

AN ABSTRACT OF THE THESIS OF

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Title: Site-specific Aflatoxin B<sub>1</sub> Adduction of Sequence-positioned Nucleosome Core Particles

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The question of how the presence of nucleosomal packing of DNA modifies carcinogen interaction at specific sites cannot be answered by studies on whole chromatin or bulk nucleosomes because of the heterogeneity of DNA sequences in the particles. This problem was circumvented by constructing nucleosomes that are homogenous in DNA-histone contact points. A cloned DNA fragment, containing a sea urchin 5S gene which precisely positions a histone octamer was employed. By using <sup>32</sup>P end-labeled DNA and genotoxins that allow cleavage at sites of attack, the frequency of adduction at every susceptible nucleotide can be determined on sequencing gels. The small methylating agent dimethyl sulfate (DMS) and the bulky alkylating agent aflatoxin B<sub>1</sub>-dichloride (AFB<sub>1</sub>-Cl<sub>2</sub>) were used to probe the influence

of DNA-histone interactions on DNA alkylation patterns in sequence-positioned core particles. I find DMS to bind equally to naked or nucleosomal DNA, in a quantitative 1:1 ratio. In contrast, AFB<sub>1</sub>-Cl<sub>2</sub> binding is globally suppressed 2.4-fold in nucleosomes, but the DNA is more accessible to AFB<sub>1</sub>-Cl<sub>2</sub> adduction in regions near the particle boundary. I observe no other histone-imposed localized changes in AFB<sub>1</sub>-Cl<sub>2</sub> sequence specificity. Further, sites of DNase I cleavage or proposed DNA bending show neither enhanced or reduced AFB<sub>1</sub>-Cl<sub>2</sub> adduction to N7-guanine. Since AFB<sub>1</sub>-Cl<sub>2</sub> binding sites lie in the major groove, nucleosomal DNA appears accessible to AFB<sub>1</sub>-Cl<sub>2</sub> at all points of analysis, but with an access which is uniformly restricted in the central 100 nucleotides of the core particle. The data available do not indicate further localized or site-specific perturbations in DNA interactions with the two carcinogens studied.

Site-specific Aflatoxin B<sub>1</sub> Adduction of  
Sequence-positioned Nucleosome Core Particles

by

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## PREFACE

In part 1 of this thesis, an overview is given of the major components of the studies described in parts 2-4. This first section contains brief reviews of chromatin structure, nucleosome reconstitution, aflatoxin B<sub>1</sub>, sequence-selective DNA adduct formation, and chromatin-carcinogen interactions, especially as they pertain to the fundamental unit of eukaryotic DNA packaging - the nucleosome core particle. Parts 2-4 then describe the influence of nucleosomal DNA-histone interactions on the alkylation of N7-guanine by dimethyl sulfate and aflatoxin B<sub>1</sub>-dichloride.

# Site-specific Aflatoxin B<sub>1</sub> Adduction of Sequence-positioned Nucleosome Core Particles

## I. INTRODUCTION

### A. CHROMATIN STRUCTURE

In a typical human cell, 20  $\mu\text{m}$  in diameter,  $6 \times 10^9$  nucleotides are packaged within the nucleus. This compaction is aided by highly conserved basic proteins, the histones. When briefly digested with micrococcal nuclease, nucleoprotein particles containing DNA from 160 to 240 bp in length are released. This DNA length, generally about 200 bp, is constant in a given cell type. The DNA linking the particles, known as spacer or linker DNA, can be trimmed by further nuclease treatment to particles with DNA 160, then 146 bp in length. This latter particle, the nucleosome core, is the fundamental unit of chromatin structure, in which 1.75 turns of DNA are wrapped around two each of the four histones H2A, H2B, H3, and H4 (Shaw, et al., 1976; Felsenfeld, 1978; McGhee and Felsenfeld, 1980). The particle is disk-shaped, with dimensions of 5.7 by 11 nm, and displays dyad symmetry. Though nucleosomal DNA is largely B-form, X-ray crystallography has revealed regions of sharp DNA bending at  $\pm 10$  and  $\pm 40$  bp from the DNA dyad, which is located at about 73 bp (Richmond, et al., 1984).

The primary sequence of bases in DNA has a major

impact on the frequency of DNA-adduct formation by a variety of alkylating agents (Muench, et al., 1983; Hartley, et al., 1986; Mattes, et al., 1986b; Boles and Hogan, 1986; and Marien, et al., 1987). The phenomenon gains great importance from the fact that many of these agents are potent carcinogens (Swenson, et al., 1975; Heidelberger, 1975; Sinnhuber, et al., 1977). However, it is unclear whether the nucleosomal conformation of DNA alters such sequence-specific alkylation. Nor do we understand how the rotational orientation of DNA on the nucleosomal surface, or nucleosomal DNA-histone contacts modulate DNA-mutagen/carcinogen interactions. It is the aim of this thesis to address these questions. In order to accomplish this, a homogenous population of nucleosomes reconstituted with a defined DNA sequence are required.

## B. NUCLEOSOME RECONSTITUTION

### 1. Reconstitution with Random Sequence DNA.

Histones and DNA fragments can be separated, then reassembled into nucleosome core particles. Tatchell and van Holde (1977) reconstituted core particles by reassociation, where the NaCl concentration was raised to 2 M, then lowered by dialysis, or by combining salt extracted histones and DNA in 1.5 M NaCl, followed by step dialysis

to 10 mM Tris, 0.7 mM EDTA, pH 7.2. All physical properties of these particles analyzed (sedimentation velocity, histone content, circular dichroism, and DNA melting) were apparently identical to native particles. Additionally, DNase I, micrococcal nuclease, and trypsin digestion patterns reflected a structure indistinguishable from native particles. Tatchell and van Holde (1979) also investigated the influence of DNA length on reconstitution by probing the  $^{32}\text{P}$  end-labeled particles with DNase I. DNA fragments 111, 125, or 161 bp in length formed particles displaying an "end effect", where one end of each fragment occupied the same position as in native 146 bp particles. Particles formed with a 177 bp fragment displayed no unique DNA positioning. These studies demonstrate the reproducibility of salt reconstitution, and the apparent native conformation of nucleosome core particles thus formed.

## 2. Nucleosome Positioning

Reports of unique positioning of nucleosomes on long DNA (>200 bp) fragments appeared soon after the description of the nucleosomal structure of DNA. Musich, et al., (1977) reported a unique nucleosome positioning in african green monkey alpha-satellite DNA (AGM DNA), determined by DNA sequence information alone. In a turbulent chapter of chromatin research, this conclusion

was questioned, refuted, reinterpreted, and finally accepted in part. The original conclusions of Musich, et al., were incorrect in the exact positioning and its uniqueness, which their subsequent reports amended (Brown, et al., 1979; Musich, et al., 1982). AGM DNA is now accepted to contain one major, and seven minor nucleosome positioning signals (Neubauer, et al., 1986).

Chao, et al., (1979) reported two unique nucleosome locations on a 203 bp E. coli lac operator fragment, and three unique nucleosome positions on a 144 bp lac operator containing fragment. Some, but not all of these positions, were likely due to end effects.

In order to prepare a completely homogenous population of sequence positioned nucleosomes, a DNA fragment is required which displays no end effects and no multiple positions. Simpson and Stafford (1983) reported such a fragment, which contains the 5S rRNA gene of the urchin Lytechinus variegatus. This fragment was used for this thesis work, and will be described in detail below.

The 5S and two other DNA fragments, from E. coli and AGM DNA have been altered by successive deletion to find the critical determinants of nucleosome positioning (Fitzgerald and Simpson, 1985; Ramsay, 1986 and Neubauer, et al., 1986). Multiple, additive DNA-histone interactions, especially in the central 40 bp of the particle, are responsible for nucleosome positioning. No

consensus signal or "nucleosome positioning box" was found. Two recent reviews (Simpson, 1986 and Travers, 1987) describe other systems in which nucleosome positioning occurs, and assess the nature of the additive DNA-histone contacts which position nucleosomes.

### C. AFLATOXIN B<sub>1</sub>

During 1960, wide-ranging outbreaks of disease and death in fowl, pigs, calves, and hatchery-reared rainbow trout were traced to toxins produced by the fungus Aspergillus flavus. Symptoms ranged from acute toxicosis characterized by hepatic necrosis and hemorrhage to hepatomas. In England, when biological transmission was ruled out, the search for a chemical agent implicated Brazilian peanut meal containing up to 20% fungal hyphae. Sargeant et al. (1961), unable to culture fungi from the sterile Brazilian meal, isolated A. flavus from a toxic sample of Ugandan peanuts. The A. flavus toxins so produced gained the moniker "aflatoxin". In the U.S., aflatoxin contaminated cottonseed meal proved to be the source of rainbow trout hepatomas (Goldblatt, 1969; Busby and Wogan, 1984).

Of the four major naturally occurring aflatoxins (Fig. I.C.1), aflatoxins B<sub>1</sub> and B<sub>2</sub> fluoresce blue under

## FIG. I.C.1 FOUR MAJOR NATURALLY OCCURRING AFLATOXINS

Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, produced by Aspergillus flavus.



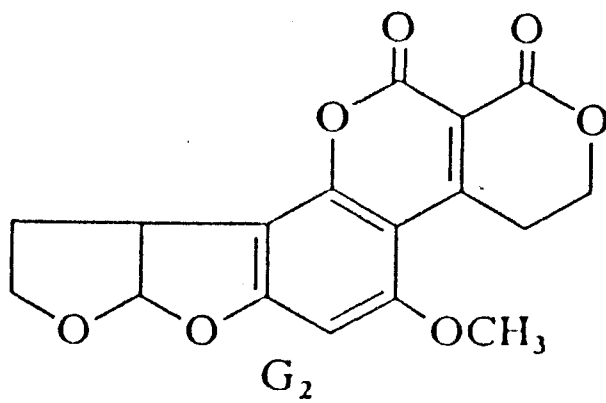
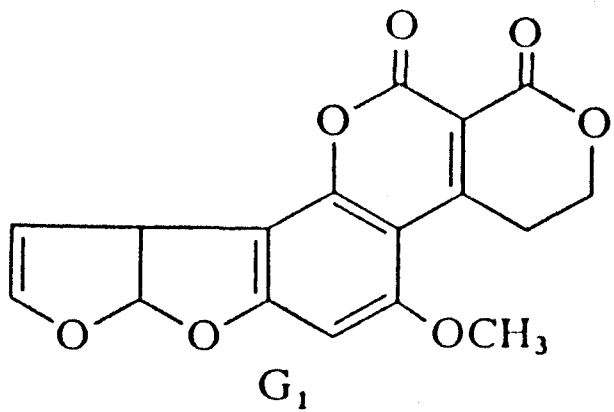
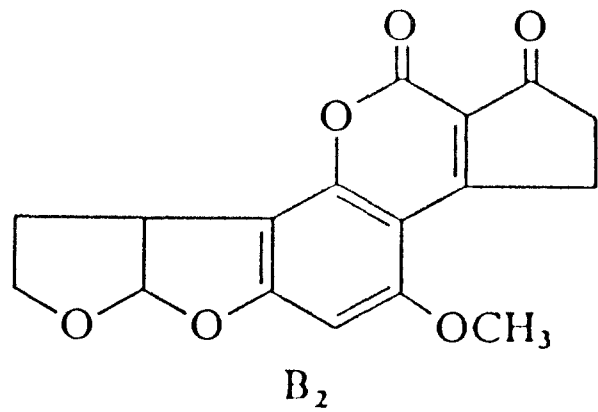
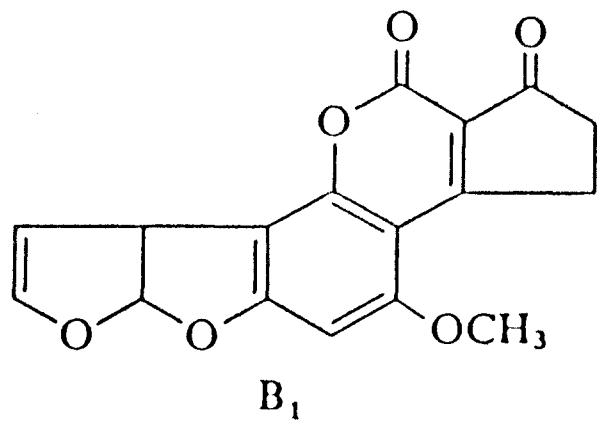


FIG. I.C.1

UV light, whereas aflatoxins G<sub>1</sub> and G<sub>2</sub> fluoresce greenish yellow. The B toxins contain a cyclopentanone ring fused to the coumarin lactone in place of the additional lactone of the G toxins. The critical moiety of these molecules is the 8,9-bond of the terminal furan ring. (Of the three extant aflatoxin numbering systems, IUPAC will be used throughout.) AFB<sub>1</sub>, with an unsaturated 8,9-bond, proved to be the primary deleterious agent in contaminated animal feeds. If this bond is saturated, as in B<sub>2</sub> and G<sub>2</sub>, the compounds are weak mutagens or carcinogens, probably due to a low level of conversion to the unsaturated bond. Wong and Hsieh (1976) assessed the mutagenicity of these four compounds with the Ames test. With AFB<sub>1</sub> set at 100%, AFG<sub>1</sub> was 3.3%, AFB<sub>2</sub> 0.2% and AFG<sub>2</sub> 0.1%. Mutagenicity was undetectable when AFB<sub>1</sub> and AFG<sub>1</sub> were hydroxylated at the 8 position to AFB<sub>8a</sub> and AFG<sub>8a</sub>.

