl chemicals, the psoralens, can be photoactivated, resulting in stable adduct formation with DNA. We show here that a significant reaction is with G residues, presumably at the N^7 position.

Aflatoxin B_1 (AFB1)[1] is a mycotoxin produced by a number of strains of the fungal genus *Aspergillus* found as a natural contaminant of the food chain (Busby & Wogan, 1979). Experimentally, AFB1 is a highly toxic substance, a powerful mutagen, and the most potent liver carcinogen described to date. In chemical terms, AFB1 is a highly substituted coumarin (Figure 1). The 8,9 double bond (previously called the 2,3 double bond) has been shown to be essential for the toxic, mutagenic, and carcinogenic properties of AFB1, since AFB2, which differs from AFB1 in not having this double bond, is less active by about 2 orders of magnitude. AFB1 requires activation before significant reaction with biological macromolecules, and activation is believed to proceed via epoxidation of the 8,9 double bond (Essignmann et al., 1977; Lin et al., 1977). Although the putative epoxide has never been isolated due to presumed reactivity, there is compelling evidence for its involvement. Activated AFB1 can react with proteins, RNA, and DNA (Miller & Miller, 1977) although the critical macromolecule is assumed to be DNA. AFB1 can be activated in vitro either by liver microsomal preparations [e.g., D'Andrea & Haseltine (1978)] or by oxidation with a mild organic oxidant like chloroperoxybenzoic acid (Martin & Garner, 1977). Recently, it has been shown that near-ultraviolet irradiation induces photobinding of AFB1 to DNA (Shieh & Song, 1980). The major stable adduct with DNA formed by the epoxide mechanism is the N^7-guanine (N^7-G) adduct (Figure 1), which alone (together with its secondary derivatives) may account for more than 90% of AFB1 stably associated with DNA. There is no direct evidence for cause–effect correlations between N^7-G modification by AFB1 and mutagenesis and carcinogenesis. However, AFB1 modification does affect the template function of DNA, as predicted for bulky DNA modifications; and since N^7-G modification is the predominant stable effect of the reaction of AFB1, biological significance has been attributed to this lesion (Miller & Miller, 1977). Moreover, the N^7 modification results in weakening the N-glycosidic bond at modified G residues such that both spontaneous and enzymatic depurination and strand scission are likely to be promoted (Singer, 1975). Both of these events are known to have biological consequences (Singer, 1975; Schaaper & Loeb, 1981).

An interesting, but largely unexplored, question on DNA modification by chemical carcinogens is the effect of nucleotide sequence environment of the target site. With reference to aflatoxin, one can ask whether all G residues react similarly on a random basis or whether the bases surrounding a given

† From the Department of Microbiology, University of Medicine and Dentistry of New Jersey—New Jersey Medical School, Newark, New Jersey 07103. *Received November 1, 1982.* This work was supported by a National Cancer Institute grant (CA27735).

[1] Abbreviations: AFB1, AFB2, AFG1, and AFG2, aflatoxin B_1, B_2, G_1, and G_2; 8-MOP, 8-methoxypsoralen; Me_2SO_4, dimethyl sulfate (DMS in figures); DMF, dimethylformamide; DS, double stranded; SS, single stranded; CT DNA, calf thymus DNA; CPB, chloroperoxybenzoic acid; bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-3351$01.50/0

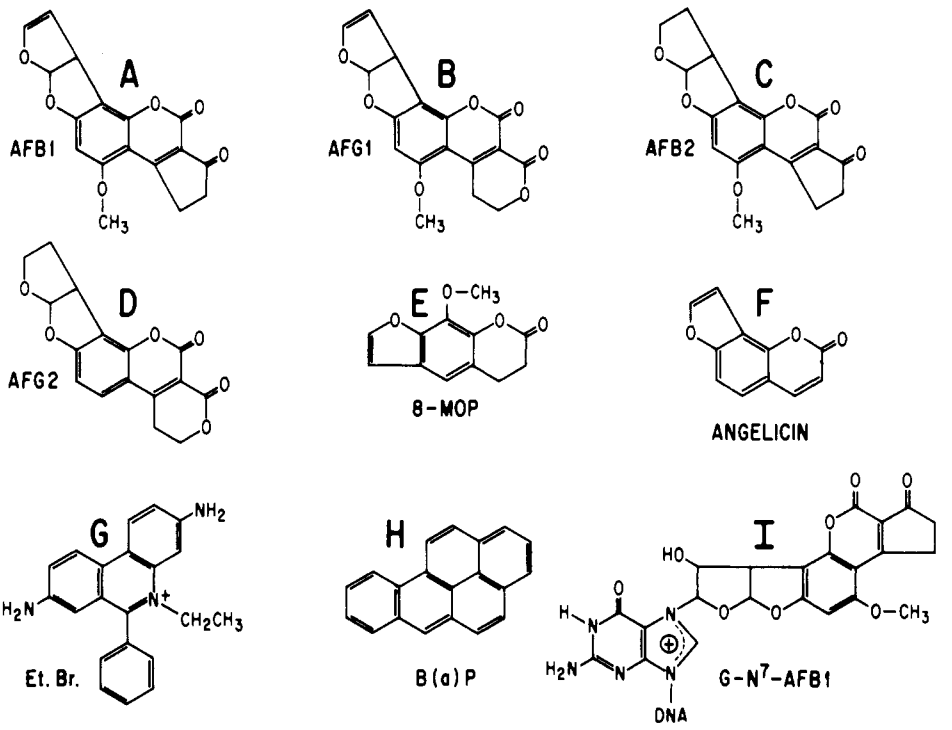

FIGURE 1: Structures of some aflatoxins and other relevant chemicals.

target ("the sequence context") have an effect on such reactions. AFB1 reacts mostly with the N^7 position of G residues to create alkali-labile sites and provides an opportunity to examine context effects on the major site of reaction of an important carcinogen. By extending methodology first described by D'Andrea & Haseltine (1978), we have initiated an investigation of this question and have reported elsewhere (Muench et al., 1983) that the relative extent of modification of G residues in double-stranded (DS) DNA is remarkably, and predictably, influenced by the sequence context. By analyzing a number of DNA fragments of known sequence, it was possible to deduce a set of empirical "rules" that predict the relative reactivity of a given G residue within a known DNA sequence (Muench et al., 1983; summarized in Table I). In general, G residues flanked by AT sequences are poor targets for AFB1-induced alkali-labile lesions while certain, *but not all*, G residues in GC bp clusters are strong targets; G residues in some sequence contexts are intermediate targets. In semiquantitative terms, intermediate targets react 2–4 times faster than poor sites while strong sites react approximately 10 times faster. While the data presented previously did not permit a deduction of the mechanistic basis for the observed specificity, certain speculations were made. Specifically, one possibility suggested was a precovalent, sequence-determined association between DS DNA and AFB1, such that mass action could account for differences in the reactivity of G residues; in this hypothesis, it is assumed that AFB1 does not diffuse freely along DNA. In the second possibility, freely diffusing AFB1 "recognizes" specific conformational features on DNA and reacts differentially with targets; it is assumed that specific noncovalent binding does not precede covalent reaction.

