Mapping the Binding Site of Aflatoxin B_1 in DNA: Systematic Analysis of the Reactivity of Aflatoxin B_1 with Guanines in Different DNA Sequences†

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ABSTRACT: The mutagenic and carcinogenic chemical aflatoxin B_1 (AFB_1) reacts almost exclusively at the N(7)-position of guanine following activation to its reactive form, the 8,9-epoxide (AFB_1 oxide). In general N(7)-guanine adducts yield DNA strand breaks when heated in base, a property that serves as the basis for the Maxam–Gilbert DNA sequencing reaction specific for guanine. Using DNA sequencing methods, other workers have shown that AFB_1 oxide gives strand breaks at positions of guanines; however, the guanine bands varied in intensity. This phenomenon has been used to infer that AFB_1 oxide prefers to react with guanines in some sequence contexts more than in others and has been referred to as "sequence specificity of binding". Herein, data on the reaction of AFB_1 oxide with several synthetic DNA polymers with different sequences are presented, and (following hydrolysis) adduct levels are determined by high-pressure liquid chromatography. These results reveal that for AFB_1 oxide (1) the N(7)-guanine adduct is the major adduct found in all of the DNA polymers, (2) adduct levels vary in different sequences, and, thus, sequence specificity is also observed by this more direct method, and (3) the intensity of bands in DNA sequencing gels is likely to reflect adduct levels formed at the N(7)-position of guanine. Knowing this, a reinvestigation of the reactivity of guanines in different DNA sequences using DNA sequencing methods was undertaken. The reactivities of 190 guanines were determined quantitatively and considered in a pentanucleotide context, 5′-WXGYZ-3′, where the central, underlined G represents the reactive guanine and W, X, Y, and Z can be any of the nucleotide bases. Methods are developed to determine that the X (5′-side) base and the Y (3′-side) base are most influential in determining guanine reactivity. The influence of the bases in the 5′-position (X) is 5′-G (1.0) > C (0.8) > A (0.3) > T (0.2), while the influence of the bases in the 3′-position (Y) is 3′-G (1.0) > T (0.8) > C (0.4) > A (0.3). These rules in conjunction with molecular modeling studies (to be published elsewhere) were used to assess the binding sites that might be utilized by AFB_1 oxide in its reaction with DNA.

In recent years evidence has been accumulating that most carcinogens are also mutagens (McCann et al., 1975b; Hsie, 1980; Milman & Weisburger, 1985). Although the details of this relationship are not completely understood, it is apparent that one (and possibly more) of the steps involved in the transformation of a normal cell into a cancerous cell involves a mutation in one (or more) particular genes (Tabin et al., 1982; Reddy et al., 1982; Zarbl et al., 1985; Bishop, 1987). Evidence also suggests a quantitative relationship between the potency of a chemical as a mutagen and as a carcinogen (Meselson & Russell, 1977). Thus, one important question in carcinogenesis is, what is the basis of the carcinogenic/mutagenic potency of a chemical, and in particular what are the factors that make some compounds more mutagenic than others?

To address this question, chemicals that are highly mutagenic and carcinogenic, such as aflatoxin B_1 (AFB_1),[1] are useful to study as models (Singer & Grunberger, 1983). An exhaustive review of the biology and chemistry of AFB_1 has appeared (Busby & Wogan, 1984). The structures of AFB_1 and several of its derivatives are given in Figure 1, which shows a paradigm to facilitate thinking about the mutagenic potency of a chemical. At several chemical junctions in Figure 1, AFB_1 species can partition either horizontally toward mutation or vertically toward detoxification. The mutagenic potency of a chemical is related to the probability that at any of these junctions the horizontal rather than the vertical path will be followed. It is known that AFB_1 itself does not react covalently with DNA, but rather must be metabolized to a chemically reactive form, the 8,9-epoxide (AFB_1 oxide) (Busby & Wogan, 1984), which is extremely unstable and has never been isolated (Essigmann et al., 1977). (Three different conventions for numbering the atoms in AFB_1 have been employed. We have adopted the IUPAC system.) The epoxide can react with a variety of nucleophiles inside the cell, including DNA, and the major adduct is formed at the N(7)-position of guanine (Essigmann et al., 1977). This adduct could give rise to mutations directly when encountered by a polymerase (Chu & Saffhill, 1983), or it might have to be processed further (e.g., to an apurinic site) before mutations could occur (Schaaper et al., 1982, 1983; Sagher & Strauss, 1983; Kunkle, 1984). Finally, SOS repair enhances AFB_1-induced mutagenesis (McCann et al., 1975a; Foster et al., 1983), while excision repair is likely to be capable of accurately repairing this lesion (Ames et al., 1973a; Sancar & Rupp, 1983; Yeung et al., 1983).

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[1] Abbreviations: AFB_1, aflatoxin B_1; AFB_1 oxide, 8,9-oxyaflatoxin B_1; AFB_1-diol, the 8,9-dihydrodiol of aflatoxin B_1; FAPY, formamidopyrimidine; HPLC, high-pressure liquid chromatography; ds, double stranded; ss, single stranded.

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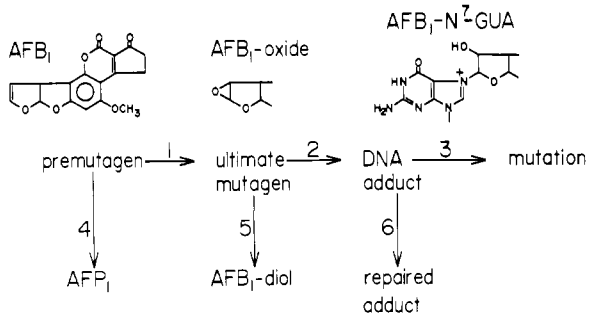

FIGURE 1: Mutagenesis paradigm using aflatoxin B_1 (AFB_1) as an example. AFB_1 can partition toward either mutagenesis (horizontally) or detoxification (vertically). Cellular enzymes can activate AFB_1 either to its epoxide (AFB_1 oxide) or to AFP_1 (demethyl-AFB_1); the latter is considerably less mutagenic and carcinogenic. AFB_1 oxide is very reactive and reacts with nucleophiles including water to give the less toxic AFB_1-diol or the major DNA adduct [AFB_1–N(7)-guanine]. This species is repaired by the excision repair pathway or is encountered by a polymerase. The latter could potentially lead to mutation.

In light of Figure 1, one of the key factors contributing to the potency of AFB_1 as a mutagen must be its ability to react readily with DNA. It is likely that this reactivity is determined, at least in part, by the ability of AFB_1 oxide to interact favorably with DNA in a noncovalent complex prior to covalent reaction at the N(7)-position of guanine. If AFB_1 oxide had a binding site (or sites) in DNA that oriented it for covalent reaction, then the entropy of reaction would be expected to be decreased (Jencks, 1975), and significant enhancement of reaction would be expected with nucleophilic centers in DNA compared to reaction with simple nucleophiles of similar basicity.

Evidence for an AFB_1 oxide/DNA binding site in double-stranded (ds) DNA includes the following: (i) AFB_1 oxide is known to react more readily with ds than single-stranded (ss) DNA, suggesting a better binding site in the former (Misra et al., 1983). (ii) AFB_1 oxide reacts much more readily with the N(7)-position of guanine in ds DNA than in guanosine, although this has not been carefully quantitated (Essigmann, personal communication; our unpublished data). (iii) Equilibrium dialysis experiments have shown that AFB_1 interacts noncovalently with DNA ($K_{assoc} = 2 \times 10^4$) (Clifford & Rees, 1969; Edwards et al., 1971). (iv) Once the AFB_1 adduct is formed in DNA, it effectively inhibits the salt-induced B to Z transition in DNA conformation, suggesting that it interacts with DNA in such a way that the B conformation is stabilized (Nordheim et al., 1983). (v) Although AFB_1 oxide reacts almost exclusively with the N(7)-position of guanine in DNA, not all guanines in a random DNA sequence appear to be equally reactive (D'Andrea & Haseltine, 1978; Misra et al., 1983; Muench et al., 1983).