AFB<sub>1</sub> must be activated via the mixed-function oxidase system before covalent attachment occurs in vivo. The major covalent adduct formed is N7-guanine (Miller and Miller, 1977; Croy, et al., 1978; and Muench, et al., 1983). The AFB<sub>1</sub> activated intermediate, the 8-9 oxide, has never been isolated due to its extreme reactivity. An electronic analogue of activated AFB<sub>1</sub>, AFB<sub>1</sub>-dichloride (AFB<sub>1</sub>-Cl<sub>2</sub>) was synthesized by Swenson, et al. (1975). AFB<sub>1</sub>-Cl<sub>2</sub> displays identical in vitro behavior as AFB<sub>1</sub>, in N7-guanine alkylation, DNA sequence-selectivity, and

replication blocks (Jacobsen, 1987), thus it was suitable for the studies herein. It should be noted that the AFB<sub>1</sub>-Cl<sub>2</sub>-DNA adduct contains a chlorine atom at the 9 position, as opposed to a 9-hydroxyl in AFB<sub>1</sub>-DNA adducts. Therefore, even though AFB<sub>1</sub>-Cl<sub>2</sub> displays the same in vitro binding characteristics as activated AFB<sub>1</sub>, the activated species of the two compounds and the ultimate adducts are not identical. AFB<sub>1</sub> sequence-specificity and AFB<sub>1</sub> chromatin binding are discussed below.

#### D. SEQUENCE EFFECTS ON DNA ALKYLATION

This section discusses two types of mutagen/carcinogen-DNA interactions; those interactions which are base specific, such as dimethyl sulfate binding to N7-guanine and N3-adenine, and those interactions which are both base specific and sequence specific, such as the 10-100 fold modulation of aflatoxin B<sub>1</sub>-N7-guanine binding, which is largely, but not wholly influenced by the bases immediately 5' and 3' (Marien, et al., 1987). Thus the term "sequence specific" will be reserved for those interactions wherein binding to a particular base is modulated by neighboring bases; all other binding to a particular base will be referred to as "base specific".

Unfortunately, the majority of DNA-base modifications

are not easily studied in a sequence-specific context. The problem is analogous to protein sequencing. A protein sample can be rather easily hydrolyzed and the percentages of its constituent amino acids determined, but an additional level of information, the order of these amino acids in the protein, must be obtained by more tedious means involving selective cleavage and purification of peptides, or sequencing in far enough to make a DNA probe. As with protein, DNA can be hydrolyzed and the bases analyzed by a variety of methods to determine which bases are altered, and at which atoms. Yet, this level of analysis does not assess the influence of neighboring bases on damage at any particular base.

In order to discern the sequence context of DNA lesions, traditional sequencing methods have been employed, with slight modifications. One technique is a variation of Sanger dideoxy sequencing, where the Klenow fragment of DNA polymerase I is used to map the location of DNA lesions. Instead of incorporation of dideoxy nucleotides, chain termination occurs at DNA lesions. The limitation of this "replication block" method is that bypass of the lesion sometimes occurs (Foster, et al., 1983; Jacobsen, et al., 1987). Such bypass may not occur at bulky adducts unless an apurinic site is first generated by the spontaneous or repair-mediated removal of the damaged base (Loeb, 1985). Until the extent of

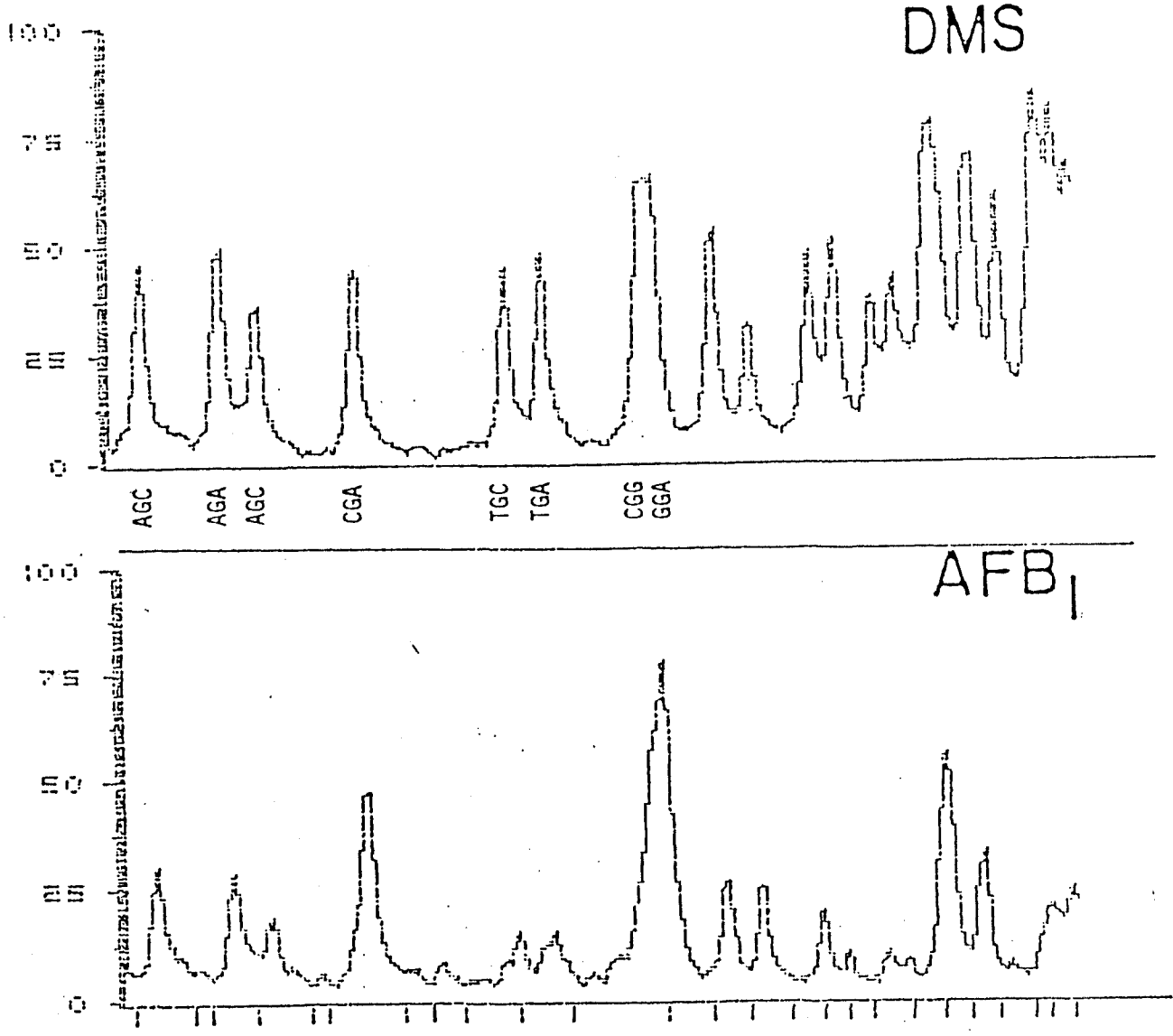
bypass is known, this method cannot be used to quantitatively determine DNA sequence-specific adduction.

Another method of studying DNA damage in a sequence context is analogous to the "G" lane chemical cleavage used in the Maxam and Gilbert (1980) method of chemical sequencing. Figure I.D.1, densitometric scans of DMS or AFB<sub>1</sub>-Cl<sub>2</sub> induced guanine specific cleavage, is an example of this method. When N7-guanine is alkylated, its glycosidic bond at N9 becomes base labile. Treatment with hot piperidine (0.3-1 M piperidine, 90°C) causes quantitative depurination and strand scission via  $\beta$ -elimination of the phosphate backbone. Treatment with hot alkali alone will not cause quantitative breakage at alkylated N7-guanines; the use of piperidine is essential, as piperidine catalyzes the removal of the formamido pyrimidine form of guanine (Mattes, et al., 1986a). Piperidine cleavage of N7-alkylguanines has been used to establish sequence specific binding for AFB<sub>1</sub> and AFB<sub>1</sub>-Cl<sub>2</sub> (D'Andrea and Haseltine, 1978; Misra, et al., 1983), AFB<sub>1</sub> and three metabolites - AFM<sub>1</sub>, aflatoxicol, and aflatoxicol M<sub>1</sub> (Marien, et al., 1987), eight nitrogen mustards (Mattes, et al., 1986b), and three chloroethylating antitumor agents plus three nitrosoureas (Hartley, et al., 1986). Additionally, quantitative cleavage of benzo(a)pyrene diol epoxide-N2-guanine adducts has been produced by laser light

FIG. I.D.1 N7-GUANINE ADDUCTION AND CLEAVAGE BY DIMETHYL  
SULFATE AND AFLATOXIN B<sub>1</sub>-Cl<sub>2</sub>

The 5S DNA fragment, W strand, was uniquely 5' end-labeled, alkylated with either DMS or AFB<sub>1</sub>-Cl<sub>2</sub>, then cleaved by piperidine treatment. Resultant bands resolved on a sequencing gel were scanned by laser densitometry. The upper portion of the gel, positions 65-145, is represented here. DNA base triplets below several cleavage sites demonstrate the marked sequence specificity of AFB<sub>1</sub>-Cl<sub>2</sub>, compared to the much less pronounced fluctuations in DMS binding.

FIG. I.D.1



(335 nm) excitation of the benzo(a)pyrene chromophore (Boles and Hogan, 1984, 1986). With the exception of two nitrogen mustards and one chloroethylating agent, all of these compounds displayed sequence-specific adduct formation, characterized most distinctively by the middle guanine of a GGG triplet being the most reactive. Other general findings from these studies include:

- (i). Guanine was less reactive when flanked by other bases, especially A and/or T.
- (ii). The next nearest neighbor (2 bases away) had little or no influence on the frequency of adduction at the middle guanine.
- (iii). The more cationic the character of the attacking species, the more pronounced the sequence-specific DNA binding.
- (iv). The more electronegative the central guanine of any triplet, the more pronounced the sequence specific binding.
- (v). Occasional, pronounced exceptions exist to any of these predictive binding "rules".

The above indicates that much remains to be elucidated about the exact nature of sequence-specific binding. For example, the pentanucleotide ATGCT was the strongest AFB<sub>1</sub> binding site in one context but one of the weakest in another (Marien, et al., 1987). It must be stressed that all such experiments have been conducted only with



isolated, purified DNA; not with chromatin or nucleosomes. Therefore, while extrapolations from naked DNA to isolated chromatin or in vivo N7-guanine alkylation levels have been suggested, there is presently no evidence that such extrapolation would be valid.

To test the influence of the first level of chromatin structure on sequence-specific DNA-adduct formation, I have treated the 5S DNA fragment, either free in solution or complexed into a nucleosome, with AFB<sub>1</sub>-Cl<sub>2</sub>, which displays perhaps the greatest range of sequence-specific DNA binding.

#### E. CHROMATIN-CARCINOGEN INTERACTIONS

In eukaryotes, it is chromatin, not naked DNA, which constitutes the target site of mutagens and carcinogens. Hence the nucleosome, the fundamental unit of chromatin, must be considered in any thorough evaluation of such insults to clarify actual physiological events. Experimental evidence indicates that genotoxic agents bind non-randomly in eukaryotic chromatin (Jahn and Litman, 1979; Kootstra, et al., 1979; Bailey, et al., 1980; Irvin and Wogan, 1984; Kurian, et al., 1985; Kootstra, 1986; Warner, et al., 1986; and Delcuve, et al., 1988).

The first definitive studies on chromosomal location of carcinogen adducts utilized micrococcal nuclease to isolate nucleosome core particles from carcinogen-labeled chromatin. By injecting trout with  $^3\text{H}$ -aflatoxin  $\text{B}_1$ , Bailey, et al., (1980) conducted in vivo analysis of  $\text{AFB}_1$  adduct concentrations among mono- and oligonucleosomal fractions containing differing percentages of core and linker DNA. These data indicated that, in the repair-deficient liver of rainbow trout, linker DNA is about 5-fold more susceptible to adduction than the histone-protected core DNA. Kootstra (1986), examining benzo(a)pyrene diol epoxide binding in human lung cell cultures, found linker to core binding ratios ranging from 14.5 to 1.7, depending on the class of mononucleosomes examined and the time of incubation after treatment, during which repair occurred.

At the gene level, rRNA gene sequences, which appear to be non-nucleosomal when examined by electron microscopy (Scheer, 1980; Labhart and Koller, 1982), are adducted 5-fold over bulk nuclear DNA (Irvin and Wogan, 1984). Irvin and Wogan (1985) also examined the repair of these  $\text{AFB}_1$  lesions, determining that the  $\text{AFB}_1$ -DNA adducts were also removed at a 5-fold higher rate, over the twelve hours measured. At the single gene copy level, histone H5 and  $\beta$ -globin genes in chicken erythrocytes are more accessible to  $\text{AFB}_1$ - $\text{Cl}_2$  modification

than vitellogenin or ovalbumin genes, which are inactive in these cells (Delcuve, et al., 1988)

All of the previous studies of carcinogen-chromatin interaction have measured only average relative accessibility, over extended gene regions or with the whole genome. To obtain a more precise understanding of the role of local chromatin structure in DNA-genotoxin interaction, the work described in this thesis utilized a monodisperse population of DNA sequence-positioned nucleosomes. The advantages of this approach are twofold: (i) an averaged population of nucleosomes is avoided and (ii) changes in global and local sequence-specific patterns of DNA alkylation, as modulated by DNA-histone interactions, can be quantitated at the single-base level.