In this paper, we describe experiments that suggest that specifically bound—rather than freely diffusing—AFB1 may be responsible for the highly sequence-specific reaction with DNA. We also show that AFB1 can be used as a chemical probe to determine the occurrence of predicted hairpin structures in single-stranded DNA at the sequence level. In other experiments, we report that a significant reaction of photoactiviated aflatoxins is the induction of alkali-labile sites at G residues.

Table I

(1) Single G residues flanked by A-T nucleotides [$(A/T)_nG(A/T)_m$ where n and $m = 1$ or more] are poor targets.
(2) In GC bp clusters flanked by A-T nucleotides [e.g., $(A/T)_nGG(A/T)_m$], the following patterns are observed.
  (a) In the dinucleotide GG the 5′ G is an intermediate target while the 3′ G is a strong site.
  (b) In the dinucleotide CG the G residue is an intermediate site.
  (c) In the dinucleotide GC the G is a poor site.
  (d) In the trinucleotide CCG the G residue is a strong site, while in GCC it is a poor site.
  (e) In the contiguous G clusters GGG and GGGG, the 5′ and 3′ residues are intermediate, and the middle residues are strong sites.
  (f) The sequence-specific effects observed in GC bp clusters at the dimer and trimer level are essentially retained in longer GC bp clusters, except for certain additive effects. For example, the 5′ G residues in the clusters *GC*, *GCC*, *GCCC* and *GCGGC* are all poor targets; similarly, the G residues in CC*G* and in CC*GCCGCCG* are all strong sites.
(3) The observed specificity is independent, within the limits of the experimental conditions used, of (a) methods of activation of AFB1, (b) source (prokaryotic or eukaryotic) of DNA, (c) dosage, (d) reaction time, and (e) ionic strength and thus appears to be an intrinsic feature of AFB1-DNA interactions (Muench et al., 1983).

Materials and Methods

Chemicals. Chemicals were from the indicated commercial sources and were used without further purification: AFB1, AFB2, AFG1, and AFG2 (Calbiochem); 8-methoxypsoralen (8-MOP) and benzo[*a*]pyrene (Sigma); angelicin (H.R.I. Associates, Emeryville, CA); chloroperoxybenzoic acid (CPB) (Aldrich); [^{3}H]AFB1, better than 98% pure, 14 Ci/mmol (Moravek Biochemicals, Brea, CA).

DNA Preparations. Phage ϕX174 replicative-form (RF) DNA and plasmid pBR322 DNA were prepared as described (Muench et al., 1983). Double-stranded (DS) DNA fragments

were prepared in specific 5′-end-labeled form as described (Humayun et al., 1977). Single-stranded (SS) DNA fragments were prepared from corresponding DS DNA fragments on strand-separation gels as described (Maxam & Gilbert, 1980). Calf thymus (CT) DNA was obtained from Sigma and was sheared by sonication to an average size of 5 kb and dialyzed against 10 mM Tris-HCl and 1 mM EDTA, pH 8, before use.

Aflatoxin Modification of DNA. A total of 1.8 nmol of [^{3}H]AFB1 and, where specified, appropriate quantities of other chemicals dissolved in CH_3OH or CH_2Cl_2 were transferred to a glass tube (10 × 75 mm) and dried down under vacuum. A total of 10–100 ng of 5′-end-labeled DNA and 1 μg of CT DNA (carrier) were dissolved in 0.2 mL of 20 mM sodium phosphate, pH 7.4, and added to the reaction tube. A 10 M excess (relative to AFB1) of CPB was added in the form of 10 μL of a freshly prepared CH_2Cl_2 solution. The reaction tube was immediately stoppered and shaken at a 45° angle at 37 °C in a water- or air-bath shaker for 30 min. The reaction was terminated by quenching with 5 μL of 2-mercaptoethanol, and the unreacted small molecules were removed by one $CHCl_3$ extraction and three successive ethanol precipitations of the DNA. Mock reactions with no CPB or no AFB1 were always carried out in an identical manner. Covalent binding of AFB1 was measured as the ^{3}H count difference between test and mock reactions. The mock reaction (no CPB) counts were usually $^1/_{40}$th or less when compared to the test. Similar protocols were used for other chemicals except that nonradioactive forms were used. The above protocol was based on the original procedure described by Martin & Garner (1977) and represents a further simplification of the modification previously described (Muench et al., 1983), in which a two-phase reaction mixture was employed. No significant differences were noticed in the results obtained by these two (Muench et al., 1983; this work) procedures. Martin & Garner (1977) and Muench et al. (1983) have reported that CPB activation results in DNA modification very similar to or identical with that caused by AFB1 activated by metabolic enzymes (microsomes) in vitro. In a few experiments, the microsome activation procedure (Muench et al., 1983) was used as indicated in legend for Figure 5.

Photobinding of Aflatoxins to DNA. Various concentrations of [^{3}H]AFB1, AFB2, AFG1, AFG2, 8-MOP, or angelicin (Figure 1) were dried down in a glass tube. A 10–100-ng aliquot of 5′-end-labeled DNA and 1 μg of CT DNA were dissolved in 0.1–1.0 mL of 20 mM sodium phosphate, pH 7.4, and added to the reaction tube. Oxygen was stripped from the solution by flushing with N_2 gas, and the contents were irrradiated at 350 nm in a Rayonet Model RPR-100 photoreactor with a $CuSO_4$ filter at an intensity of 9200 W/m^2 for various times. Mock reactions had no AFB1 or other chemicals. After the reaction, the DNA was freed from the small molecules as described above for the chemically activated AFB1 reaction, and, in the case of AFB1, the extent of binding was determined by ^{3}H counting.

Analysis of Alkali-Labile Lesions on DNA. DNA modified with various chemicals was dissolved in 1.0 M piperidine, heated at 90 °C for 30 min, and worked up for high-resolution sequence-gel analysis as described by Maxam & Gilbert (1980). Approximately equal amounts (by ^{32}P Cerenkov count) of the degraded DNAs were loaded on gels together with controls and the Maxam–Gilbert G-specific [dimethyl sulfate (Me_2SO_4) reaction] reaction products of previously unmodified DNA, the latter being used as a set of markers to identify G residues. Autoradiographs were scanned by a Zeineh soft laser scanning densitometer, Model SL-504-XL. Most of the chemicals used are highly toxic and carcinogenic (especially AFB1) and must be handled with appropriate precautions to eliminate exposure.

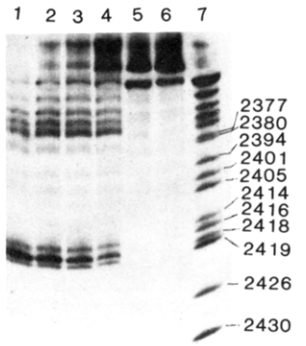

FIGURE 2: Autoradiograph of a 20% sequence gel (Maxam & Gilbert, 1980) on which AFB1-induced DNA degradation products were fractionated. Approximately 180 bp long φX DS DNA fragment was obtained by *Alu*I (φX position 2264; Sanger et al., 1978) and *Hin*fI (position 2445) digestion and was labeled in the (–) strand. The DNA was subjected to standard AFB1 modification in the absence (lane 1) or presence of 20% (lane 2), 30% (lane 3), 40% (lane 4), 50% (lane 5), and 60% (lane 6) DMF. Piperidine degradation products were fractionated along side the standard Maxam–Gilbert G track (lane 7). The numbers identify G residues while the DNA sequence of the relevant region is presented in Figure 3.