This latter observation (v), termed sequence specificity of binding, was made by use of DNA sequencing gels where AFB_1 replaced dimethyl sulfate in the Maxam–Gilbert sequencing reaction specific for guanine. N(7)-adducts of guanine have labile glycosylic bonds, which when heated are cleaved to form apurinic sites (Lawley & Brookes, 1963; Essigmann et al., 1977). If heating is performed in the presence of base, the apurinic sites give strand breaks (Maxam & Gilbert, 1980). When these reactions are performed on ^{32}P-end-labeled restriction fragments, the sites of cleavage are revealed as bands on denaturing polyacrylamide gels (DNA sequencing gels). This procedure is applicable with AFB_1 because it reacts with the N(7)-position of guanine (Essigmann et al., 1977). When this procedure was performed with AFB_1 oxide, bands were found at positions of guanine, but not all guanines showed the same band intensities (D'Andrea & Hazeltine, 1978). Careful studies of this phenomenon were carried out by Humayan and co-workers, and from this it was inferred that some sequences in DNA have better binding sites than others (Misra et al., 1983; Muench et al., 1983).

It is this final observation, sequence specificity of the reaction of AFB_1 with DNA, that we are exploiting to define the nature of the AFB_1/DNA binding site. In this paper we confirm the work of Hazeltine (D'Andrea & Haseltine, 1978) and Humayan (Muench et al., 1983; Misra et al., 1983), showing that AFB_1 oxide reacts with guanine when analyzed in DNA sequencing gels and that the intensities of the bands vary in different DNA sequences. Furthermore, we show by more direct means that these differential band intensities are likely to reflect differential levels of adduction. We also present a systematic and quantitative way of analyzing the reactivities of guanines in different DNA sequences and develop quantitative rules to describe these reactivities.

Materials and Methods

Aflatoxins are particularly dangerous compounds and were handled with great care. No reaction was removed from the fume hood until all unreacted AFB_1 had been decontaminated. Anything touching AFB_1 solutions was destroyed by treatment with bleach followed by acetone (Castgnaro et al., 1981). All solutions were handled in yellow light to avoid photochemical reactions (Büchi et al., 1982).

Aflatoxin B_1 was a gift from the laboratory of Dr. Gerald Wogan and produced by Makor Chemicals, while [^3H]aflatoxin B_1 was obtained from Moravek Biochemicals. Both were used without further purification. *m*-Chloroperoxybenzoic acid was obtained from Sigma and was used without further purification. Synthetic DNA polymers were obtained from Pharmacia and were dialyzed against 20 mM sodium phosphate (pH 6.6) and 10 mM NaCl prior to use. Calf thymus DNA was obtained from Sigma and was sonicated and dialyzed prior to use.

Analytical work was performed on a Spectra Physics Model SP8700 HPLC equipped with a Waters Model 440 detector with 254- and 365-nm filters. Analyses were done at room temperature on a C18 μBondapak column (Waters) using 1% ethanol and 0.1 M ammonium acetate (pH 5.8) as the A-solvent and 20% ethanol and 0.1 M ammonium acetate (pH 5.8) as the B-solvent. The gradient employed was 10% B-solvent for 10 min, followed by a 2-minute shift to 50% B-solvent, then a 30-min gradient from 50% to 100% B-solvent, and finally 100% B-solvent was held for an additional 18 min. In this gradient, guanine eluted at 5 min, adenine at 9 min, and the N(7)-guanine adduct of AFB_1 at 40 min. Authentic standards of this adduct and AFB_1-diol were kindly provided by Paul Donahue of the laboratory of Dr. Gerald Wogan. UV spectra were recorded on a Beckman Model 34 spectrophotometer, and scintillation counting was performed on a Beckman Model LS2800 scintillation counter.

M13mp19 DNA was prepared by the procedure of Messing (Messing, 1983). 3′-End labeling of DNA restriction fragments and DNA sequencing methods were performed with slight modifications of the procedures of Maxam and Gilbert (Maxam & Gilbert, 1980). In several instances we wished to isolate specific regions of M13mp19 that had desired DNA sequences, and this required an unconventional labeling procedure. In the first step a restriction fragment was isolated by cutting M13mp19 with a restriction endonuclease that left either blunt ends or 3′-overhanging ends. In the second step this fragment was cut with a restriction endonuclease that left 5′-overhanging ends, which were filled in with DNA polym-

Table I: Restriction Fragments Utilized in Determining Relative Guanine Reactivity

fragment	labeled end[a]	nucleotide position	unlabeled end	nucleotide position	labeled strand
A	*Eco*RI	6286	*Hha*I	6426	minus
B	*Hin*fI	2014	*Hae*III	2246	minus
C	*Hin*fI	2012	*Hae*III	1398	plus
D[b]	*Cla*I	2530	*Hae*III	2247	plus
E	*Cla*I	6884	*Rsa*I	7166	minus
F	*Hin*fI	2846	*Hae*II	3039	minus
G	*Hin*fI	2848	*Hae*II	2715	plus

[a] All fragments were labeled by the reverse-step labeling procedure except fragment A (see Materials and Methods). [b] Fragment D was labeled by first isolating an *Rsa*I fragment (2135–3468), cutting with *Hae*III, and finally cutting with *Cla*I and labeling.

erase I large fragment (Klenow) by using ^{32}P-labeled dNTPs; because the first cut did not leave 5′-overhanging ends, no label was incorporated at these ends. We refer to this method as "reverse step labeling", and it permitted a second method for placing label in a desired position of the genome. The fragments are listed in Table I.

AFB_1 was activated to AFB_1 oxide by the method of Garner (Martin & Garner, 1977). With the synthetic DNA polymers, 33 μg of DNA was added to 300 μL of water (20 mM sodium phosphate, 10 mM NaCl, pH 6.6) in a 1.5-mL, screw cap microcentrifuge tube. A 5-μL sample was removed and hydrolyzed to confirm guanine content. *m*-Chloroperoxybenzoic acid (400 μL, 3 mg/mL) was added, and, finally, [^{3}H]AFB_1 (between 30 and 300 nmol dissolved in chloroform) was added. The biphasic mixture was placed into a device that rotated the tubes, end-over-end, at 55 rotations/min; we found this method gave reasonably reproducible results. Following the initiation of the reaction, a portion of the remaining stock solution of [^{3}H]AFB_1 was added to a cuvette and the spectrum taken to determine AFB_1 concentration. Then the contents of the cuvette were transferred into a scintillation vial, and the levels of radioactivity and, thereby, specific activity were determined. The reaction was essentially completed after 2.5 h at room temperature. Two chloroform extractions followed by two ethanol precipitations were performed to remove unreacted AFB_1 and breakdown products, such as AFB_1-diol. Experiments were carried out that showed that approximately 75% of the DNA was recovered following this cleanup procedure and that DNA molecules containing larger fractions of AFB_1 adducts were not differentially removed by the procedure. Following ethanol precipitation, the DNA was resuspended in 5 mM sodium phosphate (pH 6.6) and frozen at −20 °C until analysis. Hydrolyses were performed by boiling the adducted DNA in 0.25 M HCl for 20 min, which removed the base adduct of AFB_1 and the purines. Analyses were carried out by HPLC. The detector was set at two different sensitivities of 254-nm light (to detect the purines) for 15 min, after which it was set at two different sensitivities of 365-nm light (to detect AFB_1 adducts) for the remainder of the run. Following the completion of analyses on any given day, guanine standards were run on HPLC to generate a standard curve (based on peak area) that allowed the determination of guanine concentrations by interpolation. AFB_1 concentrations were determined by radioactivity. Efficiency of counting (i.e., the relationship of dpm to cpm) was determined by adding a known amount of ^{3}H-labeled standard to each fraction and recounting. To assess the efficiency of recovery, [^{3}H]AFB_1 adduct was isolated in pure form by HPLC; then equal portions were either counted or reinjected onto the HPLC, and the adduct was reisolated and counted. The efficiency of recovery was found to be 90%, and this correction factor has been included in all data reported in this work. We feel that the method described above is subject to uncertainties and that levels of adduction were probably only reliably determined within a factor of approximately 2.