## II. MATERIALS AND METHODS

### A. CHEMICALS

Dimethyl sulfate (Aldrich) and piperidine (Sigma, St. Louis, MO.) were purchased and used without further purification. m-Chloroperoxybenzoic acid, purchased from Aldrich Chemical Co. (Milwaukee, WI), was used without further purification. AFB<sub>1</sub> (Calbiochem, La Jolla, CA.) and <sup>3</sup>H-AFB<sub>1</sub> (Moravek Biochemicals, La Brea, CA.) were purchased, then dissolved in 95% ethanol, to approx. 100 ug/ml. These stock solutions were scanned from 210 to 400 nm on a Varian DMS 100 UV-VIS spectrophotometer, and quantitated using an AFB<sub>1</sub> extinction coefficient of 21,800 at 364 nm.

### B. AFLATOXIN B<sub>1</sub>-DICHLORIDE ACTIVATION

(Aflatoxins are extremely potent hepatocarcinogens and should be used with care. See Appendix for safe handling methods.)

#### 1. Peracid activation of Aflatoxin B<sub>1</sub>

Naked DNA (salmon sperm, sheared to approx. 300 bp) or chicken erythrocyte nucleosome core particles (200 ug, as measured by A<sub>260</sub>) were combined with 20 ug AFB<sub>1</sub> in 1 ml

of sodium phosphate, pH 7.4. m-Chloroperoxybenzoic acid (CPBA) was added in 5 ul aliquots at 0, 7.5, 15, and 22.5 minutes. Each CPBA addition represented a 10 M excess, relative to AFB<sub>1</sub>. After 30 min. in a shaking water bath at 37°C, 5 ul of 2-mercaptoethanol was added to each tube to quench the reaction.

## 2. Aflatoxin B<sub>1</sub>-Dichloride Synthesis

AFB<sub>1</sub>-Cl<sub>2</sub> was synthesized as described (Swenson, et al., 1975), where AFB<sub>1</sub> was reacted with a 10% molar excess of chlorine in methylene chloride. As AFB<sub>1</sub>-Cl<sub>2</sub> exhibits a half-life of 0.5 min. in aqueous solution of 10% DMSO, pH 7.4, care was taken to oven or flame dry all glassware immediately before use, and to dry, then store all solvents under dry N<sub>2</sub>. AFB<sub>1</sub>, 10 mg (32 μmoles) in 95% ethanol, was made 10% benzene (creating an azeotropic mixture to remove water), then evaporated to dryness. Ten ml of methylene chloride, stored over Linde type 4A molecular sieves, was used to redissolve the AFB<sub>1</sub>. Chlorine gas was bubbled into a separate flask of methylene chloride for 10 s, until the solution had a slight yellowish cast. To determine the chlorine concentration, one ml of the above solution was combined with one ml of 0.1 N potassium iodide and 1 ml of a 1% w/v aqueous starch indicator solution. While stirring,

this solution was titrated by addition of 0.53 ml of 0.1 N sodium thiosulfate. Since the chlorine:methylene chloride solution was 0.053 M in  $\text{Cl}_2$ , 0.66 ml of this contained 35 mmoles of chlorine, which is a 10% molar excess to the 32 mmoles of  $\text{AFB}_1$ . After addition of chlorine to the  $\text{AFB}_1$ :methylene chloride solution, the reaction mixture was placed in the dark and incubated for five minutes at room temperature, after which excess chlorine and methylene chloride were removed under vacuum. Ten ml of methylene chloride:acetone (19:1) was used to redissolve the residue, and aliquots of this were spotted on thin-layer silica plates, along with an unreacted  $\text{AFB}_1$  standard. The plate was developed once with methylene chloride:acetone (19:1), dried, and developed again. The new compound migrated 1.4 times as fast as the parent compound  $\text{AFB}_1$ .

$3\text{H-AFB}_1\text{-Cl}_2$  was synthesized as described above for  $\text{AFB}_1\text{-Cl}_2$ . After thin layer chromatography, spots were scraped and counted on a Beckman LS 7500 scintillation counter. The yield of  $\text{AFB}_1\text{-Cl}_2$  was 59%. Both the yield and  $R_F$  values agreed with those reported by Swenson, et al. (1975).  $\text{AFB}_1\text{-Cl}_2$  was then separated from  $\text{AFB}_1$  by HPLC, before mass spectrometry analysis, as follows.

3. HPLC Separation of Aflatoxin  $\text{B}_1$ -Dichloride from Aflatoxin  $\text{B}_1$

The reaction mixture was loaded on a silica Sep-Pak (Waters Assoc., Milford, MA) and washed with increasingly polar solvent to separate AFB<sub>1</sub>-Cl<sub>2</sub> and AFB<sub>1</sub> from the slight amount (<10%) of polar contaminants. One 10 ml wash with methylene chloride was followed by one 10 ml and four 5 ml washes of methylene chloride:acetone (49:1). This was followed by two 10 ml washes of methylene chloride:acetone (47:3). A silica thin layer plate, run as described above, on all 8 collected washes, showed that most of the AFB<sub>1</sub>-Cl<sub>2</sub> eluted in the second and third wash, and that the parent compound, AFB<sub>1</sub>, eluted in the second through sixth washes. The 15 ml from the second and third wash steps were combined, then evaporated by vacuum to approx. 1 ml. This was loaded, in 200 ul portions, onto a Waters HPLC (normal phase) equipped with a uPorasil silica column. A linear gradient of 2.5% acetone in methylene chloride, at a 1 ml/min flow rate, allowed separation to baseline between the leading AFB<sub>1</sub>-Cl<sub>2</sub> peak and the trailing AFB<sub>1</sub> peak. AFB<sub>1</sub>-Cl<sub>2</sub> and AFB<sub>1</sub> were collected separately and stored at -20°C.

#### 4. Mass Spectrometry of Aflatoxin B<sub>1</sub>-Cl<sub>2</sub>

A Finnigan Model 4023 Quadrupole Mass Spectrometer-Model 4500 source, with 70 eV ionization at 140°C source temperature, was used to confirm the stoichiometry of

AFB<sub>1</sub> chlorination. AFB<sub>1</sub>-Cl<sub>2</sub> in methylene chloride was examined by direct entry probe. The molecule left the probe at approx. 300°C, and its mass spectrum, shown in Fig.III.A.2, indicated two chlorine character.

### C. GEL ELECTROPHORESIS

Chick erythrocyte and reconstituted nucleosomes were examined by SDS or native polyacrylamide mini slab gels (Idea Scientific, Corvallis, OR). To assess histone integrity, histones were denatured from nucleosomes by boiling 1 min in a 5% 2-mercaptoethanol, 2% SDS buffer, then resolved on a 0.8 mm thick 6% stacking and 15% separating mini slab gel (Laemmli, 1970). Electrophoresis was conducted at 20 V/cm until the bromophenol blue marker was ~1 cm from the gel bottom. Gels were then stained with Coomassie blue (Weber and Osborn, 1969).

To determine the extent of peracid oxidation-induced histone modifications after CPBA treatment of nucleosomes, acid-urea-Triton gels electrophoresis was conducted. Samples were lyophilized, resuspended in 5 M urea, 2% 2-mercaptoethanol, and 1.5% protamine, and heated for 30 min at 60°C (Richards and Shaw, 1982), before being applied to gels of 15% polyacrylamide, 6 M



urea and 0.5% (w/v) Triton X-100 (Bonner, et al., 1980). These gels were subjected to 25 V/cm for 1.5-2 h., then stained with Coomassie blue.

Nucleosomes or DNA fragments were analyzed on particle gels after Todd and Garrard(1977), where 1 mm thick 5% acrylamide gels, with a 1:20 bis-acrylamide to acrylamide ratio, were electrophoresed at 12.5 V/cm, 4°C, for one hour. These gels were then either stained with ethidium bromide and photographed by visualization with longwave UV light, or fixed in 5% glacial acetic acid and dried for 30 min, before autoradiography.

Single strand DNA mini-gels were used to determine DNA length in chicken nucleosome core particle preparations. These 8% polyacrylamide gels contained a 29:1 crosslinking ratio and 7 M urea, and electrophoresis was conducted as above for SDS gels. Gels were then stained and photographed as for particle gels.

For sequencing, 40 cm, 8% polyacrylamide slab gels, as described by Maxam and Gilbert (1980) were subjected to 50 W constant power for 2-4 h. These gels contained 8 M urea, and had a 19:1 acrylamide:bis-acrylamide ratio. Gels were fixed in 5% glacial acetic acid and dried for 1 h before autoradiography.

For analysis of intercalation-induced DNA supercoiling, mini-slab gels of 1% agarose in Tris-borate buffer were prepared. Samples were electrophoresed at 10

V/cm for 45 min to 1 h, then stained and photographed as above for particle gels.

#### D. MONONUCLEOSOME PREPARATION

Nucleosome core particles were prepared following Lutter (1978) with modifications as described by McMurray and van Holde (1986). Two to three chickens were purchased from the Dept. of Poultry Science at Oregon State University. Each individual was anesthetized/asphyxiated by diethyl ether, then bled by severing the carotid artery. Blood was collected into a 800 ml beaker, containing 15,000 units heparin on ice, and then filtered through four layers cheesecloth to remove feathers and clots.

Blood (50-100 ml/chicken) was centrifuged at 1500 g, 5 min in a Sorvall GSA rotor, to separate cells from serum. Cells were washed 3x in buffer containing 0.1 mM PMSF, then lysed by 3 cycles of resuspension in 0.5% NP-40 (a non-ionic detergent) and mixing with a Tekmar Model SDT tissuemizer set at lowest speed. Nuclei were collected by 1500 g centrifugation, 5 min, resuspended to 65 A<sub>260</sub>/ml and digested 2 min with 45 units/ml micrococcal nuclease (Worthington). Nuclei were lysed by resuspension in 0.2 mM EDTA, then made 0.35 M NaCl by slow addition, with stirring, of a 4 M stock. To remove histones H1, H5 and non-histone proteins, Carboxymethyl-

Sephadex C-25-120 was added to 30 mg/ml (Libertini and Small, 1980). The resultant stripped, long chromatin was again digested with micrococcal nuclease to mostly mono- and dinucleosomes, then applied to a column of Sepharose 6B-CL. Mononucleosomes were collected, pooled and trimmed again with micrococcal nuclease. By doing a test digest and then running the DNA on a single strand DNA gel, the optimal time of nuclease digestion necessary to give 146<sub>±</sub>3 bp was determined. After this third digestion, nucleosomes were fractionated on the Sepharose 6B-CL column again, and nucleosome core particles containing 146<sub>±</sub>3 bp were pooled, concentrated, and stored on ice.

#### E. PLASMID PREPARATION

E. coli HB101 containing plasmid pLv405-10 (Simpson and Stafford, 1983) was obtained from Dr. R.T. Simpson. This plasmid contains 10 tandem repeats of a 260 bp segment of Lytechinus variegatus DNA. Within the 260 bp segment are a 120 bp fragment encoding 5S rRNA, a flanking 90 bp region on the 5' side and 50 bp downstream (Fig. II.E.1). Plasmid was prepared by the methods of Sakonju et al. (1980) and Clewell and Helinski (1970). Cells were initially grown without chloramphenicol

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          -10      0      10      20      30
          |      |      |      |      |
AATTCCAACGAATAACTTCCAGGGATTTATAAGCCGATGACGTCATAACA
  GGTGCTTATTGAAGGTCCCTAAATATTCGGCTACTGCAGTATTGT

          40      50      60      70      80
          |      |      |      |      |
TCCCTGACCCTTTAAATAGCTTAACTTTCATCAAGCAAGAGCCTACGACC
  AGGGACTGGGAAATTTATCGAATTGAAAGTAGTTCGTTCTCGGATGCTGG

          90      100     110     120     130
          |      |      |      |      |
ATACCATGCTGAATATACCGGTTCTCGTCCGATCACCGAAGTCAAGCAGC
  TATGGTACGACTTATATGGCCAAGAGCAGGCTAGTGGCTTCAGTTCGTCG

          140     150     160     170     180
          |      |      |      |      |
ATAGGGCTCGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCGGGTG
  TATCCCGAGCCAATCATGAACCTACCCTCTGGCGGACCCTTATGGCCCAC

          190     200     210     220     230
          |      |      |      |      |
TTGTAGGCTTTTTTTTCTCCCCCCCCCCTCTTTGCTTCATGAAATGCC
  AACATCCGAAAAAAAGAGGGGGGGGGGAGAAACGAAGTACTTTACGG