Results

Reaction of AFB1 with G Residues in SS DNA Is both Random and Suppressed When Compared to DS DNA. Figure 2 shows an autoradiograph of a sequence gel on which AFB1-induced degradation products from a φX DS DNA fragment were analyzed. A densitometric scan of some of the lanes in Figure 2 is given in Figure 3, together with the sequence of the relevant part of the DNA fragment. Lane 7 (Figure 2) and the top trace (Figure 3) represent the standard Maxam–Gilbert dimethyl sulfate induced G pattern. Lane 1 (Figure 2) and the middle trace (Figure 3) represent the AFB1-induced degradation of the same fragment. Lanes 2–6 (Figure 2) represent degradation products obtained by including various concentrations of an inert denaturant, dimethylformamide (DMF), during AFB1 modification. The bottom trace in Figure 3 is that of lane 5 (Figure 2) and shows the effect of DMF at a concentration of 50%. Analysis of these data, with reference to the sequence-specifity rules given in Table I for AFB1 with DS DNA, permit the following conclusions: (a) With this DNA fragment, AFB1 has reacted in accordance with the rules; specifically note the striking differences between the predicted strong, intermediate, and poor reacting sites. (b) With increasing denaturant concentrations, both the extent of reaction and the striking sequence specifity are inhibited. This effect is first noticeable for this DNA fragment at 40% DMF and is virtually complete at 50%. At the latter concentration, the G-specific reaction is uniform (i.e., random) and only slightly above the background but can still be detected. The most likely explanation for the effect of the denaturant is the transition of the DS DNA into SS form. It is unlikely that DMF has a direct inhibitory effect on AFB1 reaction because (a) at "lower" DMF concentrations, there are actually substantial concentrations of DMF and yet no effect on the degradation pattern (lanes 2 and 3) and (b) the DMF concentration necessary to bring about the inhibition is different for fragments of different size and base composition (data not shown). Thus, unlike other alkylating agents that react with the N^7 position of guanine, AFB1 reaction with SS

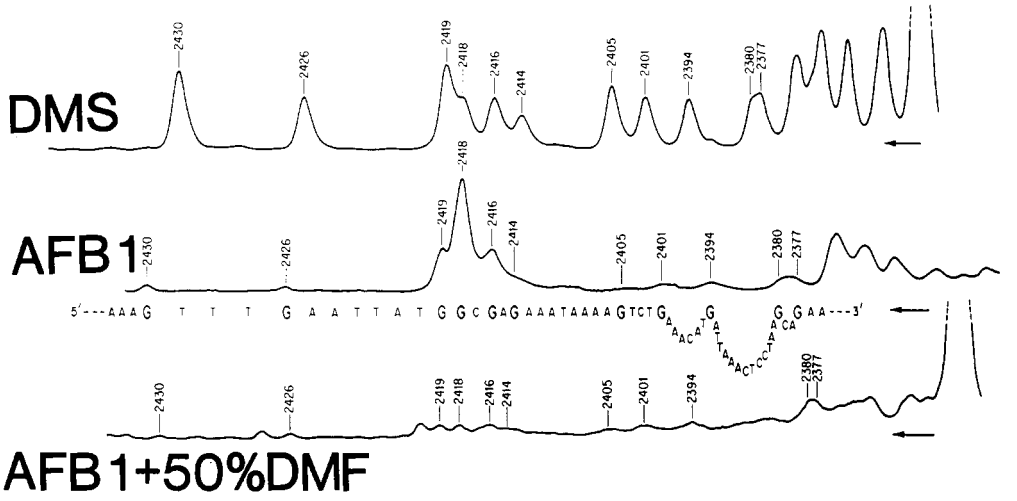

FIGURE 3: Densitometric traces of autoradiograph presented in Figure 2 superimposed on the DNA sequence of the (−) strand (written 5′ to 3′) of the relevent region of ϕX174. The top trace is that of lane 7 (Maxam–Gilbert G track) and shows the well separated peaks corresponding to G residues 2430–2377. The middle trace is that of lane 1 (no DMF) and shows that only G residues at positions 2419, 2418, and 2416 have reacted significantly, with 2418 being a strong site. The bottom trace (50% DMF) shows that AFB1 reactivity with all G residues is suppressed and is more or less random.

DNA is severely suppressed when compared to DS DNA under our experimental conditions, a situation opposite that predicted for a freely diffusing reactant. We have confirmed in similar experiments that DMF (10–80%) does not inhibit the reaction of Me_2SO_4 and that DMF by itself does not induce alkali-labile lesions in DNA (data not shown).

AFB1 Preferentially Reacts with Base-Paired G Residues in Single-Stranded DNA Capable of Hairpin Formation. We have previously reported that G residues in SS DNA incapable of forming extensive intrastrand secondary structures react more or less randomly with ABF1 (Muench et al., 1983). How does AFB1 react with SS DNA capable of forming hairpin stem–loop structure? On the basis of the information provided so far, one would predict the following: (a) base-paired G residues will react strongly; (b) base-paired G residues will react with AFB1 in accordance with rules for DS DNA; and (c) non-base-paired G residues will react poorly and more or less randomly.

The genome of single-stranded DNA phage ϕX174 has a number of DNA sequences theoretically capable of forming intrastrand base pairing. Godson et al. (1978) have specifically pointed out three possible hairpin structures that may form in three untranslated regions, occurring between genes H and A, genes J and F, and genes F and G. The H/A and J/F hairpins are implicated in transcription termination, and the H/A hairpin, in addition, contains the gene A mRNA start sequence. The F/G sequence has been implicated in the initiation of the negative-strand synthesis (Shlomai & Kornberg, 1980).

Figure 4 shows the predicted J/F and H/A stem–loop structures, as given by Godson et al. (1978). The structures of both strands in the J/F region are presented. The J/F hairpin stem is 6 base pairs (bp) long while the H/A stem is 8 bp in length. Both stems are perfectly base paired with no secondary loops or mismatches. The predictions for an AFB1 degradation analysis of the J/F upper (+) strand are the following: (1) only the four G residues (970–973) should react significantly; (2) the residues should follow one of the AFB1 rules, namely, "5′ and 3′ G residues in contiguous G trimers and tetramers should be intermediate in reactivity while the internal G residues should be highly favored"; (3) other G residues should react poorly but more or less uniformly. Similar predictions hold for the four G residues (983–980) in