Reactions of AFB_1 with ^{32}P-end-labeled restriction fragments were performed similarly to that described above for the synthetic DNA polymers with the exceptions of the concentrations of AFB_1 and DNA. AFB_1 concentrations were lower (0.8–12 nmol per reaction) in order to ensure an adduction level in the range of one adduct per restriction fragment. (Four nanomoles was used for the majority of work presented in this paper.) Typically, 20–100 ng of a restriction fragment containing 20 000–100 000 cpm was added to a reaction along with 5 μg of sonicated, carrier calf thymus DNA. Extractions were performed and the DNA was precipitated with ethanol. DNA was resuspended in 30 mM ammonium acetate (pH 6.0), and either was hydrolyzed at 100 °C for 5 min (neutral hydrolysis) to remove the ring-closed AFB_1 adduct (Essigmann et al., 1977) or was not. Finally, all samples were hydrolyzed in base (i.e., heated in 1 M piperidine for 30 min at 90 °C); the samples not heated at pH 6.0 would form the ring-opened AFB_1 adduct before hydrolysis (Lin et al., 1977). Following piperidine hydrolysis, ethanol precipitation was not performed to avoid potential complications in the analysis due to differential precipitation of truncated restriction fragments of varying sizes. Samples were evaporated to dryness on a Speedvac and resuspended in water; this was repeated an additional 2 times, except that the final resuspension was in the usual Maxam–Gilbert sequencing mixture (Maxam & Gilbert, 1980). Each sample was loaded in every other lane (to facilitate densitometry) and electrophoresed through a denaturing polyacrylamide gel for three different lengths of time. Autoradiography at −60 °C was performed using Kodak XAR film with an enhancing screen; multiple exposure times for each gel were used to permit the analysis of the wide range of band intensities. A Zeineh densitometer was used for scanning the autoradiograms, and areas under the curves were determined using a Pneumonics Corp. planimeter. Overlapping bands were resolved on a Du Pont Model 310 curve resolver. Not every guanine within a restriction fragment was assigned a value for its relative intensity (Table II). If a band was particularly poorly resolved from surrounding bands of greater intensity or had an intensity close to background, then the data for that guanine were not reported. Maxam–Gilbert sequencing was performed on all of the fragments to ensure that the sequence of our M13mp19 was identical with that reported to us from New England Biolabs. The only change we detected was a G to T transversion at position 6968 in the (+) strand. This was of no consequence to our analysis because we were working with label in the (−) strand, where the mutation was C to A in a region of the genome with no guanine nearby.

Several corrections had to be made to the intensities determined from scanning the autoradiograms before the data truly reflected relative reactivities of the individual guanines. First, sequencing gels show a phenomenon refered to as "fadeout", which is observed at the top of gels and results from the fact that when a restriction fragment receives multiple hits (i.e., multiple adductions), only intensity resulting from the hit closest to the labeled end of the restriction fragment appears in the gel. Thus, the hits that occur further from the labeled end (which appear near the top of the gel) are underrepresented. Second, a method had to be devised to interrelate the data derived from different DNA restriction fragments. We assumed that all of the data in one fragment could be related

Table II: Relative Reactivity of the N(7)-Position of Guanine toward AFB_1 Oxide[a]