          240
          |
TCTTGG
  AGAACCTTAA

```

FIG. II.E.1 SEQUENCE OF THE CLONED L. VARIEGATUS 5S rRNA GENE SEGMENT

The underline shows the nucleosome-binding region, and the arrows depict the NciI site at 175.

amplification, as this may have been the source of plasmid nicking due to ribose substitution. Later preparations included chloramphenicol amplification, and the "nicking problem" did not persist. Cells were pelleted by centrifugation, resuspended in 25% (w/v) sucrose, 50 mM TRIS, pH 8.0, then lysed by addition of lysozyme to 3 mg/ml. After 30 min on ice, EDTA was added to 25 mM, then the solution was made 0.5% Brij 58 and 0.2% deoxycholate. After 5 min on ice, 5 min at 37°C, then 15 more min on ice, the solution was subjected to centrifugation at 17,000 rpm in a Sorvall SS34 rotor for 80 min. The supernatant was decanted, made 15% polyethylene glycol 8000, 0.75 M NaCl, then incubated on ice for 30 min. Plasmid was then precipitated by centrifugation at 12,000 rpm in a SS34 rotor, for 15 min. Pellets were resuspended in 5 ml of 10 mM Tris (pH 7.2), 1 mM EDTA. Volumes were adjusted to 13.5 ml before addition of 14.0 g optical grade CsCl and 1.35 ml of 10 mg/ml ethidium bromide. Exactly 4.4 ml were transferred into Sorvall 12.7 X 50.8 mm polyallomer tubes and inserted into a Sorvall TV 865 vertical rotor. (Care was taken to shield samples from light while ethidium bromide was present.) Samples were centrifuged at 45,000 rpm, for 17 h, at 4°C. The bottom DNA band was collected with a 1 ml syringe. Ethidium bromide was extracted from samples with CsCl-saturated isopropanol until the

solution was clear. Plasmid was precipitated by addition of 3 volumes of distilled water, then 2.5 volumes of 95% ethanol. After precipitation, DNA was extracted with phenol:chloroform, followed by chloroform. After addition of 2.5 volumes 95% ethanol, DNA was stored at 20°C. Subsequent agarose gel electrophoresis showed >90% supercoiled plasmid, with a fraction of circular, but no linear plasmid.

#### F. PREPARATION OF END LABELED 5S DNA FRAGMENTS

Plasmid pLv405-10 was cleaved with EcoRI. For 5'-end labeling, ends were desphosphorylated with bacterial alkaline phosphatase, then labeled with gamma-<sup>32</sup>P-ATP (ICN, 6000 Ci/mmol) by T4 polynucleotide kinase. Labeling at the 3' end was performed by addition of Klenow fragment of DNA polymerase I and alpha-<sup>32</sup>P-ATP (New England Nuclear, 3000 Ci/mmol). Free label was removed by centrifugation of the reaction mixture through small columns of Sephadex G50-150 (Maniatis, et al., 1982). Secondary restriction was performed with either NciI or RsaI, to generate 195 or 165 bp fragments, respectively, and ClaI, to cleave a similar size plasmid fragment. A 1 mm wide particle gel was prepared with the comb inserted upside down, to make a trough. The labeled

fragments were made 5% glycerol, then applied evenly over the top of the gel, which was run at 20 V/cm for one h. The  $^{32}\text{P}$ -labeled bands were visualized by brief exposure to Kodak X-OMAT AR film. The band of interest was then excised, placed in a 5 ml tube, and the DNA eluted by gentle, overnight shaking in 1 volume of 10 mM Tris, pH 7.4, 1 mM EDTA. The next morning, this solution was removed, and 0.5 volumes of buffer were placed in the tube, which was shaken 1 hour. The two solutions containing extracted DNA (90-95% yield) were pooled, then concentrated by butanol extraction or by Centricon (Amicon) centrifugation. The remaining 100-200 ul was extracted with phenol:chloroform (1 volume), then chloroform, before ethanol precipitation.

#### G. RECONSTITUTION

Nucleosomes and free,  $^{32}\text{P}$  end-labeled 5s DNA were combined in 300 ul of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (TE), and 0.8 M NaCl (Fig. II.G.1). To achieve a ratio of 1:200 to 1:250 pieces of 5S DNA to core particles, 81 to 100 ng of 195 bp fragment was added to 15 ug of core particles, as determined by absorbance at 260nm on either a Cary 2200 or a Bausch and Lomb 601 spectrophotometer. After 20 minutes on ice, during which exchange of DNA

FIG. II.G.1 RECONSTITUTION OF SEQUENCE POSITIONED  
NUCLEOSOMES

The 260 bp fragment, containing the sea urchin 5S gene, is 5'  $^{32}\text{P}$  end-labeled with T4 kinase. The arrow at position 195 denotes Nci I cleavage, which renders a uniquely end-labeled fragment. Chicken erythrocyte nucleosomes and 5S DNA are combined in a 200:1 or 250:1 ratio, in 0.8 M NaCl. Exchange of histones occurs between the chicken nucleosomes and 5S DNA, then the NaCl mixture is lowered to 0.6 M, and finally 0.05 M by dialysis. The 5S gene precisely positions a histone octamer, between 20 and 165 bases from the labeled end.





occurs with histones, dialysis tubing (3500 dalton cutoff) was placed over the tube, secured, and the tube inverted in 200 ml of TE, 0.6 M NaCl, and 1 mM PMSF, stirred at 4°C for >12h, then transferred to 200 ml of TE, 0.05 M NaCl, 1mM PMSF for >3h (Tatchell and van Holde, 1977; Simpson and Stafford, 1983; Yager, 1984). After each reconstitution, a small amount of these particles were resolved on mini 3.5% PAGE, as described above. Gels of reconstituted particles containing the 5S rRNA gene were dried, then exposed to film for 12-16 h, usually without the aid of intensifying screens. Bands were quantitated by laser densitometry, demonstrating that 90-97% of the original 5S DNA incorporated into core particles of appropriate size, with no detectable free 5S DNA.

#### H. DNA ALKYLATION

Control DNA was purified from nucleosomes by Proteinase K (Boeringer Mannheim) digestion (0.5 mg/ml), phenol and chloroform extraction, and ethanol precipitation (Britten, et al., 1974). For alkylation, DNA samples were dissolved in 0.2 ml of 50 mM NaCl, 10 mM Tris (pH 7.5), and 1 mM EDTA. Equivalent amounts (3.5 ug) of DNA, either naked or in reconstituted nucleosomes,

were treated in parallel with dimethyl sulfate (DMS) or aflatoxin B<sub>1</sub>-Cl<sub>2</sub> (AFB<sub>1</sub>-Cl<sub>2</sub>), on ice. DMS was added to a final concentration of 200  $\mu$ M, and the reaction quenched after one minute by addition of dithiothreitol to 2 mM. AFB<sub>1</sub>-Cl<sub>2</sub> in methylene chloride was added to tubes and dried under a gentle stream of dry nitrogen before addition of buffer and DNA or nucleosomes, then dissolved by vigorous shaking. Final concentration of AFB<sub>1</sub>-Cl<sub>2</sub> was 1.7  $\mu$ M. After five minutes on ice, the reaction was quenched with dithiothreitol as above. An aliquot of each sample was resolved on a particle gel to ensure particle integrity after alkylation. Samples were then made 0.5% SDS, 0.5 mg/ml Proteinase K and incubated 30 min. at 37°C. NaCl was added to a final concentration of 0.2 M. After extractions with 1 volume each of phenol, phenol:chloroform, and chloroform, the DNA was ethanol precipitated, then dissolved in 0.1 ml of glass distilled H<sub>2</sub>O. Each sample was made 3% (v/v) piperidine, then placed in a 90°C heating block. After 20 min., each tube was pierced with a needle, frozen, and lyophilized. Each pellet was redissolved with 10-20  $\mu$ l water, and counted in a Packard Tri-carb liquid scintillation counter. After two additional lyophilizations, sufficient 90% (v/v) formamide (2-20  $\mu$ l) was added to make each sample the same activity per unit volume (usually 10,000 cpm/ $\mu$ l). Equivalent volumes (usually 2  $\mu$ l) of each