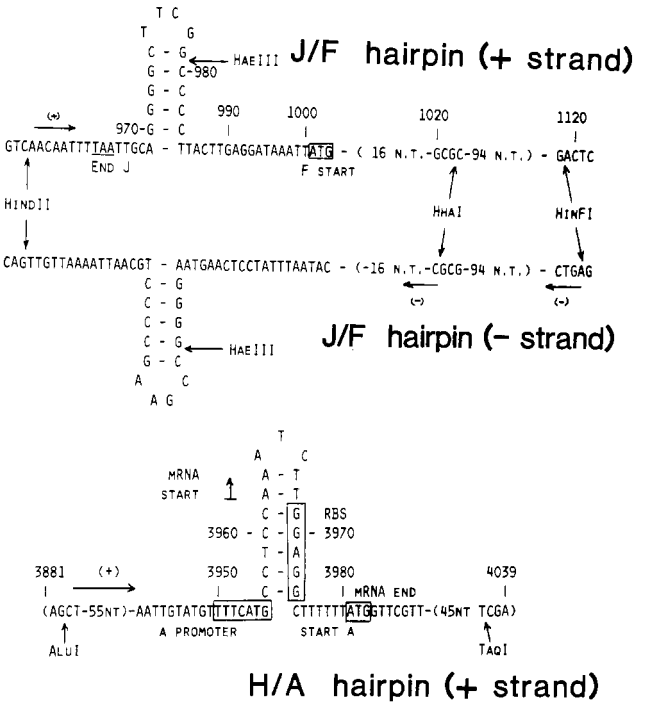

FIGURE 4: Nucleotide sequences of gene H/A and J/F intergenic regions of ϕX174 viral DNA showing predicted hairpin structures [adapted from Godson et al. (1978)]. Some relevant regulatory signals and restriction sites are shown. RBS is ribosome binding site.

the J/F (−) strand. In the H/A (+) strand, the G residues at positions 3956, 3969, 3970, 3972, and 3973 should react significantly. In addition, following the rule "in G dimers the 5′ residue is intermediate in reactivity while the 3′ residue is a strong reactor", the G residues at positions 3970 and 3973 should be the strongest sites.

Figure 5A, lane 1, shows the Maxam–Gilbert G track (Me_2SO_4 degradation) for DS DNA labeled in the (+) strand in the J/F region of ϕX, with the G residues identified by position numbers. Lane 2 shows the same DNA region in the single-stranded form subjected to Maxam–Gilbert Me_2SO_4 degradation and, as expected, shows all the G residues. Lane 3 shows AFB1 degradation of the same DNA as in lane 2. Only the four G residues at positions 970–973 have reacted

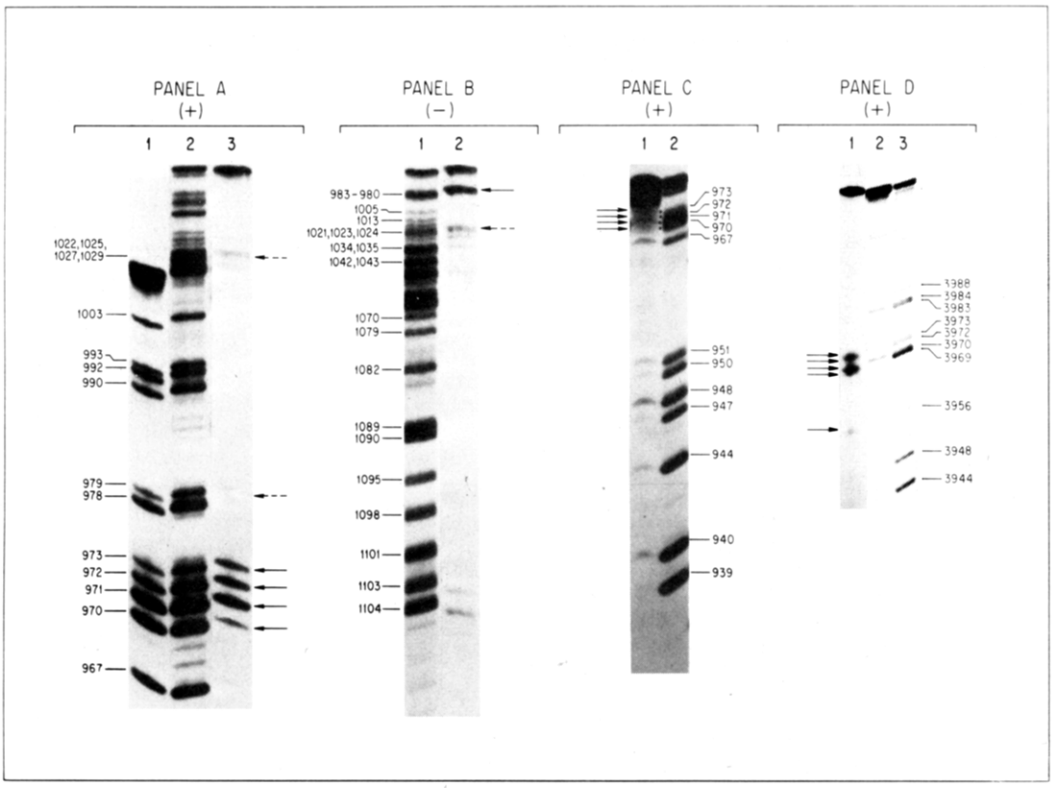

FIGURE 5: Composite autoradiograph of sequence gels on which AFB1- and Me_2SO_4-induced degradation products derived from ϕX DS or SS DNA were analyzed. The symbols (+) or (−) at the top of each panel denote the labeled strand. Panels A, B, and C represent analysis (20% sequence gels) of the J/F intergenic region while panel D (8% sequence gel) shows the data on the H/A intergenic region. (Panel A) (Lane 1) 69-bp ϕX DS DNA fragment [*Hin*dII and *Hha*I digestion, positions 951 and 1020, respectively; see Godson et al. (1978) and Figure 4] subjected to Me_2SO_4 degradation; (lane 2) 167-nucleotide (nt) ϕX SS DNA fragment (*Hin*fI and *Hin*dII; positions 1118 and 951) subjected to Me_2SO_4 degradation [this SS DNA fragment was isolated by separating the strands from the corresponding DS DNA fragment (Maxam & Gilbert, 1980)]; (lane 3) the same SS DNA fragment as in lane 2, subjected to AFB1- (microsomes) induced degradation. (Panel B) (Lane 1) 167 nt long (−)-strand SS DNA fragment subjected to Me_2SO_4 degradation (this fragment is the complementary strand of the DNA used in lanes 2 and 3, panel A, and was isolated in the same manner); (lane 2) AFB1 (microsomes) degradation analysis of the same fragment. (Panel C) (Lane 1) 50-nt (+)-strand SS DNA fragment (*Hha*I and *Hae*III digestion; positions 927 and 978) subjected to AFB1- (microsomes) degradation as above; (lane 2) the same fragment subjected to Me_2SO_4 degradation. It should be noted that lane 1 in panel C has been overexposed to show the low level of G degradation caused by AFB1. In contrast to the four G residues 970–973 in panel A (lane 3) that stand out against the background, the same G residues in the shorter DNA fragment shown in panel C (lane 1) do not stand out in comparison to other G residues in the same track. (Panel D) (Lane 1) 158-nt (+)-strand SS DNA fragment (*Alu*I and *Taq*I digestion, positions 3881 and 4039, respectively), and strand separation subjected to AFB1 (CPB) degradation; (lane 2) the same DNA as in lane 1 in double-stranded form subjected to AFB1 (CPB) degradation; (lane 3) the same DS DNA subjected to Me_2SO_4 degradation. Solid arrows indicate observable degradation at position 3956 and significant degradation at positions 3969–3970 and 3971–3973 although the bands are not well resolved.

significantly and follow the rule referred to above. This specific degradation at positions 970–973 is inhibited at DMF concentrations greater than 30%, presumably due to melting of the stem–loop structure (data not shown).