	sequence[b]	P_i[c]		sequence[b]	P_i[c]		sequence[b]	P_i[c]
				Fragment A				
1	6296 CGGCC	0.0532	15	6343 AAGTT	0.0169	29	6365 CTGGC	0.0335
2	6297 ACGGC	0.107	16	6349 GCGAT	0.0417	30	6368 CAGCT	0.00895
3	6300 ACGAC	0.0199	17	6351 AGGCG	0.169	31	6372 ACGCC	0.0922
4	6308 TTGTA	0.0284	18	6352 AAGGC	0.0681	32	6380 TCGCT	0.0419
5	6310 ACGTT	0.160	19	6356 CTGCA	0.0287	33	6398 GGGCC	0.0414
6	6313 ACGAC	0.0581	20	6359 GTGCT	0.0138	34	6399 CGGGC	0.190
7	6318 CAGTC	0.0334	21	6362 ATGTG	0.0336	35	6400 GCGGG	0.175
8	6327 GGGTT	0.0555	22	6354 GGGAT	0.0489	36	6402 GTGCG	
9	6328 AGGGT	0.222	23	6355 GGGGA	0.134	37	6404 CGGTG	0.207
10	6329 CAGGG	0.0573	24	6356 GGGGG	0.212	38	6405 TCGGT	0.123
11	6333 ACGCC	0.0864	25	6357 AGGGG	0.302	39	6409 GCGAT	0.0476
12	6338 GGGTA	0.218	26	6358 AAGGG	0.0609	40	6411 GGGCG	0.0551
13	6339 TGGGT	0.251	27	6362 GCGAA	0.0273	41	6412 AGGGC	0.180
14	6340 TTGGG	0.0270	28	6364 TGGCG	0.0859			
				Fragment B				
1	2030 AAGTA	0.00749	13	2101 TTGAG	0.00760	25	2140 AGGAG	0.0661
2	2034 ATGAA	0.00364	14	2106 GTGCC	0.0104	26	2141 CAGGA	0.0504
3	2040 CTGAA	0.00553	15	2108 CAGTG	0.0404	27	2147 ATGAT	
4	2055 CGGAA	0.147	16	2112 GGGTC	0.0551	28	2150 TTGAT	
5	2056 TCGGA	0.126	17	2113 GGGGT	0.0955	29	2156 TGGCT	0.0546
6	2065 CTGCC	0.0133	18	2114 CGGGG	0.173	30	2157 ATGGC	0.0302
7	2072 ATGCC	0.00845	19	2115 ACGGG	0.175	31	2166 GCGTC	0.0912
8	2078 CAGTT	0.0258	20	2123 AAGTT	0.0264	32	2168 AAGCG	0.0342
9	2087 CCGTA	0.218	21	2130 TGGTA	0.152	33	2172 CAGTA	0.0370
10	2091 GTGCC	0.0133	22	2131 CTGGT	0.0651	34	2178 CCGTT	0.0712
11	2093 CAGTG	0.0429	23	2136 GTGTA	0.0273			
12	2099 GAGTA	0.0323	24	2138 GAGTG	0.0206			
				Fragment C				
1	1977 GAGCA	0.0125	10	1927 CGGGC	0.102	19	1886 TGGCG	0.0735
2	1975 CTGAG	0.00758	11	1926 CCGGG	0.112	20	1885 GTGGC	0.0494
3	1970 TGGTA	0.131	12	1912 GTGAT	0.00942	21	1883 GGGTG	0.130
4	1969 CTGGT	0.0894	13	1910 CGGTG	0.220	22	1882 AGGGT	0.209
5	1965 CCGCC	0.0406	14	1909 ACGGT	0.359	23	1881 GAGGG	0.108
6	1955 CGGCA	0.0893	15	1905 GAGTA	0.0296	24	1879 CTGAG	
7	1954 ACGGC	0.118	16	1903 CTGAG	0.0117	25	1874 CGGTT	0.0899
8	1951 TCGAC	0.0168	17	1889 CGGTA	0.218	26	1873 GCGGT	0.126
9	1928 GGGCT	0.0281	18	1888 GCGGT	0.181			
				Fragment D				
1	2519 CGGTG	0.136	10	2479 CTGAC	0.00732	19	2440 GGGCT	0.0388
2	2518 ACGGT	0.123	11	2475 CAGTC	0.0347	20	2439 GGGGC	0.102
3	2512 CTGAT	0.00728	12	2469 GCGCT	0.164	21	2438 GGGGG	0.228
4	2506 TCGCT	0.0331	13	2467 ACGCG	0.0565	22	2437 AGGGG	0.286
5	2503 CTGTC	0.0204	14	2461 ATGAA	0.00849	23	2436 AAGGG	0.0774
6	2496 TTGAT	0.00405	15	2458 CCGAT	0.0689	24	2428 ACGCT	0.0989
7	2489 AGGCA	0.0713	16	2455 ATGCC	0.00684	25	2423 TGGCA	0.113
8	2488 AAGGC	0.0299	17	2449 CCGAA	0.0413	26	2421 ATGGC	0.0298
9	2482 ACGCT	0.145	18	2445 ATGAC	0.0111			
				Fragment E				
1	6901 TGGAG	0.0674	12	6941 GAGAG	0.0201	23	6979 TAGCT	0.0188
2	6902 CTGGA	0.0221	13	6943 TTGAG	0.00469	24	6986 CCGTT	0.149
3	6906 GAGTC	0.0151	14	6952 TAGCT	0.0182	25	6996 ATGAT	0.0143
4	6908 GAGAG	0.00943	15	6955 GGGTA	0.172	26	7016 CAGTC	0.0780
5	6910 CTGAG	0.0121	16	6956 AGGGT	0.208	27	7019 GAGAC	0.00789
6	6914 TTGCC	0.00762	17	6957 GAGGG	0.0542	28	7021 CGGAG	0.0999
7	6920 AGGTC	0.145	18	6959 GAGAG	0.0106	29	7022 CCGGA	0.127
8	6921 CAGGT	0.0304	19	6961 CGGAG	0.0996	30	7026 AGGCC	0.0764
9	6928 AGGCT	0.0771	20	6962 CCGGA	0.170	31	7027 AAGGC	0.0431
10	6929 AAGGC	0.0319	21	6966 ATGCC	0.0129	32	7030 GAGAA	0.0100
11	6939 GAGAT	0.0169	22	6976 CTGAT	0.0195			
				Fragment F				
1	2877 CGGAA	0.0510	9	2907 CAGAA	0.0173	17	2951 AAGCC	0.0163
2	2878 ACGGA	0.0916	10	2914 AAGTT	0.0130	18	2954 CCGAA	0.0666
3	2889 ACGCA	0.0798	11	2921 CCGAA	0.0521	19	2965 TAGCT	0.0223
4	2894 AGGAA	0.0533	12	2924 TAGCC	0.0155	20	2971 TAGCA	0.0335
5	2895 GAGGA	0.0249	13	2928 CAGAT	0.0221	21	2977 ATGAA	0.0118
6	2897 CCGAG	0.0377	14	2931 AAGCA	0.0340	22	2985 AAGAA	0.0184
7	2902 AGGAA	0.0714	15	2935 AAGTA	0.0245	23	2989 GAGCA	0.0272
8	2903 AAGGA	0.0318	16	2940 AAGAA	0.0123	24	2991 AAGAG	0.0253
				Fragment G				
1	2837 TTGCT	0.00484	5	2805 TTGCC	0.00718	9	2770 GTGTC	0.0295
2	2835 ACGTT	0.0921	6	2794 ATGTT	0.0260	10	2768 TGGTG	0.106
3	2825 ATGTA	0.0457	7	2778 GCGTT	0.0952	11	2767 GTGGT	0.0514
4	2809 ATGTA	0.0359	8	2776 TTGCG		12	2765 CCGTG	0.0477

[a] Refer to Table I for restriction fragment designation. [b] The numbers refer to the genomic position in M13mp19 for the central reactive guanine, which is surrounded by four bases and can be generalized 5′-WXGYZ-3′ (see text). [c] The relative probability of reaction for the guanine of interest. Four nanomoles of AFB_1 was used in each of these experiments; hydrolysis was in base (see text). Three figures are reported for each data point, but the accuracy of the data warrants fewer significant figures.

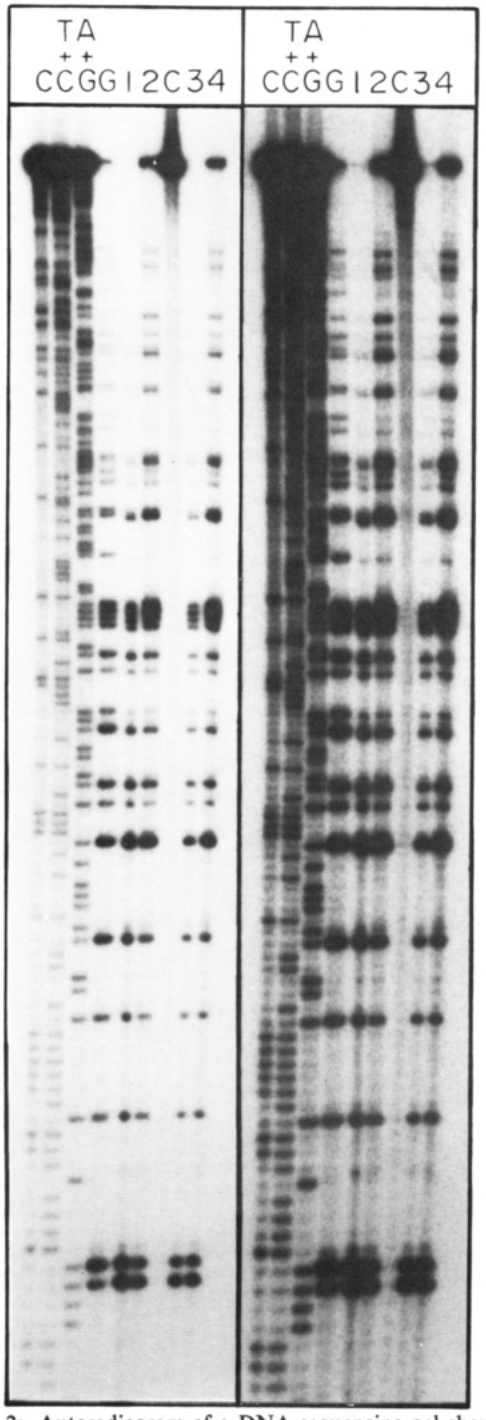

FIGURE 2: Autoradiogram of a DNA sequencing gel showing the pattern of reaction of AFB_1 oxide with DNA. The method of preparation of the ^{32}P-end-labeled restriction fragment (C in Table I) and of adduction and analysis is described under Materials and Methods. The autoradiogram shows the same DNA sequencing gel after two different times of exposure. The lanes—designated C, T+C, A+G, and G—represent Maxam–Gilbert sequencing reactions for those bases. The reactions contained 12 (lanes 1 and 3) or 4 nmol of AFB_1 and were either hydrolyzed in base (lanes 1 and 2) or at neutrality (lanes 3 and 4). Lane 3 is a control where the restriction fragment was carried through the entire procedure without AFB_1. The lowest doublet in lanes 1–4 in the autoradiogram corresponds in M13mp19 to genome positions 2055 and 2056, while the prominent quartet corresponds to genome positions 2112, 2113, 2114, and 2115.

to the data from another fragment by a simple scaling factor. Correction for fadeout and the determination of the scaling factors were accomplished with a computer program that runs on Digital Equipment Corp. computers. The program is available upon request. A detailed description of the data manipulation will be presented elsewhere.