sample were applied to sequencing gels for resolution of fragments.

#### I. QUANTITATION OF DMS OR AFB<sub>1</sub>-Cl<sub>2</sub> BINDING

Sequencing gels were fixed, dried, and then exposed to film for 12 to 96 h, in order to obtain a range of film intensities. Care was taken to scan within the linear range of the film. In order to eliminate errors due to irregularities of band shape or width, films were turned ninety degrees and scanned across the two lanes at bands being compared. This also allowed more accurate quantitation of less intense bands, as scanner gain could be adjusted to maximize signal to noise ratios - settings which often put adjacent darker bands far off scale. A few weakly adducted AFB<sub>1</sub>-Cl<sub>2</sub> cleaved sites were judged insufficiently above film background to be included in the cleavage map (Fig. III.D.2). Additionally, adjacent pairs or triplets of binding sites toward the right side of the particle (Fig. III.D.2), beginning with 100,101 on the W strand, and 32 on the C strand, were scanned and quantitated as a single ratio of DNA:core binding, and are depicted accordingly.

AFB<sub>1</sub>-Cl<sub>2</sub>:DNA ratios were chosen so that less than one adduct formed per DNA fragment or nucleosome, which is equivalent to less than 0.5 adducts formed per individual DNA strand. This was readily established by determining

densitometrically the amount of uncut DNA in AFB<sub>1</sub>-Cl<sub>2</sub> treated samples, compared to control DNA. The probability (P) that no cuts occurred in the treated samples was obtained by dividing the area of the trace from the treated sample by that of the untreated sample. The average number of cuts per strand (Y) was then calculated using the Poisson distribution function

$$P = \exp (-Y)$$

(Boles & Hogan, 1984, Marien, et al., 1987). At less than 0.5 cleavages per strand, Poisson statistics indicate less than 10% of the fragments cut twice, preventing a significant bias toward shorter fragments.

#### J. PLASMID SUPERCOILING

DNA topoisomers can be prepared by adding increasing amounts of the known intercalator ethidium bromide to aliquots of plasmid in the presence of Topoisomerase I (Peck, et al., 1982). After incubation, EtBr and Topo I are simultaneously extracted, producing EtBr-induced supercoiled plasmid. This method was used to determine if the precovalent mode of AFB<sub>1</sub> binding induced DNA supercoiling via intercalation. Supercoiled plasmid pUC 8 was relaxed with Topoisomerase I (Bethesda Research Labs) alone, or in the presence of ethidium or AFB<sub>1</sub>.

All reactions contained 1 ug of plasmid in 50 ul of 50 mM Tris (pH 7.4), 10 mM MgCl<sub>2</sub>, 50 mM KCl and 1 mM dithiothreitol. Two controls, one without Topo I, and one with Topo I, were prepared in parallel with each series of EtBr or AFB<sub>1</sub> incubations. Plasmid, plus 9 units Topo I, was incubated in 10:1, 100:1, and 1000:1 molar ratios of EtBr:plasmid, or 10:1, 100:1, 1000:1 and 10,000:1 molar ratios of AFB<sub>1</sub>:plasmid for 1 h at 37°C. All samples were then extracted twice with chloroform, and ethanol precipitated. After resuspension, samples were resolved on 1% agarose gels as described above.

## III. RESULTS

A. AFLATOXIN B<sub>1</sub> - ACTIVATION AND DNA BINDING

AFB<sub>1</sub> requires biological activation to be carcinogenic or mutagenic; this is accomplished in vivo by the mixed-function oxidase system. One can isolate the "S9" or microsomal liver fraction to activate AFB<sub>1</sub> in vitro, as was done to demonstrate AFB<sub>1</sub> mutagenicity in the Ames test (Ames, et al., 1973). A major disadvantage of this microsomal fraction for AFB<sub>1</sub> activation is extensive nuclease and protease contamination, which seriously compromises attempts to study extracellular DNA-protein complexes. Although purified enzyme (cytochrome P-450) may be used, the effect of these membranous protein fractions on purified nucleosomes is unknown. Chemical activation of AFB<sub>1</sub> with peracid was performed, but when it proved unacceptable, causing significant nucleosomal DNA dissociation and altered gel mobilities of histones H3 and H2B, AFB<sub>1</sub> dichloride, an activated AFB<sub>1</sub> analogue, was synthesized.

1. m-Chloroperoxybenzoic Acid Activation of AFB<sub>1</sub>

Martin and Garner(1977) showed that peracid oxidation of AFB<sub>1</sub> with m-chloroperoxybenzoic acid (CPBA) yielded the same AFB<sub>1</sub>-DNA chromatographic profiles as

microsomally activated AFB<sub>1</sub>. Therefore, studies were begun with CPBA to determine AFB<sub>1</sub> binding levels to naked DNA. As this system would soon be extended to AFB<sub>1</sub>-nucleosome binding, it was imperative to determine the effects of CPBA on histones.

Nucleosome core particles (200 ug, as measured by absorbance at 260 nm) were combined with 20 ug AFB<sub>1</sub> in 1 ml of sodium phosphate buffer, pH 7.4. CPBA was added in 5 ul aliquots at 0, 7.5, 15, and 22.5 minutes. Each CPBA addition represented a 10 M excess, relative to AFB<sub>1</sub>. After 30 min. in a shaking water bath at 37°C, 5 ul 2-mercaptoethanol was added to each tube to quench the reaction. The samples were resolved on a native particle gel, and ethidium bromide staining revealed a small (approx. 10%) dissociation of those core particles treated with CPBA. Those cores treated with AFB<sub>1</sub> or 2% dichloromethane (final concentration, used as the CPBA carrier) alone were not dissociated. Histones were resolved on an acid-urea-Triton gel, which revealed altered mobilities of histones H3 and H2B.

In light of this dissociation of DNA from nucleosomal particles and histone modification upon CPBA treatment, another method of AFB<sub>1</sub> activation was chosen. (It was recently noted that CPBA cleaves DNA, in a nonrandom manner (Jacobsen and Humayun, 1986).)



## 2. Aflatoxin B<sub>1</sub> Dichloride Synthesis

The putative active electrophile of AFB<sub>1</sub>, AFB<sub>1</sub>-8,9-oxide, has proven refractory to isolation, presumably due to its extreme reactivity. Swenson, et al. (1975) synthesized AFB<sub>1</sub>-Cl<sub>2</sub> as a model of this epoxide (Fig. III.A.1). The chlorine at carbon 8 is an efficient leaving group, forming a resonance stabilized carbonium ion. Indeed, AFB<sub>1</sub>-Cl<sub>2</sub> is a potent mutagen and carcinogen, displaying the same sequence-binding specificity as the activated parent compound (Swenson, et al, 1975; Muench, et al., 1983; Refolo, et al. 1987).