Figure 5B, lane 1, shows the Maxam–Gilbert Me_2SO_4-induced G track of the (−) strand covering the J/F hairpin region. Lane 2 shows AFB1-induced degradation of the same fragment. Although the four G residues (980–983) predicted to be involved in the stem are not well resolved [among themselves and from other immediately neighboring G residues; see Sanger et al. (1978) for sequence] in this part of the gel, strong degradation has occurred only at these positions, but not at other G residues. Figure 5C compares the AFB1 and Me_2SO_4 degradation patterns for a (+)-strand fragment from the same region as that in Figure 5A except that the fragment had been enzymatically dissected such as to interrupt the proposed hairpin stem. This dissected 50 nucleotide long SS DNA fragment had the same (+)-strand sequences up to position 979 [site of *Hae*III digestion is between 979 and 980; see Figure 4, J/F hairpin (+) strand], but because of the loss of C residues at 980–983, the hairpin cannot form. It can be seen that all G residues in lane 1 (AFB1) have reacted poorly, including the residues shown in the undissected fragment (Figure 5A, lane 3) to be involved in intrastrand base pairing.

Figure 5D, lane 3, shows the Me_2SO_4-induced G track of double-stranded DNA in the H/A region, while lane 2 shows an AFB1-induced pattern of the same DNA. Lane 1 displays the AFB1 pattern of the same DNA in single-stranded form and reveals that significant degradation has only occurred at positions 3956, 3969, 3970, 3972, and 3973; moreover, as predicted, reaction at 3970 and 3973 is stronger than at other sites. It is worth noting that in panels A (lane 3) and B (lane 2) of Figure 5, although most non-base-paired Gs have reacted so poorly that they are not detectable under the exposure conditions, some G residues are detectable (broken arrows indicate the stronger secondary residues). We assume that these reflect the formation of less stable secondary structures in these DNAs. Accordingly, we note that in the J/F (+) strand, the complement for the pentanucleotide TCGGC (976–980) occurs at position 1022–1026 (GCCGA; Sanger et al., 1978) and potential for base pairing between these two sequences exists. The stronger secondary G bands in Figure 5A (broken arrows) correspond to these regions. Moreover, in the (−) strand (Figure 5B), some of the stronger secondary bands (broken arrow) correspond to the G residues 1023 and 1024, which are a part of the same sequence. It should be noted that this secondary reaction must compete with the major J/F hairpin since a part of the TCGGC sequence is also

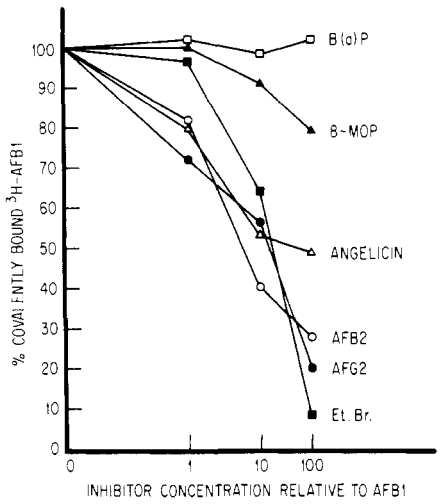

FIGURE 6: Effect of various chemicals on covalent binding of [^{3}H]-AFB1 to DNA. A 174 bp long pBR322 DNA fragment (*Eco*RI and *Hae*III digestion, positions 0 and 174, respectively; Sutcliffe, 1978) 5′ end labeled in the upper strand with ^{32}P together with 1 μg of CT DNA was used in these experiments. The reactions were carried out as described under Materials and Methods. 100% represents the amount of activated [^{3}H]AFB1 bound in the absence of additional ligands. Under the experimental conditions, 100% reaction represented 5–6 pmol of total bound [^{3}H]AFB1.

a part of the J/F hairpin stem depicted in the (+) strand in Figure 4. Even if such secondary interactions are unstable and infrequent, it is interesting that such interactions are demonstrable.

The data presented in Figure 5 experimentally verify the predictions made and support the differential and sequence-specific covalent reaction of AFB1 with DS as compared SS DNA under our experimental conditions. In addition, these data suggest that reagents like AFB1 might provide a useful approach to detect intrastrand secondary structures at the sequence level.

Certain AFB1 Structural Analogues and the Intercalative Dye Ethidium Bromide Inhibit Reaction of AFB1 with DS DNA. AFB2 is identical in structure with AFB1 except that it does not have the 8,9 double bond found in AFB1 (Figure 1). AFB2 cannot be activated to a significant extent by chemical or enzymatic procedures in vitro (unpublished observations) and is in effect a nonreacting analogue for AFB1. If the sequence specificity exhibited by AFB1 is due to mass action as a result of preferential association with sequences before covalent reaction, including AFB2 may lead to one or both the following effects (without excluding other possibilities) on the reaction of AFB1 with DS DNA. (1) An inhibition of the extent of AFB1 covalently bound and (2) a reduction or abolition of sequence specificity. Similarly, the related chemical AFG2 may be able to compete for binding sites on DNA, while the more distantly related ligands angelicin and 8-MOP may be less effective and the unrelated carcinogen benzo[*a*]pyrene may be ineffective (Figure 1). Figure 6 shows the effect of including these chemicals on the extent of binding of [^{3}H]AFB1 to defined DNA fragments. Analyses of these data show that AFB2, AFG2, and angelicin do inhibit (approximately 75% at the maximal concentration for AFB2 and AFG2; 50% for angelicin) the reaction of AFB1 with DS DNA. 8-MOP, another psoralen, is less effective, displaying only 20% inhibition, while benzo[*a*]pyrene is seen to be an ineffective competitor.

When ethidium bromide, the classic example of an intercalating ligand, is included in the reaction mixture, AFB1 reaction with DNA is inhibited. While it is not possible to conclude from the effect of ethidium bromide that AFB1 binds by intercalation, it is clear that intercalation of an unrelated ligand is sufficient to perturb the reaction of AFB1 with DNA.

Alkali-degradation analysis of DS DNA fragments modified with AFB1 in the presence of various ligands (data not shown) suggested the following. With increasing inhibitor concentrations (AFB2, AFG2, and ethidium), there was a progressive inhibition of alkali lability such that more and more DNA remained undegraded. At maximal concentrations used, ethidium also abolished AFB1 sequence specificity, while AFB2 and AFG2 reduced such specificity.