Table III: Effect of Changing AFB_1 Concentration and Hydrolysis at Neutral pH on Band Intensities in DNA Sequencing Gels[a]

		P_i[c]		
	sequence[b]	higher AFB_1[d]	lower AFB_1[e]	neutral hydrolysis[f]
1	2519 CGGTG	0.138	0.0680	0.0937
2	2518 ACGGT	0.125	0.0626	0.0946
3	2512 CTGAT	0.00740	0.00691	0.0129
4	2506 TCGCT	0.0336	0.0234	0.0330
5	2503 CTGTC	0.0207	0.0181	0.0237
6	2496 TTGAT	0.00412	0.00387	0.0144
7	2489 AGGCA	0.0725	0.0611	0.0647
8	2488 AAGGC	0.0304	0.0298	0.0251
9	2482 ACGCT	0.147	0.0902	0.0963
10	2479 CTGAC	0.00744	0.0108	0.0102
11	2475 CAGTC	0.0352	0.0304	0.0331
12	2469 GCGCT	0.167	0.107	0.107
13	2467 ACGCG	0.0575	0.0492	0.0528
14	2461 ATGAA	0.00864	0.0106	0.00833
15	2458 CCGAT	0.0700	0.0642	0.0523
16	2455 ATGCC	0.00696	0.00892	0.00751
17	2449 CCGAA	0.0420	0.0367	0.0369
18	2445 ATGAC	0.0113	0.0146	0.0125
19	2440 GGGCT	0.0394	0.0522	0.0400
20	2439 GGGGC	0.104	0.132	0.0775
21	2438 GGGGG	0.232	0.217	0.122
22	2437 AGGGG	0.291	0.276	0.179
23	2436 AAGGG	0.0787	0.0779	0.0639
24	2428 ACGCT	0.101	0.0969	0.0737
25	2423 TGGCA	0.115	0.159	0.0947
26	2421 ATGGC	0.0303	0.0582	0.0325

[a] All data are from restriction fragment D (Table I). [b] The numbers refer to the genomic position in M13mp19 for the central reactive guanine, which is surrounded by four bases and can be generalized 5′-WXGYZ-3′ (see text). [c] The relative probability of reaction for the guanine of interest. Three figures are reported for each data point, but the accuracy of the data warrants fewer significant figures. [d] Four nanomoles of AFB_1 was used in this experiment; hydrolysis was in base (see text). The data in this table for restriction fragment D do not exactly match the same values presented in Table II, because the calculations for this table included data for fragment D a total of 3 times (one for each of the three data sets in the table), while in Table II data for fragment D was included only once. [e] Eight-tenths nanomole of AFB_1 was used in this experiment; hydrolysis was in base (see text). [f] Four nanomoles of AFB_1 was used in this experiment; hydrolysis was at pH 6.0 (see text).

RESULTS

Figure 2 shows typical autoradiographical results for AFB_1-induced strand cleavages when analyzed on a DNA sequencing gel, where AFB_1 oxide was generated by the chemical activation procedure of Garner (Martin & Garner, 1977). This reaction was performed on seven, ^{32}P-end-labeled restriction fragments (Table I). All of the resulting autoradiograms were scanned with a densitometer, and a typical scan of a portion of the gel in Figure 2 is presented in Figure 3. The data for the band intensities of 190 guanines were determined (see Materials and Methods) and are presented in Table II, where the sequence surrounding the reactive guanine is included in a pentanucleotide context. The intensities of bands can vary by as much as a factor of approximately 100. Guanines that were poorly resolved were not included in the analysis. Table III shows that the relative intensities do not vary significantly as the number of adducts per restriction fragment was varied by lowering the amount of AFB_1 in an adduction.

Band intensities on DNA sequencing gels were merely an indirect measure of levels of adduction. Several concerns about the relationship between band intensity and adduct level were considered before an analysis of these data was performed.

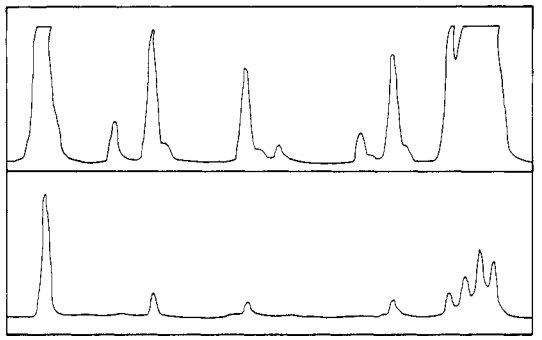

FIGURE 3: Densitometric scans of portions of the sequencing gel shown in Figure 2 for restriction fragment C (Table I). The scans in the upper and lower panels were from the autoradiograms with longer and shorter exposures, respectively. The peaks in the upper panel correspond to (from left to right) genome positions 2087 (off scale), 2091, 2093 (shoulder), 2099 (shoulder), 2101, 2106, 2108 (shoulder), and the quartet at positions 2112, 2113, 2114, and 2115 (off scale). Shoulders correspond to band intensities at non-guanine genome positions and were not included when peak area was determined.

(*A*) *Does Hydrolysis of AFB$_1$ Adducts in Hot Alkali Lead to Artifacts*? Hydrolysis of AFB$_1$ adducts in base, as performed during the strand cleavage reaction in Maxam–Gilbert sequencing, could lead to band intensities that are not reflective of levels of adduction for several reasons. First, the imidazole ring in N(7)-adducted guanines opens in base, and the ring-opened formamidopyrimidine derivative (AFB$_1$–FAPY) is not expected to hydrolyze as rapidly in base as its precursor (Lin et al., 1977). Thus, AFB$_1$ adducts might remain in DNA. Second, additional, unknown chemistry might occur at elevated pH, which could result in a nonquantitative conversion of AFB$_1$ adducts to apurinic sites and/or, subsequently, to strand breaks. To test these concerns, AFB$_1$ adducts were hydrolyzed to give apurinic sites at neutral pH (6.0) prior to carrying out the strand cleavage reaction (i.e., heating in 1 M piperidine). At this pH, ring opening of the adducted guanine adduct is a minor reaction. The results for neutral hydrolyses are shown in lane 4 of Figure 2, and no large differences in guanine intensities are observed when compared to the same reaction when hydrolyzed in base (lane 2). This was confirmed quantitatively (Table III). Thus, hydrolysis in base did not appear to be a reason for concern. In certain instances, neutral hydrolysis yielded bands of low intensity at bases other than guanine, which did not appear with base hydrolysis. These bands appeared to be due to the presence of *m*-chloroperoxybenzoic acid in the activation procedure, and not from AFB$_1$ oxide itself (Jacobsen & Humayun, 1986). Thus, the data reported in Table II were derived from the samples hydrolyzed with piperidine that, in general, were cleaner.

(*B*) *Is the Same DNA Adduct Formed in All DNA Sequences*? AFB$_1$ oxide showed a high degree of specificity (>95%) for reaction at the N(7)-position of guanine (Essigmann et al., 1977, 1983). Nevertheless, it was important to show that a single adduct was predominantly formed in different DNA sequences; a minor adduct might conceivably have been preferentially formed in certain sequences. To approach this question, synthetic, alternating, DNA copolymers of different sequence were reacted with AFB$_1$ oxide. Following hydrolysis, adduct profiles were analyzed on HPLC. A typical chromatogram is shown in Figure 4 for the reaction of AFB$_1$ oxide with the polymer poly(dG-dT)·poly(dC-dA). A single adduct peak was observed, which comigrated with the authentic, N(7)-guanine adduct of AFB$_1$ (data not shown). Reactions were carried out on a total of four synthetic polymers (listed in Table IV), and in each case this adduct predominated (>80%). Furthermore, it is unlikely that another adduct would form based on the limited number of binding sites that are available to AFB$_1$ oxide in ds DNA (unpublished experiments).