After <sup>3</sup>H-AFB<sub>1</sub>-Cl<sub>2</sub> synthesis, samples were subjected to silica thin layer chromatography. Spots were visualized by UV light, scraped and collected. Upon liquid scintillation counting, the yield of AFB<sub>1</sub>-Cl<sub>2</sub> was determined to be 59%, compared to a 58% yield obtained by Swenson, et al. (1975). AFB<sub>1</sub>-Cl<sub>2</sub> was subjected to normal phase HPLC on a silica uPorasil column with a 47:3 methylene chloride:acetone linear gradient. AFB<sub>1</sub>-Cl<sub>2</sub> was collected, then an aliquot was subjected to HPLC again to insure the absence of parent compound, AFB<sub>1</sub>. AFB<sub>1</sub>-Cl<sub>2</sub> was then subjected to mass spectrometry (Fig.III.A.2), which confirmed that the compound contained only two chlorines. The spectra matched closely those values given by Swenson, et al., except that their sample appeared to contain more parent compound.

FIG. III.A.1 SYNTHESIS OF AFLATOXIN B<sub>1</sub>-DICHLORIDE

AFB<sub>1</sub> was dissolved in dichloromethane, and a 10% molar excess of Cl<sub>2</sub> (as determined by iodometric titration) was added. After 5 min at room temperature, the solvent was evaporated and the residue dissolved in dichloromethane. In aqueous solution, the chlorine at carbon 8 leaves, forming a carbo-cation analogous to that in activated AFB<sub>1</sub>.

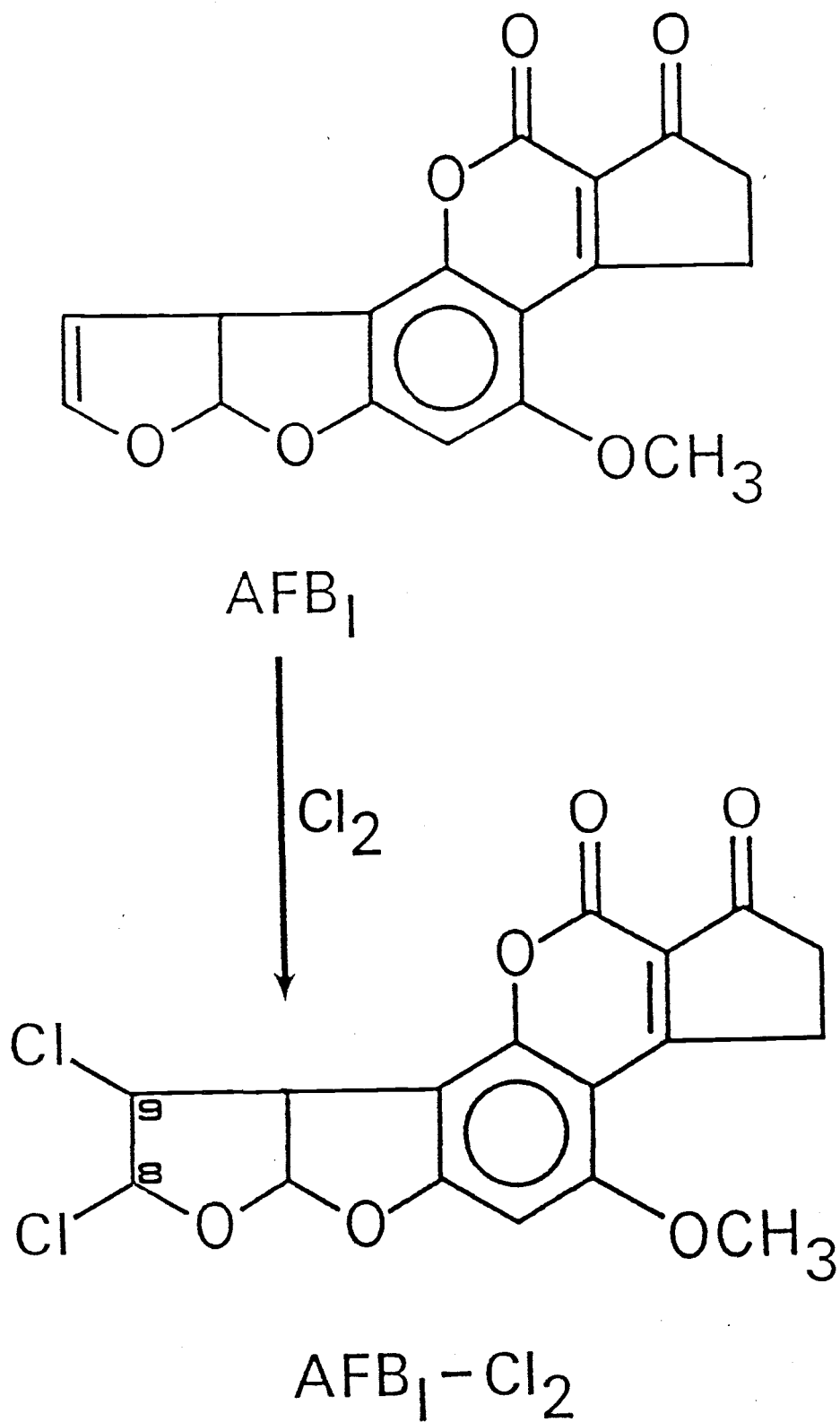


FIG. III.A.1

FIG. III.A.2 MASS SPECTRUM OF AFLATOXIN B<sub>1</sub>-DICHLORIDE

A Finnigan Model 4023 Quadrupole Mass Spectrometer-Model 4500 source, with 70 eV ionization at 140°C source temperature, was used to confirm the stoichiometry of AFB<sub>1</sub> chlorination. AFB<sub>1</sub>-Cl<sub>2</sub> in methylene chloride was examined by direct entry probe. The molecule left the probe at approx. 300°C, and its mass spectrum indicates two chlorine character.

MASS SPECTRUM  
05/24/88 22:33:00 + 9:57  
SAMPLE: EI PROBE  
COND.: EI PROBE  
0594 TO 0601 SUMMED

DATA: AFLATOX B597  
CALL: FC43B B3

BASE P/E: 311  
R/C: 1075190.

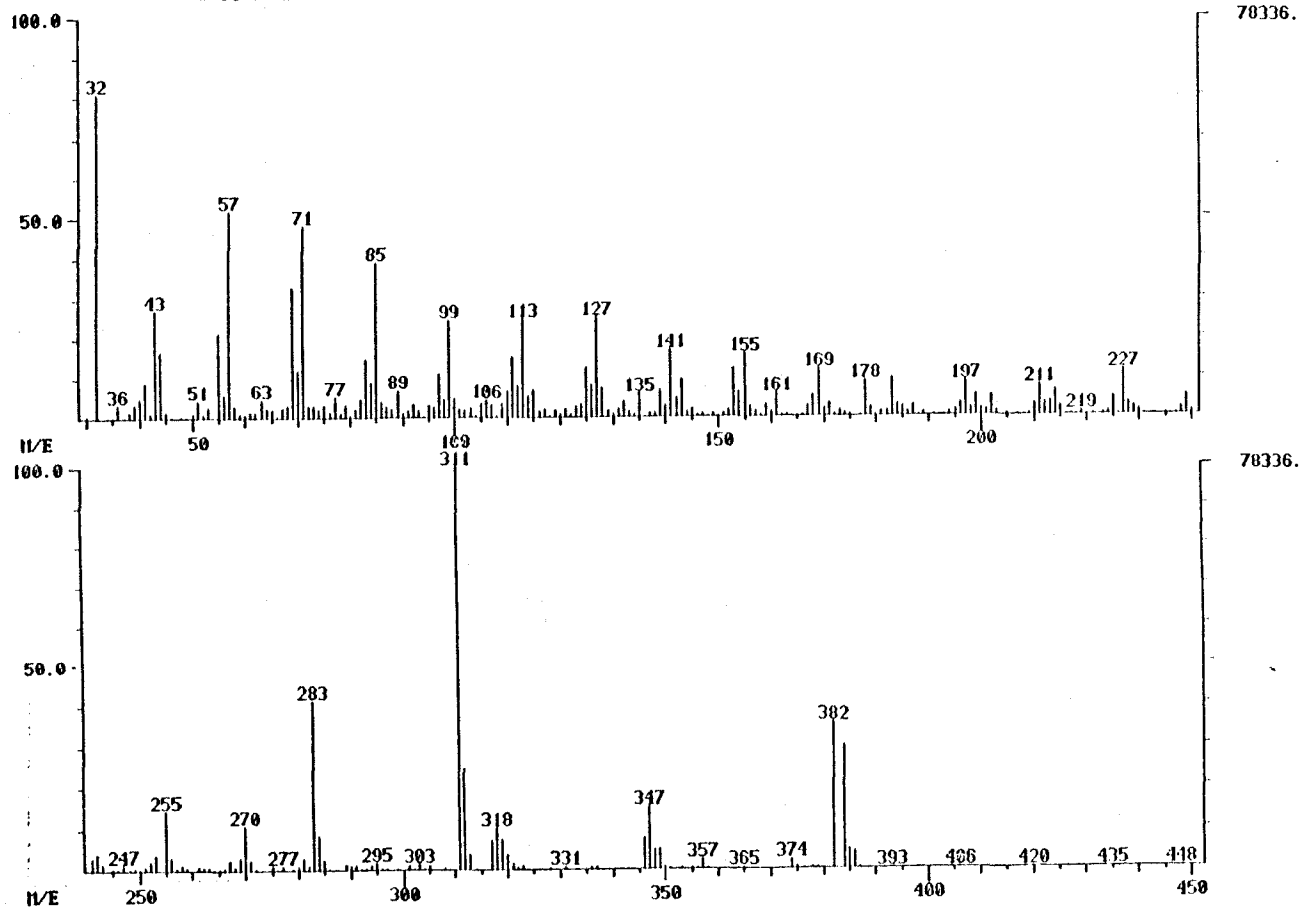


FIG. III.A.2

### 3. AFB<sub>1</sub> Binding to Supercoiled DNA

To determine if the mode of AFB<sub>1</sub> pre-covalent DNA-binding was intercalation, supercoiled plasmid pUC 8 was relaxed with Topoisomerase I (Topo I) alone, or in the presence of increasing amounts of ethidium bromide or AFB<sub>1</sub>. After simultaneous ethidium and Topo I extraction, an ethidium:plasmid molar ratio of 1000:1 produced a fully supercoiled plasmid, as in the control which lacked both Topo I and ethidium (Fig. III.A.3, lanes 5 and 1, respectively). Even at a 10,000:1 molar ratio of AFB<sub>1</sub>:plasmid, the plasmid remained in the relaxed state after AFB<sub>1</sub> and Topo I extraction (Fig. III.A.3, lane 10). This indicates that the pre-covalent DNA-binding mode of AFB<sub>1</sub>, and by inference, AFB<sub>1</sub>-Cl<sub>2</sub>, is not intercalation, but a type of groove binding.

### B. NUCLEOSOME RECONSTITUTION

Nucleosome reconstitution was performed by adding trace amounts of <sup>32</sup>P end-labeled 5S DNA to erythrocyte nucleosome core particles, transiently raising the NaCl concentration to or above 0.7 M, then lowering the ionic strength by dialysis to 0.1 M or below (Tatchell and van Holde, 1977). The exact conditions used evolved over

FIG. III.A.3 TREATMENT OF TOPO I RELAXED PLASMID WITH ETHIDIUM BROMIDE OR AFB<sub>1</sub>

Supercoiled plasmid pUC 8 was relaxed with Topo I alone, or in the presence of ethidium or AFB<sub>1</sub>. All reactions contained 1 ug of plasmid in 50 ul of 50 mM Tris (pH 7.4), 10 mM MgCl<sub>2</sub>, 50 mM KCl and 1 mM dithiothreitol. Two controls, one without Topo I (lanes 1 and 6), and one with Topo I (lanes 2 and 7), were prepared in parallel with each series of EtBr or AFB<sub>1</sub> incubations. (Supercoiled plasmid, lanes 1, 5, and 6, migrates ahead of relaxed plasmid.) Plasmid, plus 9 units Topo I, was incubated in 10:1, 100:1, and 1000:1 molar ratios of EtBr:plasmid (lanes 3-5), or 100:1, 1000:1 and 10,000:1 molar ratios of AFB<sub>1</sub>:plasmid (lanes 8-10) for 1 h at 37°C. All samples were then extracted twice with chloroform, and ethanol precipitated. Samples were resuspended, then resolved on 1% agarose gels. After simultaneous extraction of Topo I and ethidium or AFB<sub>1</sub>, plasmid becomes supercoiled to the degree that intercalation occurred. Ethidium intercalation causes plasmid supercoiling (lane 5), but AFB<sub>1</sub>, even at 10-fold higher molar ratios, does not (lane 10).

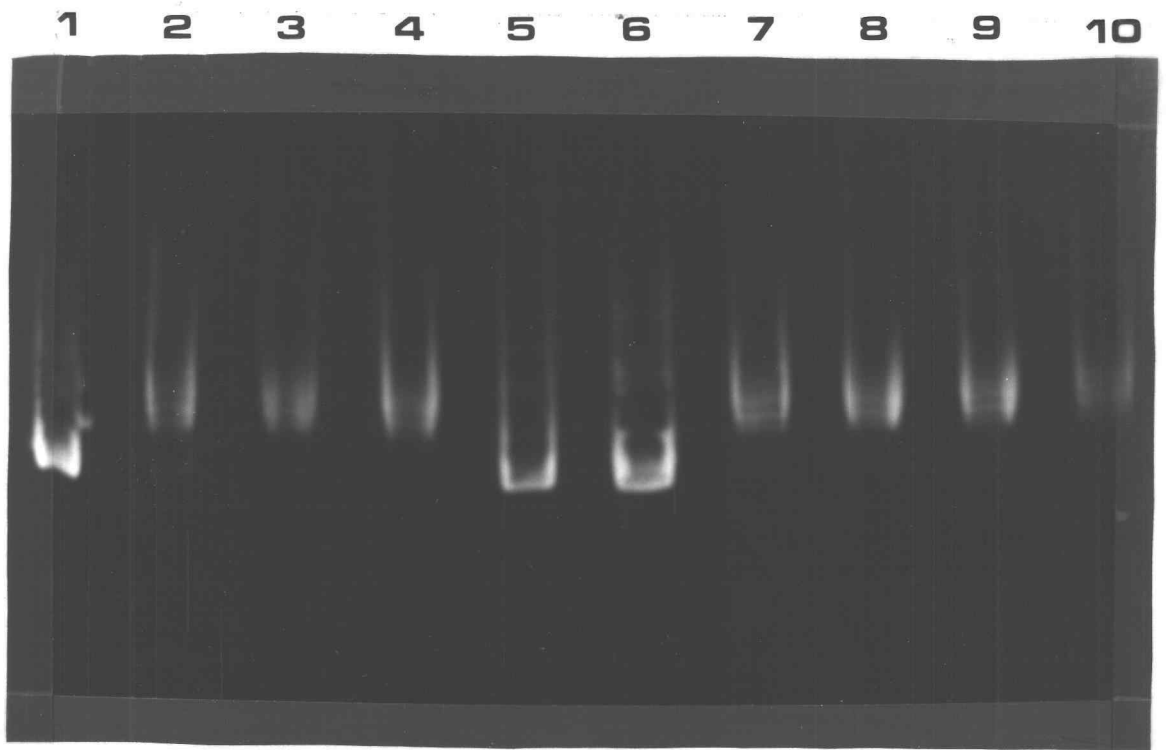


FIG. III.A.3



time, in order to maximize the quantity and quality of reconstituted nucleosomes.

Reconstitutions were usually done in triplicate, as the yield of reconstituted core particles in each sample varied slightly, and occasionally significantly. It was, therefore, imperative that the mixture of free DNA, core particles, and larger molecular weight particles after each reconstitution be resolved on a particle gel, analyzed by autoradiography and scanned, to determine the percentage of each component. All DMS or AFB<sub>1</sub>-Cl<sub>2</sub> binding studies, as well as DNase I digestions, were conducted on reconstitutions wherein the core particle represented greater than 90% (usually 95-98%) of all <sup>32</sup>P-labeled species, and the free DNA component less than 1% and often undetectable. The remaining labeled fragment, which migrates above the core particle, is likely a core particle with additional histones attached.

#### 1. Reconstitution Conditions

The <sup>32</sup>P-labeled, 195 bp fragment of 5S DNA and 1.5ug "cold" chicken cores were combined in 30 ul TE buffer at 0.7 M NaCl, at ratios between 1:100 and 1:200. After dialysis to 0.1 M NaCl, the resultant reconstituted particles contained free DNA and three other species. After re-examining other methods of reconstitution by

salt-induced exchange (Tatchell and van Holde, 1977; Yager, 1984; Simpson et al., 1983, 1985; Drew and Travers, 1985) and a discussion with Joel Gottesfeld about optimal labeled-DNA to core ratios, reconstitutions were conducted at ratios of 1:200 or 1:250 and at 2M NaCl. At these ratios free labeled DNA was no longer detectable and core yields could approach 90%. Frequently, however, larger particles were still associated with 25-50% of the available labeled DNA. In order to minimize formation of these larger particles, which were likely core particles with additional attached histones, reconstitutions were conducted at 0.8M NaCl. A free-DNA core ratio of 1:250 and 0.8M NaCl for exchange proved to be the optimal conditions of those examined for reconstitutions of high core particle yield and reproducibility. Figure III.B.1 shows a typical reconstitution under these conditions. Note the complete absence of free DNA below lanes 2-4, which would comigrate with the DNA in lane 1. The larger particles above the major core particle band represent approx. 5% of the total input 195 bp fragment. When this same gel was exposed to film twice as long, a trace of aggregates at the top of the gel appeared, but there still was no discernable free DNA band, meaning that all of the input DNA was incorporated into DNA-histone complexes.