A Significant Consequence of AFB1 Photobinding to DNA Is the Creation of Alkali-Labile Sites at G Residues. Like the skin-sensitizing psoralens, AFB1 is a coumarin derivative, and like the psoralens, AFB1 has been shown to be capable of photobinding to DNA. Photobound AFB1 has been shown to block the template function of DNA. The available data (Shieh & Song, 1980) suggest that the 8,9 double bond is essential for photobinding and that AT sequences are more reactive compared to GC sequences. In analogy with the psoralens, a photocycloaddition reaction with pyrimidine bases has been proposed for the photobinding of AFB1. However, proof for such an adduct is not available, and other products are not eliminated (Shieh & Song, 1980).

Figure 7a (lanes 2 and 3) shows that the reaction of photoactivated AFB1 with defined DNA sequences leads to alkali-labile sites at G residues, and Figure 7b shows that the reaction is clearly dependent on the UV dose. The resulting G-specific degradation shows elements of sequence specificity although the number of sequences examined is too low to determine whether the same rules as those followed by epoxidized AFB1 are followed.

Similar results were obtained with AFG1 (not shown). In order to examine whether the 8,9 double bond is necessary for the creation of G-specific alkali lability, identical reactions were carried out with AFB2 and AFG2 (Figure 7a, lanes 4–7) and angelicin and 8-MOP (not shown). From the results, both AFB2 and AFG2 were found to be capable of inducing alkali lability at G residues while neither of the psoralens was capable of inducing such lesions (data not shown).

Discussion

Context Effects in Carcinogen–DNA Interactions. It is clear that all DNA in a higher organism exposed to chemical carcinogens is not equally affected. For example, many carcinogens exhibit tissue or organ specificity in both DNA modification and tumor induction. More recent evidence suggests that even at the subcellular level, damage is not randomly distributed in DNA. We have attempted below to list the types of nonrandom DNA damage observed.

(*A*) *Locus Specificity.* Several carcinogens, including benzo[*a*]pyrene (Backer & Weinstein, 1980) and AFB1 (Niranjan et al., 1982), preferentially react with mitochondrial DNA. It has been argued that chromosomal DNA attached to the nuclear membrane may represent a preferred target for radiation-induced damage (Cole et al., 1980).

(*B*) *Domain Specificity.* The simplest level of chromatin architecture is the nucleosome, which creates "linker" and "core" domains of DNA. A number of carcinogens have been shown to preferentially react with linker domains [for a brief recent review, see Bailey et al. (1980)]. It has been reported that benzo[*a*]pyrene prefers transcriptionally active regions over untranscribed regions (Arrand & Murray, 1982), and this can be cited as an example of domain specificity.

(*C*) *Intrinsic or Sequence Specificity.* The most basic level at which the reaction of a carcinogen could deviate from

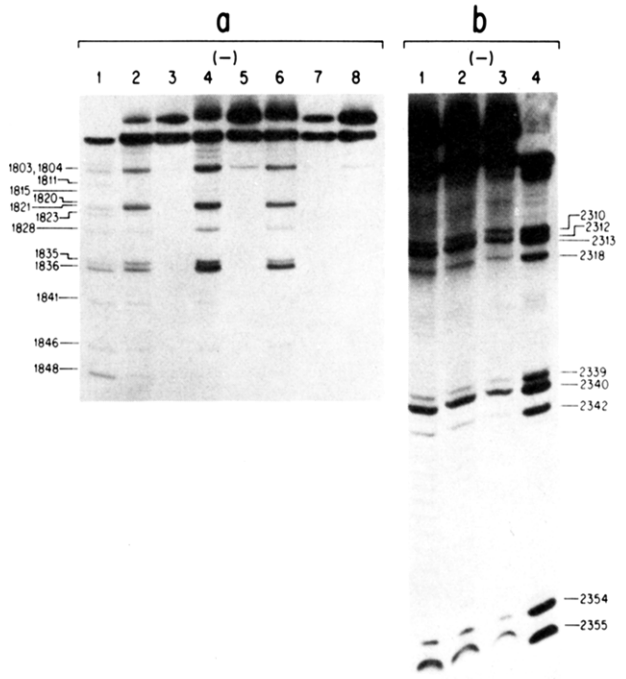

FIGURE 7: Effect of photoactivated aflatoxins on DNA. (−) at the top indicates the labeled strand. (Panel a) A 88 bp long ϕX DS DNA (*Hae*III, 1776; *Hha*I, 1864) fragment together with 1 μg of carrier DNA subjected to photoadduct formation with AFB1, AFB2, and AFG2. The irradiation was for 1 h in the presence of 27 nmol of the appropriate aflatoxin in a volume of 1 mL as described under Materials and Methods. The reacted DNA was subjected to base degradation as described, and the fragments were resolved on 20% sequence gels. (Lane 1) The Maxam–Gilbert G track; (lanes 2, 4, and 6) photoreactions with AFB1, AFB2, and AFG2, respectively; (lanes 3, 5, and 7) unirradiated controls with AFB1, AFB2, and AFG2 respectively; (lane 8) an irradiated control in the absence of any aflatoxin. (Panel b) Irradiation dose–response of AFB1-induced G degradation of a 101 bp long ϕX DS DNA fragment (*Hin*fI, 2264; *Hha*I, 2365) together with 1 μg of carrier DNA. The reaction was carried out in 0.1 mL. (Lane 4) Maxam–Gilbert G track; (lanes 3, 2, and 1) photoreaction in the presence of 1.8 nmol of AFB1 with irradiation times of 1, 3, and 5 h, respectively. The enhanced degradation after longer irradiation times correlated with increased levels of covalently bound [^{3}H]AFB1. Thus, the picomoles of [^{3}H]AFB1 found at these irradiation times were 14, 39.5, and 49.8, respectively, per microgram of DNA.

randomness is at the sequence level. Thus, if one considers the N^7 atom in guanine ("the target"), certain guanine residues may be preferred over others in a manner specified by the nucleotide sequence context. It is reasonable to expect such nonrandom reactivity on the basis of the current understanding of the chemistry of carcinogens and the three-dimensional structure as well as the dynamic nature of DNA. For example, greater reactivity of the N^7 atom with (freely diffusing) carcinogens has been predicted for the Z DNA conformation, which itself is influenced by DNA sequence (Wang et al., 1979). Similarly, dynamic properties such as breathing, localized ligand-induced structural changes, and hairpin formation at inverted repeat sequences could affect carcinogen action.

Experimentally, sequence-specified variations in covalent and noncovalent binding of dyes, antibiotics, and chemotherapeutic agents have been described [e.g., Neidle (1979), Lloyd et al. (1979), Bennett (1982), and Tullius & Lippard (1982)]. Despite previous speculation [e.g., Pegg (1977)], a largely unexplored question has been the effect of nucleotide sequence environment on the reaction of carcinogens. The data of Muench et al. (1983) and the present work make it clear that strong context effects do operate on the reaction of the carcinogen AFB1 with DNA. Indirect evidence also suggests that the acridine/half-mustards ICR191 and ICR170 prefer certain sequences [for recent data and review, see Calos & Miller (1981)]. Examination of this question with reference to other carcinogens is of considerable interest.