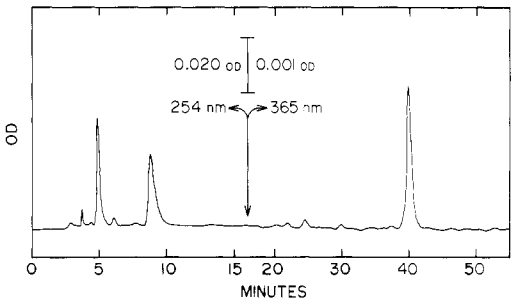

FIGURE 4: HPLC chromatogram of acid-hydrolyzed material following reaction of AFB$_1$ oxide with poly(dG-dT)·(dA-dC). The method of adduction and the separation conditions are described under Materials and Methods. Before 17 min, the detector was set at 254 nm on 0.1 OD full scale; after 17 min, the detector was set at 365 nm on 0.005 OD full scale. The peaks eluting at 5, 9, and 40 min correspond to guanine, adenine, and AFB$_1$–N(7)-guanine, respectively. The chart speed was faster in the first 17 min; this facilitated the quantitation of the guanine, which was accomplished by determining peak area.

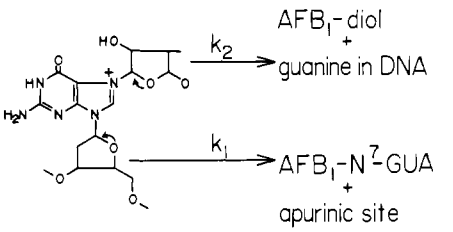

FIGURE 5: Two potential pathways by which the major AFB$_1$ adduct can break down when in DNA. After breakdown by k_1, the DNA molecule contains an apurinic site at the original position of the adduct and yields AFB$_1$–N(7)-guanine. In contrast, breakdown by k_2 would restore guanine in DNA and give AFB$_1$-diol.

Table IV: Formation of the N(7)-Guanine Adduct of AFB$_1$ in Different Synthetic DNA Polymers

synthetic DNA polymer	expt 1[a]	expt 2[a]	expt 3[a]
poly(dG-dC)·(dG-dC)	0.17 (0.10)	0.19 (0.12)	0.013 (0.011)
poly(dG-dMeC)·(dG-dMeC)[b]	0.29 (0.17)	0.12 (0.061)	0.024 (0.020)
poly(dG-dT)·(dA-dC)	0.14 (0.078)	0.21 (0.11)	0.017 (0.012)
poly(dG-dA)·(dT-dC)	0.009 (0.006)	0.010 (0.009)	0.0010 (0.001)
calf thymus DNA	ND[c]	0.29 (0.16)	0.027 (0.026)

[a] The ratio of nanomoles of the N(7)-guanine adduct of AFB$_1$ to nanomoles of guanine in the reaction mixture. Nanomoles of adduct were determined by radioactivity in the adduct peak as analyzed by HPLC, while nanomoles of guanine were determined in the same HPLC run on the basis of peak area when compared to a standard (see Materials and Methods). The numbers in parentheses are the ratio of peak area for the N(7)-guanine adduct measured at 365 nm divided by the peak area for guanine at 254 nm from the same run. The highest value observed for the ratio in parentheses for the five DNA species reported in this table were (top to bottom) 0.237, 0.489, 0.695, 0.085, and 0.420 (data not shown). The ratio in parentheses is 0.68 on average compared to the ratio not in parentheses and reasonably constant for all experiments (as expected). [b] MeC = 5-methylcytosine. [c] ND = not determined.

(*C*) *Can Breakdown of the N(7)-Adduct in DNA during Hydrolysis Occur by Different Pathways in Different DNA Sequences*? Figure 5 shows two pathways for the breakdown of the N(7)-adduct in DNA. Breakdown could either result in the formation of an apurinic site in DNA (k_1) or lead to the restoration of a guanine in DNA at the original site of the adduct (k_2). Both chemistries are similar, but breakdown by k_1 ultimately would lead to strand cleavage, while breakdown by k_2 would not. If the ratio of k_1/k_2 varied in different DNA sequences, then the intensities of AFB$_1$-generated bands in

DNA sequencing gels might not reflect the relative levels of adduct formation. The ratio of k_1/k_2 was evaluated in different DNA sequences by using the synthetic polymers. Breakdown by k_1 led to the formation of the N(7)-guanine adduct, while breakdown by k_2 led to the formation of the diol of AFB_1 (AFB_1-diol). The latter eluted at a characteristic time (approximately 4 min prior to the adduct) under our chromatographic separation conditions. Only negligible amounts (<5%) of AFB_1-diol were observed in any of the DNA polymers studied (e.g., see Figure 4). Thus, the ratio k_1/k_2 appeared to be large in all sequences, and predominantly apurininc sites were formed when the adduct was hydrolyzed. This result is sensible because breakdown by both pathways is expected to occur by way of an oxocarbonium ion (Cordes & Bull, 1974; Young & Jencks, 1977; unpublished experiments), and k_2 would be slower because the electron-withdrawing oxygens in the AFB_1 moiety are closer to the developing positive charge at the reactive center than are the oxygens in the deoxyribose moiety. The hydrolysis presented in Figure 4 was performed in 0.25 N HCl. It is conceivable that the partitioning would change as a function of pH. Partitioning would be affected by ionizations near the site of adduction; however, the only ionizations that were expected to affect k_1/k_2 were at phosphate oxygens. At higher pH, these oxygens would be ionized, which would preferentially stabilize oxocarbonium ion formation at the deoxyribose and serve to increase k_1/k_2. Hydrolysis in base leads to the formation of AFB_1–FAPY (Lin et al., 1977); the dominant isomer of this species has a better leaving group at N(9) (aromatic amine) than at N(7) (aromatic amide), which would also disfavor AFB_1-diol formation. In keeping with these suppositions, AFB_1-diol formation was low when samples were hydrolyzed at neutrality at 100 °C or at 90 °C in base (data not shown).

(*D*) *Can Sequence Specificity Be Demonstrated More Directly*? We wished to corroborate the sequence specificity observed in sequencing gels by a method that more directly determined the level of adduction in different DNA sequences. Table IV gives the levels of adduction in the four different synthetic DNA polymers, and the results indicated that levels varied with sequence. The relative levels of adduction in the different polymers could have reflected either extent of binding at saturation of adduction or relative reactivities. To probe this, the initial [AFB_1] was lowered in experiment 3 (Table IV) and adduct levels were accordingly lowered in comparison to experiments 1 and 2 for each synthetic polymer (Table IV). Thus, the data in experiment 3 clearly reflected relative reactivities of guanines in different sequence contexts. We note that the synthetic polymer poly(dG)·poly(dC) could not be studied because it precipitated during the adduction procedure.