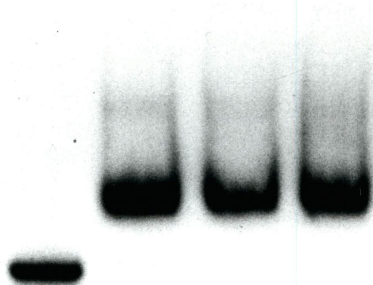


FIG. III.B.1 NUCLEOSOME CORE PARTICLE RECONSTITUTION

Free  $^{32}\text{P}$ -labeled 5S DNA, 195bp (Lane 1). Reconstituted core particles (lanes 2-4) were prepared by mixing 15  $\mu\text{g}$  (DNA wt.) chicken core particles and 81 ng of DNA (a 250:1 ratio), in 10 mM Tris, pH 7.4, 1 mM EDTA (TE), and 0.8 M NaCl. After 20 min. incubation on ice, samples were dialyzed against 0.6 M NaCl in TE for 18 hr, then 3-4 hr against 0.05 M NaCl in TE. All buffers contained 1 mM PMSF. Samples were loaded on a mini 3.5% particle gel, and run for one hour at 100V. The gel was fixed in 5% acetic acid, dried, and then exposed to X-ray film 16 hr. Note the absence of free DNA and negligible amount of species larger than the core particle.

Reconstitutes, once made, were stored on ice and used within two weeks. Particles were examined by gel electrophoresis after each alkylation experiment, demonstrating particle integrity over this time. Care was taken to prevent even transient warming of the particles to room temperature, as this could cause up to 10% dissociation. In order to avoid this temperature increase, gel electrophoresis was conducted at low voltage (12 V/cm) in a 4°C room. Both the gel buffer and the glass Hamilton syringe used for sample application were precooled. Additionally, microfuge tubes and reaction buffer were precooled on ice, before core particles were added in alkylation experiments. (Though some of these steps may seem extreme, particle manipulations under these conditions resulted in no detectable DNA dissociation.)

## 2. Influence of Alkylation on Core Particle Integrity

As depicted in Fig. III.B.2, core particles are not detectably altered in mobility, nor does an average of one DMS or 0.5 AFB<sub>1</sub>-Cl<sub>2</sub> adducts formed per particle cause detectable DNA dissociation. Also, as described above, care was taken to keep the particles at 0-4°C during preparation and through alkylation steps, until the alkylating agents were quenched with dithiothreitol. By examining the particles on a gel immediately after each

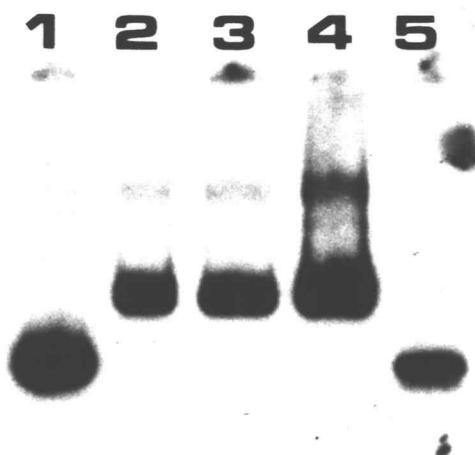


FIG. III.B.2 RECONSTITUTED NUCLEOSOMES - TREATMENT WITH DMS OR AFB<sub>1</sub>-Cl<sub>2</sub>

Outer lanes are <sup>32</sup>P-labeled 5S DNA; inner 3 lanes of reconstituted particles are control, 10 mM DMS for 1 min., and 1.1 uM AFB<sub>1</sub>-Cl<sub>2</sub> for 5 min, respectively, followed by immediate loading on particle mini-gels as described in text. AFB<sub>1</sub>-Cl<sub>2</sub> lane, overloaded, shows absence of any detectable free DNA caused by dissociation.

alkylation (Fig. III.B.2), both particle integrity, and the absence of a compromising free DNA signal were assured.

AFB<sub>1</sub>-Cl<sub>2</sub>-induced nucleosomal-DNA dissociation was a matter of concern, for questions remained as to whether intercalation was the mode of AFB<sub>1</sub>-Cl<sub>2</sub> pre-covalent binding. Recent work by McMurray and van Holde (1986) demonstrated that ethidium intercalation in nucleosome core particles, a highly cooperative event, causes DNA dissociation. We subsequently demonstrated that the pre-covalent association of parent AFB<sub>1</sub> is not intercalation between DNA bases (Section III.A.3). (AFB<sub>1</sub>-Cl<sub>2</sub> is very reactive in aqueous conditions, which prevents pre-covalent association studies.)

### 3. Precision of Reconstitution

Not only is it essential that free DNA be absent, but the positioning of the histone core on the cloned DNA fragment must be precise and uniform. Multiple positions or phases would give mixed results on the influence of DNA-histone interactions on DNA alkylation, making single-base analysis difficult or impossible. As shown in Fig. III.B.3, DNase I cutting of free DNA and the reconstituted particle, W strand, demonstrates the unique positioning of the particle utilized in this study. Arrows at 0 and 145 represent presumed particle

FIG. III.B.3 DNASE I DIGESTION OF RECONSTITUTED CORE PARTICLE VS. FREE DNA

All three samples are 195bp W strand, labeled at the 5' end. The left lane was treated for 1 minute with 5 uM dimethyl sulfate, then cleaved with piperidine to give a G ladder for calibration. In the center lane, 4 ug of reconstituted particles were treated with 8 units DNase I, on ice, in 50 mM NaCl, 10 mM Tris, pH 7.4, and 1 mM EDTA. After one minute, the reaction was quenched with an excess of EDTA. The right lane represents 5 ug of free DNA with a trace (50 ng) of labeled 5S DNA, in the same buffer, but only 2 units DNase I were used and the reaction was quenched after 30 s.

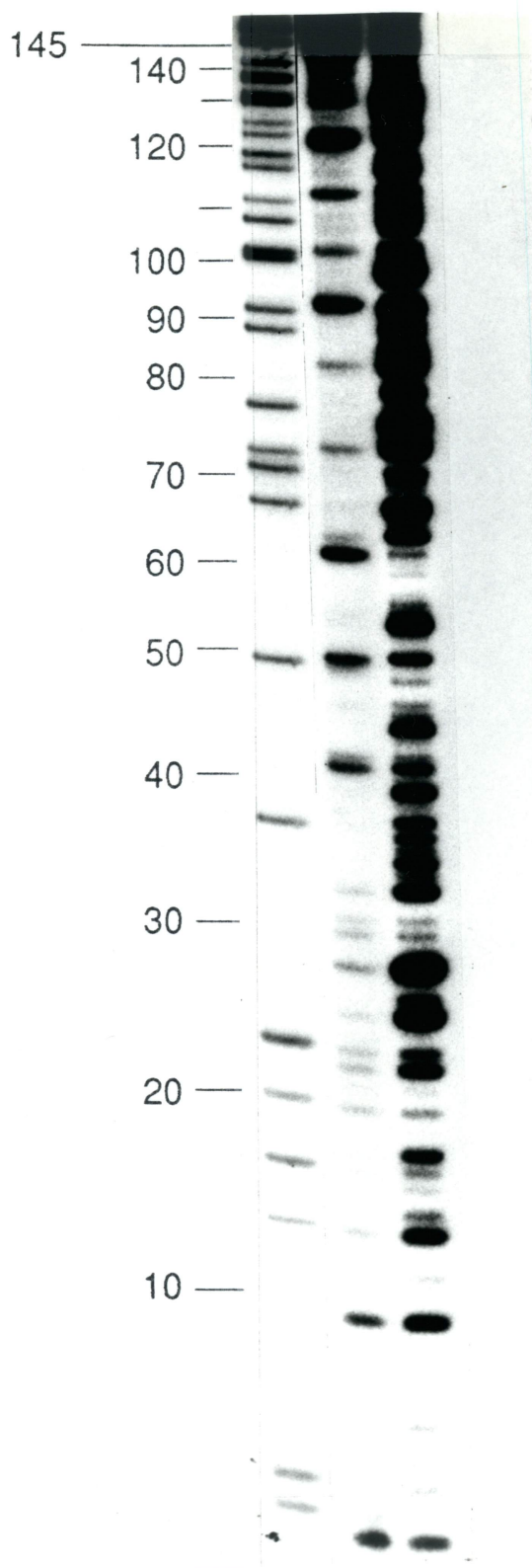


FIG. III.B.3

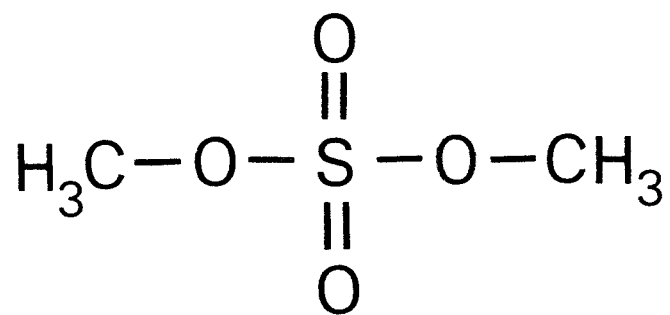


boundaries. The 195 bp W strand, 5' end-labeled, is treated with DMS (left lane) as a marker. The center lane represents DNase I cleavage of the reconstituted core particle, while the right lane is DNase I cleavage of free DNA. Protected regions (approx. every 10bp) in much of the core particle were observed, as opposed to frequent, sequence-selective cutting throughout the naked DNA. The fact that several regions of preferred cutting appear as single bands is evidence for high precision in positioning. Also informative are those sites where strong cleavage occurs in free DNA that is absent in the core particle, i.e., approx. 39, 43, 52, 64, 75, 85, 95, etc. Such cleavage patterns are indicative of a homogenous population of uniquely positioned particles, wherein these sites are consistently shielded from DNase I attack by DNA-histone contacts. In the first 30 bases of the particle (positions 0-30) the cleavage sites parallel those of free DNA, though the frequencies of cutting are clearly lowered in the core particle DNA. At approx. 145 bp (Fig. III.B.3., arrow) and above, the cleavage patterns are coincident, indicating the particle boundary. Additionally, AFB<sub>1</sub>-Cl<sub>2</sub> cutting frequencies reflect a boundary at about 145 bp (see below). Taken together, these results indicate a histone octamer located between 20 and 165 bp from the labeled end, in full agreement with Simpson and Stafford (1983).

### C. DIMETHYL SULFATE TREATMENT OF FREE DNA VS. RECONSTITUTED CORE PARTICLE DNA

Dimethyl sulfate, a small methylating agent compared to AFB<sub>1</sub>-Cl<sub>2</sub> (Fig.III.C.1), was exposed to free DNA or reconstituted particles in parallel with AFB<sub>1</sub>-Cl<sub>2</sub>, as DMS was known to be equally accessible to the minor and major grooves of naked and bulk nucleosomal DNA prepared from rooster erythrocytes (McGhee and Felsenfeld, 1979). Thus it furnished a good internal control for AFB<sub>1</sub>-Cl<sub>2</sub> treatments of naked and nucleosomal DNA. Additionally, McGhee and Felsenfeld observed a site of enhanced DMS activity in the core particle. Our system, utilizing a discrete instead of averaged particle, allowed a more precise examination of this and any other sites of altered DMS binding.