Mechanism of AFB1 Sequence Specificity. How does AFB1 "choose" particular sequences? We have previously speculated on two possibilities. (a) AFB1 diffuses freely along DNA and recognizes structural features peculiar to certain sequences. Alternatively, initial modification of G residues in certain contexts makes neighboring residues more (or less) accessible to further modification. Thus, all G residues are not inherently equally reactive. (b) All G residues are equally reactive with AFB1, and this accounts for the level of modification of poor targets. However, AFB1 does not diffuse freely along the DNA but specifically binds to particular DNA sequences prior to covalent reaction such that mass action will determine enhanced reactivity at intermediate and strong sites. Such binding must be both sequence specific and orientation specific for the enhanced reaction to occur, since all G residues within GC bp clusters are *not* equally reactive. For example, the G residue in the sequence 5′-GCC-3′ is a poor target while in 5′-CCG-3′ it is a strong target. In other words, a specific lock-and-key fit of AFB1 at particular sequences is possible.

The data presented in this paper are consistent with the latter possibility, although proof requires further investigation. First, these data suggest that in SS DNA, G residues react randomly and, compared to DS DNA, weakly. It is difficult to explain this result if AFB1 diffused freely along DNA since the effect of single-stranded conformation on other N^7-G alkylating agents is the opposite, i.e., enhancement of reactivity (Singer & Kusmierek, 1982). The differential reactivity of AFB1 with SS DNA has been shown in the presence of a denaturing inert solvent and by using SS DNA capable of intrastrand base pairing. Second, nonreactive structural analogues of AFB1 inhibit its reaction with DNA and reduce sequence specificity. At the same time, a more distantly related coumarin derivative (8-MOP) and the unrelated carcinogen benzo[*a*]pyrene are less effective or ineffective, although both react with DNA under appropriate conditions. The simplest interpretation of these data is that the same binding sites are occupied by the interfering ligands. On the other hand, ethidium bromide, an efficient DNA intercalator, is likely to inhibit AFB1 reaction because of changes induced in the three-dimensional structure of DNA as a result of intercalation. The effect of the inefficiently activated analogues (AFB2 and AFG2) and the unrelated intercalator ethidium provide examples of interactions among carcinogens at the molecular level [cf. Tullius & Lippard (1982)].

It is interesting to consider the relationship between DNA "breathing" effects and reactivity of G residues. Here, one could suggest that not only GC-rich but also AT-rich sequences have specific noncovalent binding sites for AFB1. However, because of facilitated breathing of the latter sequences, binding near G residues flanked by A and T sequences is less stable and unproductive. Several preliminary observations argue against correlations between breathing effects and poor targets. First, certain G residues in GC by clusters are poor targets (e.g., *G*C, *G*CC, *G*CCC, *G*CGCC, etc.; Muench et al., 1983); in other words, poor targets are not always correlated with AT sequences. Second, the size of the AT cluster in which a G residue is embedded does not affect the reactivity; thus, the expectation that the larger clusters (i.e., greater propensity to breathe) will lead to lower binding and smaller clusters (a single A or T on both sides of G) will lead to greater binding

is not fulfilled [see Muench et al. (1983)]. There are interesting anticorrelations between known stacking preferences and observed reactivities. Thus, in the GC sequence, bases are more extensively stacked when compared to the CG sequence, and this might, in part, account for the relative unreactivity of the former.

Binding of Photoactivated Aflatoxins to DNA. We have presented evidence to show that a significant consequence of photobinding of ABF1 to DNA is the induction of alkali-labile sites at G residues. Our data do not permit a deduction of the fraction of photobound AFB1 responsible for damage to G residues or indeed whether any AFB1 is bound to G residues at all. Shieh & Song (1980) have suggested that a photocycloadduction to pyrimidine is likely, and we assume that a substantial proportion of the bound AFB1 may be of the latter type. At the same time, it is commonly held that the major type of damage leading to an alkali-labile G residue is N^7 modification. If the photoactivated AFB1 in fact does bind at this position, it is curious that a preexisting 8,9 double bond is not necessary for this reaction. However, neither angelicin nor 8-MOP, both of which have the coumaryl double bond, gives the same reaction under identical conditions. Shieh & Song (1980) have suggested that the photoreactive state is the AFB1 triplet. In any event, it is clear that, in the case of AFB1, both metabolic activation and photoactivation lead to significant reaction at G residues, probably at the N^7 position. The photoreaction of AFB1 raises the possibility that skin could be a target tissue for DNA modification by this carcinogen.

Reaction of AFB1 with Hairpin Structures. The reaction of AFB1 with SS DNA capable of stable intrastrand base pairing deserves further comment. Nucleotide sequence data accumulated over the past decade show the ubiquitous presence of inverted repeat sequences with a potential for forming intrastrand Watson–Crick base pairing to generate the so-called hairpin structures. Such sequences are frequently associated with origins of DNA replication (Shlomai & Kornberg, 1980) and termination of transcription (Platt, 1981) and are thus thought to be of regulatory significance. However, such structures also occur apparently unassociated with identified functions.

Thermodynamic considerations permit the derivation of rules for estimating the probability of hairpin formation in single-stranded nucleic acids (Tinoco et al., 1973). There is compelling experimental evidence for the formation of hairpin structures in single-stranded as well as double-stranded nucleic acids. In general, experimental approaches for their demonstration exploit the differences in the physical characteristics of single-stranded vs. double-stranded nucleic acids or exploit nuclease specificities and are nonspecific in the sense that the actual nucleotide pairs formed are not demonstrable. Electron microscopy, often in conjunction with chemical cross-linking, permits the demonstration of hairpin stem–loop formation [see, e.g., Shen et al. (1979)]. However, this approach is not applicable to short hairpins and is imprecise at the sequence level. Hairpin formation in DS DNA is demonstrable indirectly, for example, by nicking of the loop regions with single-strand specific enzymes (Lilley, 1980). A direct experimental approach for demonstrating intrastrand base pairing at the sequence level is not available but is useful.

The data presented here suggest that AFB1 and similar chemicals can be exploited for demonstrating the existence of hairpins and for studying the parameters affecting such structures. Although AFB1 can only be used as a probe for GC but not AT bp formation, most proposed hairpins do have some GC base pairing. A corollary of these observations is that DNA present in single-stranded form in vivo (e.g., as the loop of a hairpin) is less likely to be a target for AFB1, quite irrespective of its sequence. An interesting recent hypothesis (Groudine & Weintraub, 1982) has linked transcriptional activity to stable, heritable single-stranded regions in higher eukaryotic cells. A caveat in this speculation is the unknown effect of any protein(s) bound to DNA. Finally, the enhanced reactivity of DS vs. SS DNA sequences suggests a method for site-enhanced mutagenesis of sequences cloned into M13 vectors.

Potential Significance of AFB1 Sequence Specificity. The observed sequence preference (this work and Muench et al., 1983) provides a basis for predicting AFB1-induced mutational hot spots if a DNA sequence is known. For example, certain amino acid codons (glycine, GGN, and proline, CCN) are likely to be more frequently affected. However, a positive correlation between N^7 modification and mutation has not been established, and any predictions are not warranted at this time. Nevertheless, N^7-G modification can lead to apurinic sites that may be mutagenic (Schaaper & Loeb, 1981).