Discussion

The data presented in Figure 2 and Tables III and IV suggested that (i) hydrolysis in basic conditions did not give rise to problems in the analysis of levels of adduction at guanine using DNA sequencing gels, (ii) predominantly one heat-labile adduct was formed in all DNA sequences, (iii) a single breakdown pathway predominated in all DNA sequences, and (iv) sequence specificity in the reaction of AFB_1 could be demonstrated more directly. There is an obvious caveat to the final three of these assertions; only a small subset of all possible sequences was studied by using the synthetic DNA polymers, and we cannot be absolutely confident that some of these concerns might not be realized in other DNA sequences. However, the subset of sequences studied with the synthetic polymers is representative of what might be expected in other sequences (e.g., the bases A, C, and T were found on both the 5′- and 3′-sides of the reactive guanine in the set of polymers employed), and the formation of other adducts did not appear to be feasible given the structure of AFB_1 and double-stranded DNA (unpublished experiments). Lastly, we agree with the assessment of Humayan (Misra et al., 1983; Muench et al., 1983) that sequence-specificity effects are not likely to be solely due to differential nucleophilicity of the N(7)-position of guanine in different DNA sequences, because dimethyl sulfate (DMS), which also reacts predominantly at the N(7)-position of guanine, did not show the same pattern of sequence specificity. These considerations gave us confidence that a systematic analysis of the reactivity of AFB_1 oxide with DNA could yield reactivity rules, which, in turn, could be used to probe the structure of the AFB_1 oxide binding site(s) in DNA (unpublished experiments).

Table V: Matrix Analysis of the Relative Reactivity Data[a]

X (5′-side)	Y (3′-side) A	C	G	T
A	0.080	0.12	0.24	0.16
	11	10	12	15
	1.12	0.88	0.48	0.73
	0.23	0.21	0.22	0.26
C	0.24	0.40	0.72	0.57
	10	10	13	8
	0.49	0.18	−0.161	−0.025
	0.23	0.32	0.13	0.28
G	0.37	0.34	1.00	0.78
	8	13	15	13
	0.23	0.28	−0.35	−0.21
	0.16	0.23	0.21	0.23
T	0.045	0.051	0.20	0.16
	16	10	8	7
	1.45	1.38	0.59	0.73
	0.27	0.21	0.22	0.11

[a] Data points were considered in a pentanucleotide context, 5′-WXGYZ-3′, and analyzed by a matrix analysis in a pairwise fashion (i.e., W vs X, W vs Y, etc.). The matrix that proved to be most predictive of guanine reactivity was the X/Y matrix (see text), whose results are summarized in this table. The values in each group are, top to bottom, the average reactivity, the number of data points (data points were removed based upon the Student *t*-test), the relative energies, and the standard deviation of the latter (see text).

Systematic Analysis of Relative Reactivity. The pentanucleotide context (Table II) for each reactive guanine can be generalized as

5′-WXGYZ-3′

where the central, underlined G represents the reactive guanine and W, X, Y, and Z can be any of the four nucleotide bases. Four-by-four matrices were constructed, where (for example) the four possible bases in the X-position (the base on the immediate 5′-side of the adducted guanine) were placed horizontally and the four possible bases in the Y-position (the base on the immediate 3′-side) were placed vertically (i.e., as arranged in Table V). For example, if a reactive guanine in the sequence 5′-ACGAC-3′ was of relative reactivity 0.020, then 0.020 was considered along with all other data points from DNA sequences having a cytosine in the X-position and an adenine in the Y-position. Six different four-by-four matrices (W/X, W/Y, W/Z, X/Y, X/Z, and Y/Z) were possible, and the data from Table II were placed into each of these matrices.

The reaction of AFB_1 oxide with guanine in DNA is a pseudo-first-order reaction because the guanines are in excess (i.e., the ratio of adducted to nonadducted guanines in the reaction was always low). For first-order reactions, the ratio of products is directly proportional to the ratio of the first-order

Table VI: Standard Deviations, Number of Data Points within a Factor of 1.5 of the Average, and Spread from Different Matrices

matrix[a]	sum SD[b]	data points within 1.5 of av[c]	spread[d]
X/Y	3.5	125/190	X = 5.0–8.3 Y = 2.7–4.3
W/X	5.3	87/190	W = 1.6–2.3 X = 4.5–10.3
X/Z	5.4	91/190	X = 5.3–10.9 Z = 1.5–2.4
W/Y	6.7	71/190	W = 1.2–3.9 Y = 2.5–20.0
Y/Z	6.8	67/190	Y = 3.4–6.1 Z = 1.7–2.3
W/Z	8.0	66/190	W = 2.6–3.4 Z = 1.7–10.0

[a] The relative reactivity data were considered in a pentanucleotide context, 5′-WXGYZ-3′, and the data set was evaluated by a matrix analysis, where two of the bases (W, X, Y, and Z) were considered at a time. For example, the analysis of the X and Y bases is presented in the matrix in Table V and is designated the X/Y matrix. [b] Sum of the standard deviations for the energies; e.g., for the X/Y matrix (Table V) this is the sum of the 16 values at the bottom of each matrix element. [c] Number of data points that have a value that is within a factor of 1.5 of the average value for that particular data point. [d] The spread is best illustrated by example from data in Table V (i.e., for the X/Y matrix). Consider the spread for X (=5.0–8.3) in the X/Y matrix. The largest value for the spread of X (=8.3) is found when adenine is in the Y-position and is equal to the ratio of the average reactivity when guanine is in the X-position (0.37) divided by the average reactivity when thymine is in the X-position (0.045). The smallest value for the spread of X (=5.0) is found when guanine is in the Y-position and is equal to the ratio of the average reactivity when guanine is in the X-position (1.00) divided by the average reactivity when thymine is in the X-position (0.20).

rate constants for the formation of those products (Jencks, 1969). Thus, sequence-specificity data, which reflected relative product yield, gave information about the relative rates of reaction and, thereby, about the transition states. Thus, pairwise differences in the relative reactivities obey the relationship, $\Delta\Delta G^{\ddagger} = -RT \ln (k_m/k_n)$, and comparisons between different data points must be made in reference to the relative free energies of the transition states (i.e., to $\Delta\Delta G^{\ddagger}$). This point and the details of the mathematical treatment of the data will be presented elsewhere.

The average values for the different matrix elements for the X/Y matrix, which proved to be of greatest significance (see below), are given in Table V. The four values in each matrix element are (from top to bottom) the average relative intensity (underlined values), the number of data points considered, the average free energy (on a relative scale), and the standard deviation of the latter.

We believe that a pentanucleotide context is not an unreasonable size in which to consider sequence-specific effects, because model building (unpublished experiments) suggested that AFB_1 could only interact directly with two bases on either side of the guanine to which it was bound. Nevertheless, larger sequence contexts might have to be considered in certain cases. We note that Boles and Hogan (1986) have also employed the matrix approach to the analysis of sequence-specific effects for reactions of another mutagen with DNA. Finally, we took the qualitative data from the work of Humayan (Muench et al., 1983), evaluated it by matrix analysis, and found that it agreed qualitatively with our results.

Data codified in a matrix format evaluated the influence that two bases had on the energy of reaction for a guanine, independently of the sequence of other bases in the vicinity. An analysis of the six different, four-by-four matrices revealed that the bases on the immediate 5′-side of the reactive guanine (i.e., in the X-position) and on the immediate 3′-side of the reactive guanine (i.e., in the Y-position) had the greatest influence on the reactivity of AFB_1 oxide with the N(7)-position of guanine in DNA. This was ascertained in three ways. First, the sum of the standard deviations in each matrix (Table VI) was determined, and the one with the lowest sum was the one for the X- and Y-positions, i.e., from the matrix derived from considering all data in the context, 5′-XGY-3′. This indicated that the X/Y matrix was the most predictive of sequence-specificity results. Second, it is possible that another matrix might be generally as predictive as the X/Y matrix except for a small number of aberrant data points, in which case considering only the sum of the standard deviations might be misleading. Thus, we also determined the number of data points that were within a factor of 1.5 of their average value (1.5 was chosen as a reasonable but arbitrary value). This number was greatest for the X/Y matrix (Table VI). Finally, the values of the reactivity for each matrix element in the X/Y matrix varied the most within a row or a column, implying greatest influence (Table VI). We did not conclude from this analysis that the X- and Y-positions were the only positions that influenced reactivities, but merely that they had the greatest influence.