Assuming that the primary stable AFB1 lesion (N^7-G) or a secondary derivative [such as the open-ring triaminopyrimidine derivative; e.g., Groopman et al. (1981)] is the critical lesion responsible for toxicity, mutagenesis, and carcinogenesis raises an interesting question. Why is AFB1 modification at N^7-G so deleterious when comparatively large doses of other chemicals (e.g., certain alkylating agents; benzanthracenes, etc.) do not have the same intensity of effect? From previously available information, one can make two speculations. (a) Relatively more AFB1 is actually available to react with DNA at a given dosage in the target tissue, and as a result, low dosage has an effect equivalent to that of a very large dose of another, weakly carcinogenic, chemical. Thus, differences can be explained on the basis of pharmacological, physiological, and metabolic considerations. (b) An inherent property of the AFB1 adduct makes this N^7 substitution more deleterious. For example, as a bulky adduct, AFB1 could more strongly affect template function (Miller & Miller, 1977). Alternatively, one can speculate whether sequence specificity is a contributory or even a critical factor. Theoretically, there should be important differences in the consequences of modifying a given DNA sequence to the same extent (i.e., the same number of hits) by a random N^7-G modifying agent and by a sequence-specific reagent. AFB1, by strongly preferring certain sequences like 5′-CCG-3′ and the complement 5′-CGG-3′, should theoretically increase the chances of generating DNA damage on both strands at closely set sites, as compared to a nonspecific alkylating agent where such damage would be randomly distributed throughout both the strands. One can propose that a sequence-determined clustering of N^7-G adducts could lead to lethal or deleterious DS DNA breaks as a result of depurination and strand scission occurring either spontaneously or by an enzymatic process. Thus, a consequence of the sequence preference shown by AFB1 could be the increased hazard of DS DNA breaks, although two caveats should be mentioned: (a) sequence specificity has not yet been demonstrated in vivo and (b) whether prior AFB1 modification affects the reactivity of a closely set G residue in the complementary strand of *the same DNA molecule* has not yet been examined.

It is interesting to note that AFB1 does indeed cause a high level of DNA breakage in vivo, although it is not clear whether the breaks are single or double stranded (Spadari et al., 1982). One can think of at least two different deleterious effects on

surviving cells as a result of double-stranded breaks induced by such mechanisms: (a) The bases at which scission occurred may be destroyed in a significant number of cases such that rejoined molecules may have a micromutation (small deletion); (b) Since intracellular DNA is usually in a compact form, it is not difficult to imagine the joining of two unrelated ends, resulting in gross chromosomal rearrangements. Either possibility may contribute to the genotoxicity of AFB1. In any event, whether AFB1 would induce DS DNA breaks in vitro and in vivo in defined DNA sequences at predicted sites should be experimentally testable.

Acknowledgments

We thank W. Olson for pointing out anticorrelations between stacking preferences and AFB1 reactivities and S. Marotti for preparing the manuscript.

Registry No. AFB1, 1162-65-8; AFB2, 7220-81-7; AFG1, 1165-39-5; AFG2, 7241-98-7; 8-MOP, 298-81-7; angelicin, 523-50-2.

References

Arrand, J. E., & Murray, A. M. (1982) *Nucleic Acids Res. 10*, 1547–1555.

Backer, J. M., & Weinstein, I. B. (1980) *Science (Washington, D.C.) 215*, 73–75.

Bailey, G. S., Nixon, J. E., Hendricks, J. S., Sinnhuber, R. O., & van Holde, K. E. (1980) *Biochemistry 19*, 5836–5842.

Bennett, G. N. (1982) *Nucleic Acids Res. 10*, 4581–4594.

Busby, W. F., & Wogan, G. N. (1979) in *Food Borne Infections and Intoxications* (Feimann, H. P., & Bryan, R. L., Eds.) 2nd ed., pp 519–610, Academic Press, New York.

Calos, M. P., & Miller, J. H. (1981) *J. Mol. Biol. 153*, 39–66.

Cole, A., Meyn, R. E., Chen, R., Corry, P. M., & Hittelman, W. (1980) in *Radiation Biology in Cancer Research* (Meyn, R. E., & Withers, H. R., Eds.) pp 33–58, Raven Press, New York.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4120–4124.

Essignmann, J. M., Croy, R. G., Nadzan, A. M., Busby, W. F., Reinhold, V. N., Buchi, G., & Wogan, G. N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1870–1874.

Godson, G. N., Fiddes, J. C., Barrell, B. G., & Sanger, F. (1978) in *The Single-Stranded DNA Phages* (Denhardt, D. T., Dressler, D., & Ray, D. S., Eds.) pp 51–86, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Groopman, J. D., Croy, R. G., & Wogan, G. N. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5445–5449.

Groudine, M., & Weintraub, H. (1982) *Cell (Cambridge, Mass.) 30*, 131–139.

Humayun, M. Z., Jeffrey, A., & Ptashne, M. (1977) *J. Mol. Biol. 112*, 265–277.

Lilley, D. M. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6468–6472.

Lin, J., Miller, J. A., & Miller, E. C. (1977) *Cancer Res. 37*, 4430–4438.

Lloyd, R. S., Haidle, C. W., & Robberson, D. L. (1979) *Gene 7*, 289–302.

Martin, C. N., & Garner, R. C. (1977) *Nature (London) 267*, 863–865.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Miller, J. A., & Miller, E. C. (1977) in *Origins of Human Cancer* (Hiatt, H. H., Watson, J. D., & Winsten, J. A., Eds.) pp 605–627, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Muench, K. F., Misra, R. P., & Humayun, M. Z. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6–10.

Neidle, S. (1979) *Prog. Med. Chem. 16*, 151–221.

Niranjan, B. G., Bhat, N. K., & Avadhani, N. G. (1982) *Science (Washington D.C.) 215*, 73–75.

Pegg, A. E. (1977) *Adv. Cancer Res. 25*, 195–269.

Platt, T. (1981) *Cell (Cambridge, Mass.) 24*, 10–23.

Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchinson, C. A., III, Slocombe, P. M., Smith, M., Drouin, J., Friedmann, T., & Smith, A. J. H. (1978) in *The Single-Stranded DNA Phages* (Denhardt, D. T., Dressler, D., & Ray, D. S., Eds.) pp 659–669, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schaaper, R. M., & Loeb, L. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1773–1777.

Shen, J. C., Ikoku, A., & Hearst, J. E. (1979) *J. Mol. Biol. 127*, 163–175.

Shieh, J.-C., & Song, P.-S. (1980) *Cancer Res. 40*, 689–695.

Shlomai, J., & Kornberg, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 799–803.

Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 219–284, 310–312.

Singer, B., & Kusmierek, J. T. (1982) *Annu. Rev. Biochem. 52*, 655–693.

Spadari, S., Taningher, M., Boero, P., & Santi, L. (1982) *Mutat. Res. 93*, 1–24.

Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.

Tinoco, I., Jr., Borer, P. M., Dengler, B., Levine, M. D., Uhlenbeck, C., Crothers, D. M., & Gralla, J. (1973) *Nature (London), New Biol. 246*, 40–44.

Tullius, T. D., & Lippard, S. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3489–3492.

Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.