Table VII: Reactivities Normalized to the Reactivity of Guanine in the X-Position[a]

X (5′-side)	Y (3′-side) A	C	G	T
A	0.21	0.35	0.24	0.20
C	0.64	1.19	0.72	0.73
G	1.00	1.00	1.00	1.00
T	0.12	0.15	0.20	0.20

[a] The data points in Table V (top value) were normalized to the reactivities of the guanine in the X-position (i.e., to the guanine on the 5′-side of the reactive guanine).

Comparison of Results for Synthetic Polymers and DNA Sequencing Gels. The results presented in Table IV for the reaction of AFB_1 oxide with synthetic polymers gave a relative reactivity of approximately 1.0:1.0:0.06 for poly(dG-dC)·(dG-dC):poly(dG-dT)·(dA-dC):poly(dG-dA)·(dT-dC). These results can be compared to the ratios determined from DNA sequencing gels by utilizing the data presented in Table V, for 5′-CGC-3′:5′-TGT-3′:5′-AGA-3′, 1.0:0.41:0.19. A more sophisticated analysis that took into account the entire pentanucleotide context for determining the ratios from the results with DNA sequencing gels (based on data not shown) gave a ratio of 1.0:0.55:0.13 for 5′-GCGCG-3′:5′-GTGTG-3′:5′-GAGAG-3′. The agreement between these two methods is within our estimated experimental error. However, if the differences are real, they might be attributable to unusual structures that could form with alternating copolymers. Nevertheless, the results suggest that the synthetic DNA polymers are not totally unreasonable models for the structures that are found for the same sequences in natural DNA.

Quantitative Analysis of Relative Reactivity. The data in Table V could be analyzed further to provide a quantitative assessment of the influence that specific bases on the 5′- and 3′-sides had on the relative reactivities. For example, the data in Table V revealed that in going from a cytosine to an adenine on the 5′-side (X-position) of the reactive guanine, the reactivity was decreased independently of the base on the 3′-side (Y-position). This observation could be quantitated by normalization of all of the data to the most reactive base, guanine. Table VII shows the influence that the 5′-side (X-position) base had on guanine reactivity. Similarly, the data in Table

Table VIII: Reactivities Normalized to the Reactivity of Guanine in the Y-Position[a]

X (5′-side)	Y (3′-side) A	C	G	T
A	0.33	0.50	1.00	0.65
C	0.33	0.56	1.00	0.79
G	0.37	0.34	1.00	0.78
T	0.23	0.26	1.00	0.78

[a] The data points in Table V (top value) were normalized to the reactivities of the guanine in the Y-position (i.e., to the guanine on the 3′-side of the reactive guanine).

Table IX: Summary of the Normalized Reactivities

	X (5′-side)[a]	Y (3′-side)[b]
A	0.25	0.32
C	0.82	0.42
G	1.00	1.00
T	0.17	0.75

[a] The data points in Table VII were averaged for all of the bases in the Y-position when the indicated base was in the X-position. [b] The data points in Table VIII were averaged for all of the bases in the X-position when the indicated base was in the Y-position.

V were normalized to show the influence that the 3′-side (Y-position) had on guanine reactivity; normalization in this case was done to the reactivity of guanine in the 3′-position (Table VIII).

Several observations could be made from the results in Tables VII and VIII. The first is that, in general, the change of a base on the 5′-side from any one base to any other base resulted in approximately the same quantitative change in reactivity independently of the base that was found on the 3′-side (Table VII). Similarly, the change of one base for another on the 3′-side resulted in the same change in reactivity independently of the base of the 5′-side (Table VIII). These results are summarized in Table IX. Furthermore, the results imply that the reactivity of a particular guanine could be reasonably approximated by a simple equation, namely

$$\text{reactivity} = X \cdot Y$$

Sequence specificity of binding is not unique to AFB_1. Evidence indicates that the diol epoxide of benzo[*a*]pyrene (Sage & Haseltine, 1984; Boles & Hogan, 1984, 1986; Chen, 1985; Lobanenkov et al., 1986), *N*-acetylaminofluorene (Bases et al., 1983; Fuchs, 1984), dimethyl sulfate (Maxam & Gilbert, 1980), CC-1065 (Hurley et al., 1984), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Hashimoto & Shudo, 1983), and various other antitumor drugs [reviewed by Dervan (1986)] also show sequence preferences in their reactions with DNA. In fact, due to the constraints imposed by the structure of DNA, it would be more surprising not to find sequence specificity in the reaction of a substance with DNA.

Conclusion

We have evaluated the use of DNA sequencing gels to analyze levels of adduction of AFB_1 oxide with guanines in DNA and concluded that it is reliable in many sequences and is likely to be reliable in all sequences. We have developed a systematic way of analyzing these data and have developed quantitative rules to describe the reactivities of AFB_1 oxide with guanines in different DNA sequences. In summary the base pairs on the immediate 5-′ and 3′-sides of the reactive guanine have the greatest influence on reactivity. The most reactive sequence (5′-GGG-3′) is approximately 20 times more reactive than the least reactive sequence (5′-TGA-3′) on average. Molecular modeling of the N(7)-guanine adduct of AFB_1 in different DNA sequences, which will be presented elsewhere, suggests that the sequence-specificity data can be rationalized on the basis of a single DNA binding site in which AFB_1 is bound externally in the major groove of B-DNA.

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Registry No. AFB_1 oxide, 90358-59-1; poly(dG-dC)·(dG-dC), 36786-90-0; poly(dG-dMeC)·(dG-dMeC), 51853-63-5; poly(dG-dT)·(dA-dC), 55684-99-6; poly(dG-dA)·(dT-dC), 29627-66-5; guanine, 73-40-5.

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Expression and Properties of the Regulatory Subunit of *Dictyostelium* cAMP-Dependent Protein Kinase Encoded by λgt11 cDNA Clones†

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ABSTRACT: λgt11 phages harboring five different cDNA fragments for the regulatory (R) subunit of *Dictyostelium discoideum* cAMP-dependent protein kinase (CAK) directed the synthesis of this protein in *Escherichia coli* cells. Crude bacterial extracts were probed with an antiserum against the *Dictyostelium* R subunit. The presence of specific epitopes for the R subunit in a given extract was compared with high-affinity cAMP-binding activity and with the ability to inhibit the catalytic (C) subunit through protein–protein interaction. The expression and the biochemical properties of these proteins were correlated with their cDNA nucleotide sequence. The results show that the *Dictyostelium* R subunit can be functionally expressed in *E. coli* cells either as a fusion protein with β-galactosidase or as a nonfusion protein. In both cases, the products of cDNA clones containing the entire coding sequence retained high-affinity cAMP-binding activity and the capacity to interact with the catalytic subunit. One of the fusions, lacking the 94 N-terminal residues, failed to inhibit catalytic activity, although it bound cAMP with an affinity similar to that of the native R protein from *D. discoideum*.

Dictyostelium has long been considered a model organism for the study of cell differentiation and morphogenesis [for review, see Loomis (1982)]. Moreover, the cellular slime molds may represent one of the most primitive forms of eukaryotic life, since they diverged from the main eukaryotic branch at the earliest point yet characterized by molecular phylogeny (McCaroll et al., 1983; Lane et al., 1985).

cAMP plays an important role in the control of cellular function in prokaryotic and eukaryotic cells. In bacteria, the nucleotide affects gene regulation directly via binding to the

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