Figure III.C.2, lanes 2 and 3, displays DMS cleavage of free DNA or particle DNA respectively, and represents one of three replicate experiments, using different chicken nucleosome core particle preparations for two of the three reconstitutions. For this the W strand, the average ratio of DMS-DNA binding to free DNA vs. core



DMS

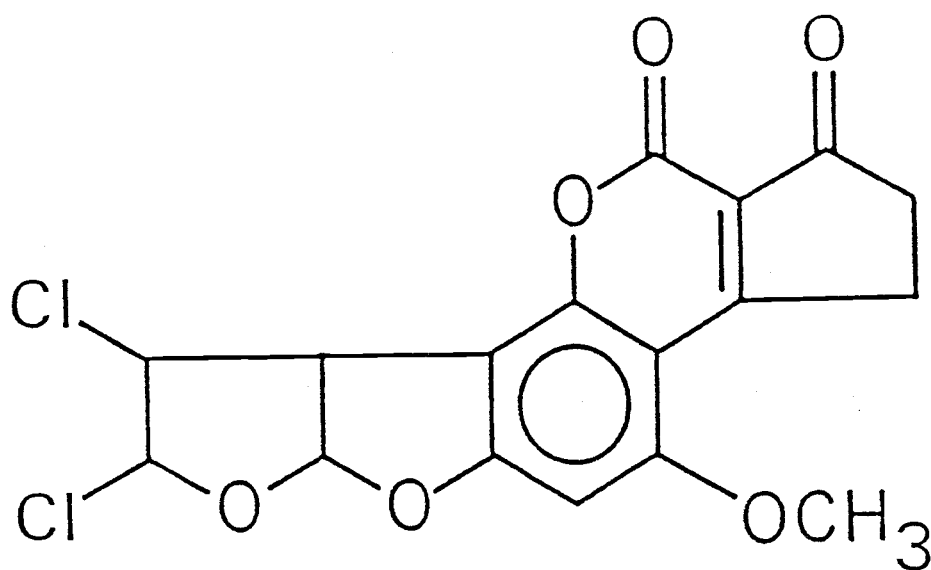
AFB<sub>1</sub>-Cl<sub>2</sub>FIG.III.C.1 DIMETHYL SULFATE AND AFLATOXIN B<sub>1</sub>-DICHLORIDE

FIG. III.C.2 DIMETHYL SULFATE, AFLATOXIN B<sub>1</sub>-Cl<sub>2</sub>, OR  
DNASE I CLEAVAGE OF FREE DNA AND RECONSTITUTED CORE  
PARTICLES

W strand, 195 bp, prepared by cutting plasmid pLv405 with EcoRI, <sup>32</sup>P 5' end labeling with T4 kinase, and secondary restriction with NciI. Control (lane 1) was carried through all steps but alkylation. The fragment, either as "naked" DNA (lanes 2, 4, and 7) or incorporated into nucleosomes by salt exchange (lanes 3, 5, and 6), was treated in parallel with 25 mM DMS, for one min, or 1.1 uM AFB<sub>1</sub>-Cl<sub>2</sub>, for five minutes, on ice. Lanes 6 and 7 are core particles or free DNA fragment, respectively, cleaved with DNase I.

DMS  $B_1-Cl_2$  DNASE I  
1 2 3 4 5 6 7

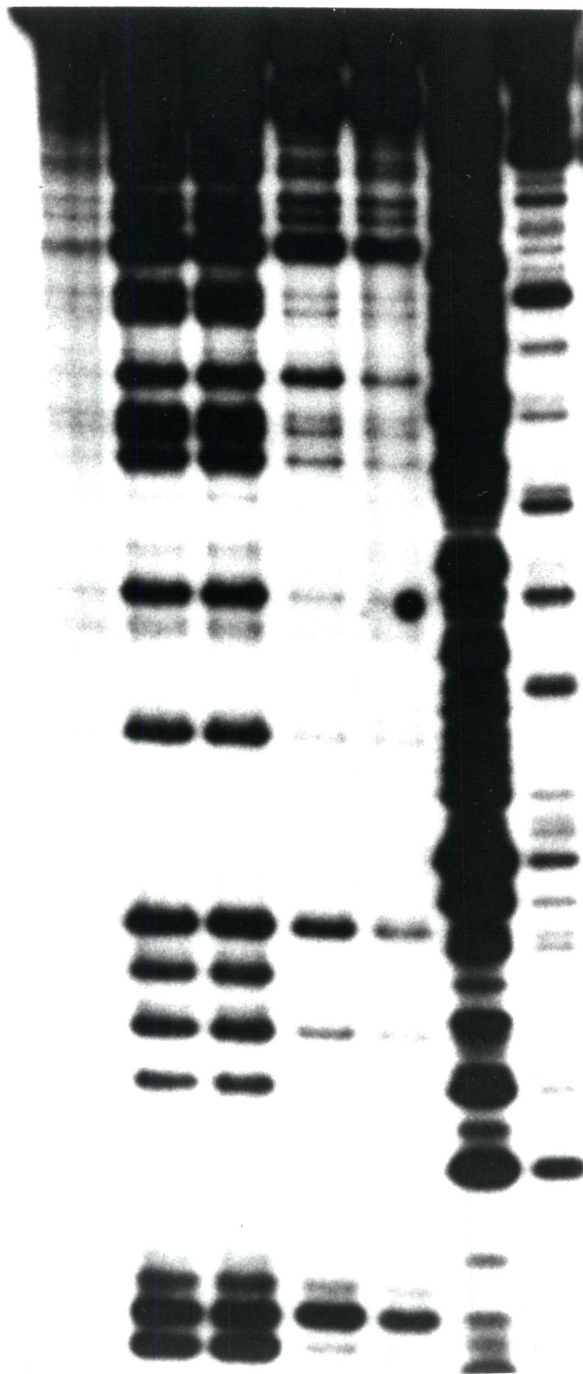


FIG. III.C.2

particles for 25 scanned sites within the particle is  $0.98 \pm 0.08$ . The highest ratio of binding, 1.06, occurs at position 19, and the low, 0.87, occurs at position 3 within the particle, as depicted in Figure III.C.3.

Figure III.C.4 displays DMS binding to the C strand. The average DNA-binding ratio for 32 scanned sites is  $1.03 \pm 0.05$ . The ratio of binding ranges from 1.16 at position 137 to 0.86 at position 24. Thus a nucleosomal conformation neither inhibits or promotes DMS methylation of N7-guanine. McGhee and Felsenfeld(1979), using "native" rooster erythrocyte core particles of heterogenous sequence, also observed a generally uniform pattern of N7-guanine-DMS accessibility, though site 62 in their bulk nucleosomes displayed enhanced reactivity.

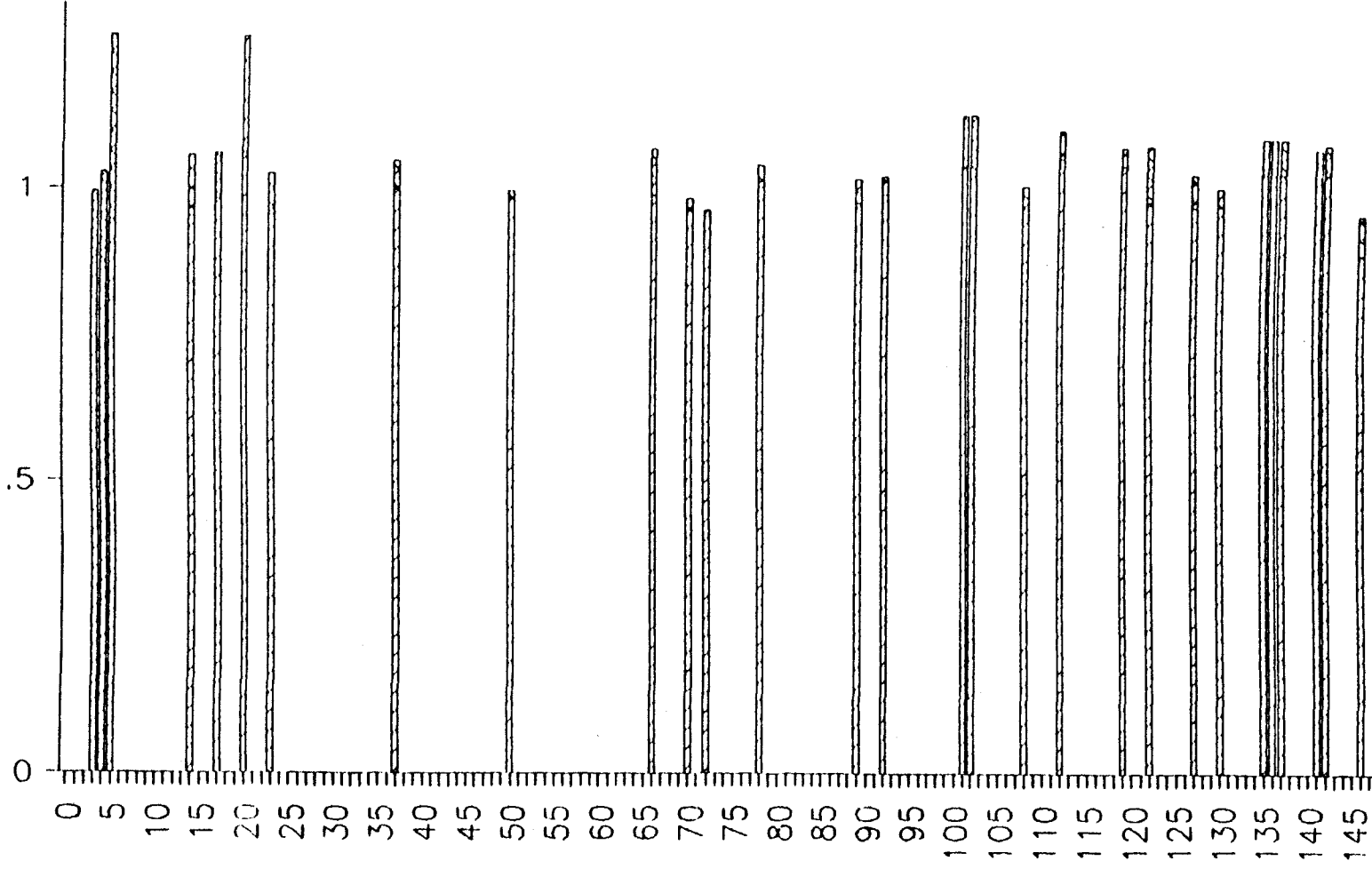
#### D. AFLATOXIN B<sub>1</sub>-Cl<sub>2</sub> BINDING TO FREE DNA VS. RECONSTITUTED CORE PARTICLE DNA.

In Fig.III.C.2, lanes 4 and 5 represent the results of AFB<sub>1</sub>-Cl<sub>2</sub> binding to free 5S DNA or reconstituted core particles, respectively. Unlike DMS, AFB<sub>1</sub>-Cl<sub>2</sub> binding to nucleosomal DNA is hindered. Surprisingly, the marked sequence specificity of AFB<sub>1</sub>-Cl<sub>2</sub> (Misra, et al., 1983; Muench, et al., 1983; Marien, et al., 1987; and Benasutti, et al., 1988) is unchanged by DNA-histone

FIG. III.C.3 FREE DNA:CORE DMS DNA BINDING RATIOS - W  
STRAND

The W strand of the 195 bp fragment was 5' end labeled, then reconstituted into core particles or mixed with deproteinized DNA from chicken core particles. A ratio of one represents equivalence in DMS binding at that site. Average binding and standard deviations were pooled from laser densitometry scans of three separate experiments.

FIG. III.C.3

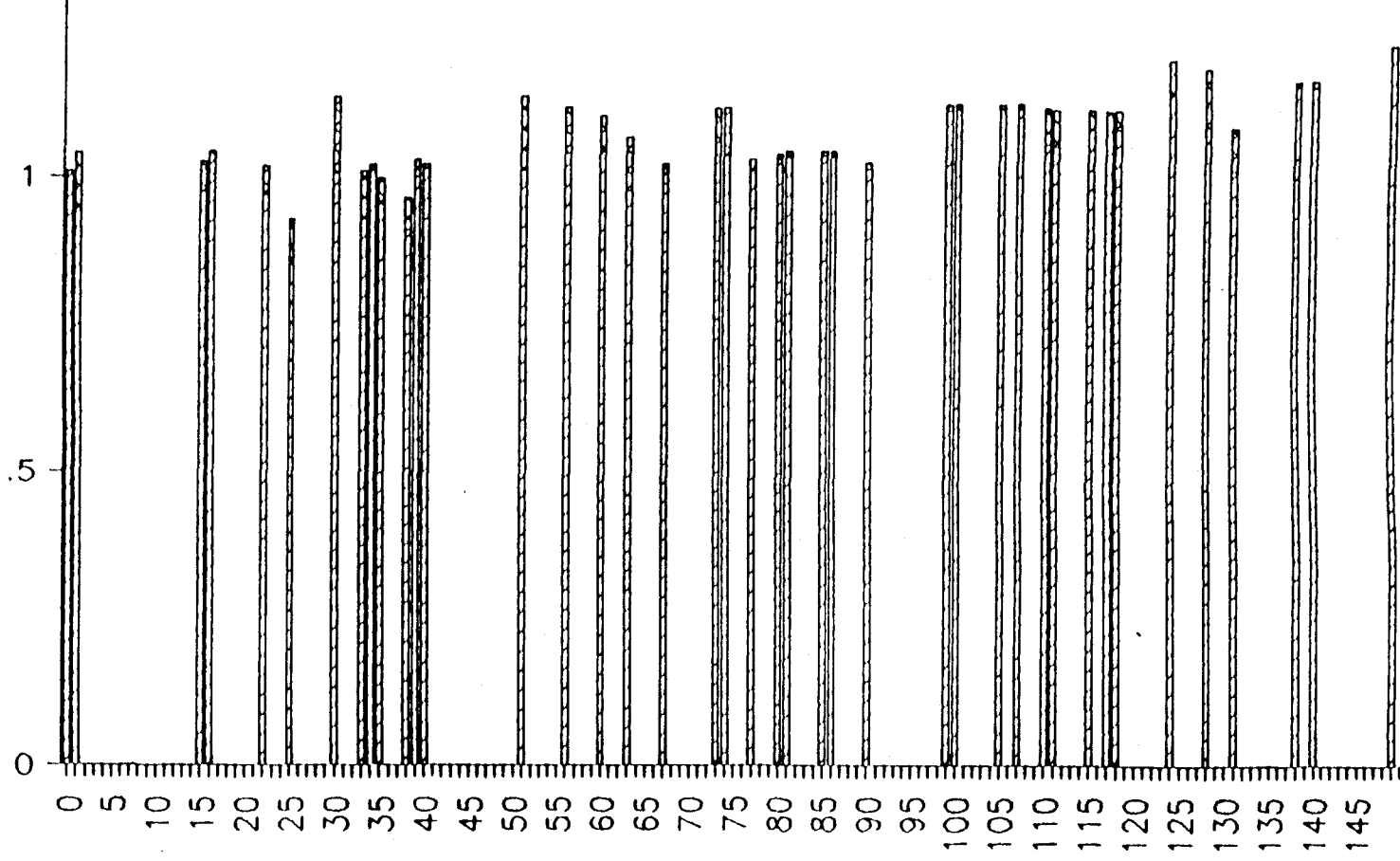




## FIG. III.C.4 FREE DNA:CORE DMS BINDING RATIOS - C STRAND

The C strand of the 195 bp fragment was 5' end labeled, then reconstituted into core particles or mixed with deproteinized DNA from chicken core particles. A ratio of one represents equivalence in DMS binding at that site. Average binding and standard deviations were pooled from scans of two separate experiments.

FIG. III.C.4



contacts. In Fig.III.D.1, scans of lanes 4 and 5 from Fig.III.C.2 were superimposed, demonstrating that although AFB<sub>1</sub>-Cl<sub>2</sub> binding is generally suppressed by the nucleosomal core particle structure, the sequence specificity of AFB<sub>1</sub>-Cl<sub>2</sub> is unchanged.

These results are more fully depicted in Fig.III.D.2. For the W strand (Fig.III.D.2, upper), AFB<sub>1</sub>-Cl<sub>2</sub> binding to free vs. nucleosomal DNA averages  $2.57 \pm .34$  to position 116. Site 137 is 1.07 and site 148, just beyond the particle boundary, is 1.00.

The C strand (Fig.III.D.2, lower) has a AFB<sub>1</sub>-Cl<sub>2</sub> binding ratio near unity for position -1 and 0, then averages  $2.17 \pm .54$  to position 116. At site 137, the ratio is 1.07 and site 148, just beyond the particle boundary, is 1.00.

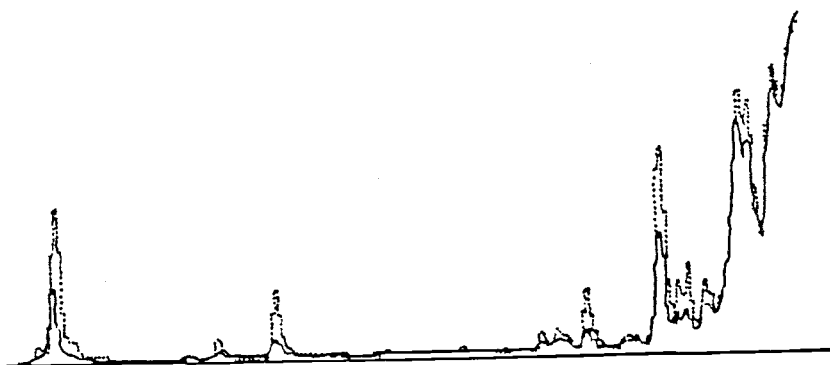


FIG. III.D.1 AFB<sub>1</sub>-Cl<sub>2</sub> SEQUENCE SPECIFIC BINDING TO FREE  
VS. NUCLEOSOMAL DNA

Superimposed scans of AFB<sub>1</sub>-Cl<sub>2</sub> binding to free DNA  
and core particles - lanes 4 and 5 of Fig.III.C.2.

FIG. III.D.2 FREE DNA: CORE AFLATOXIN B<sub>1</sub>-Cl<sub>2</sub> BINDING RATIOS

The W strand of the 195 bp fragment was 5' end labeled, or alternately, the C strand was 3' end labeled, then reconstituted into core particles. Position 0 is the left boundary of the particle; the terminal <sup>32</sup>P label (not shown) is at -20. The box (center) represents the position of the particle.

UPPER: AFB<sub>1</sub>-Cl<sub>2</sub> binding ratios for free DNA vs. particle. A ratio of one (dotted line) represents equivalence in AFB<sub>1</sub>-Cl<sub>2</sub> binding at that site. Average binding was calculated from scans of three separate experiments. Sites where AFB<sub>1</sub>-Cl<sub>2</sub> was insufficiently above film background to be quantitated are represented by triangles.

LOWER: DNase I fractional cleavages of reconstituted particles.

AFB1-CL2 BINDING - DNA: CORE

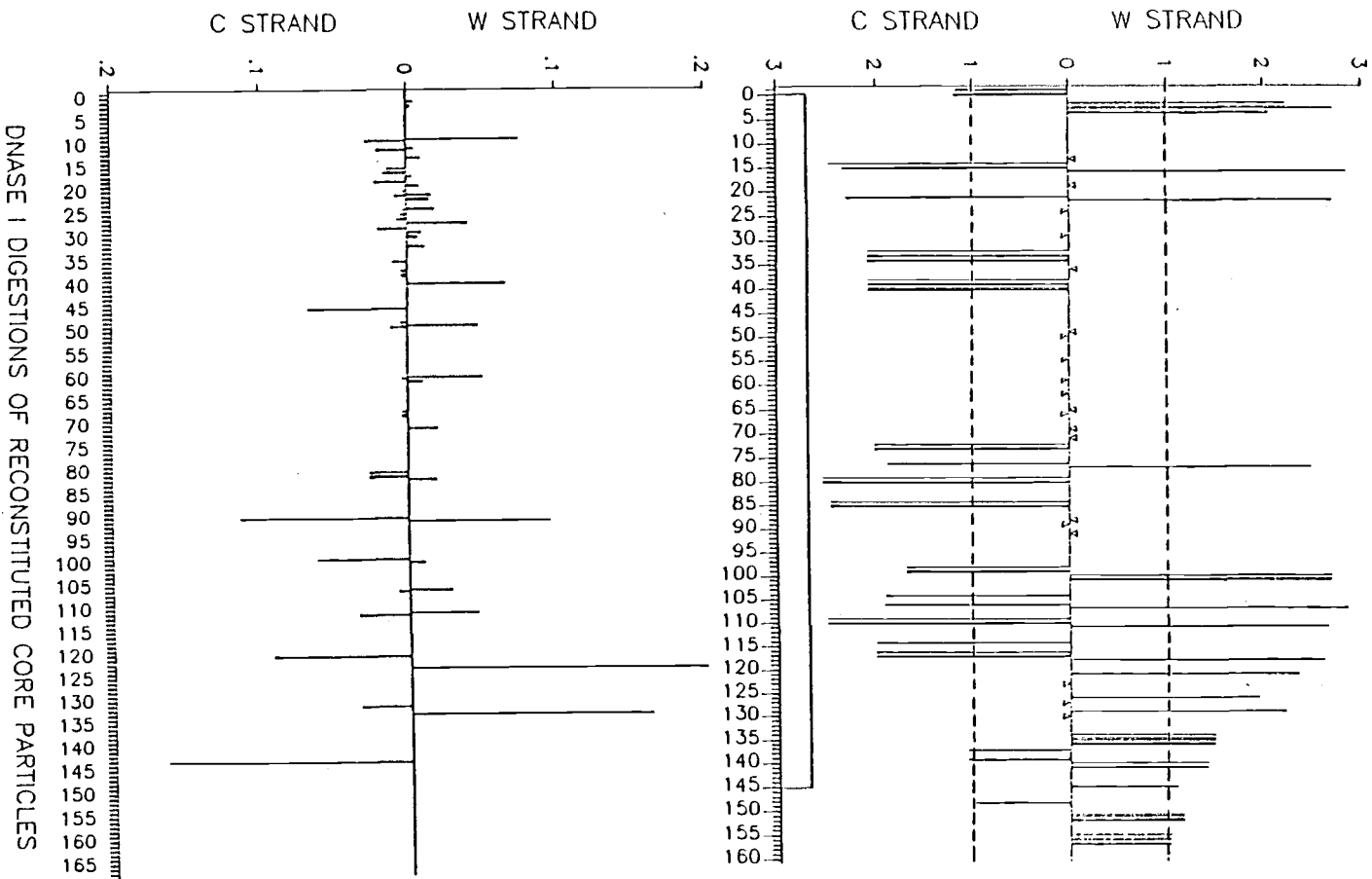


FIG. III.D.2

## IV. DISCUSSION

## A. DNASE I CLEAVAGE PATTERNS

For the majority of reconstitutions I performed with the 5S DNA fragment, a fraction was taken and tested by DNase I digestion. These cleavage patterns varied only in the intensity of bands, due to differing extents of digestion. The cleavage positions were invariant from one reconstitution to the next, attesting to the reproducibility of reconstitution with this DNA fragment.

DNase I cleavage patterns of the reconstituted nucleosome particles utilized in these studies (Fig. III.B.3) closely parallel those obtained by Simpson and Stafford (1983). Minor differences can be observed, though. In their Fig. 5, laser densitometry scans of the W and C strands labeled at the left end of the particle are presented. The W strand is cleaved to the extent that little uncut material is left at the top of their gels (their Figs. 2 and 4), indicative of multiple cleavages per strand. This necessarily increases the shorter strands at the expense of the longer, and may give the appearance, falsely, of preferred cutting toward the labeled end. Indeed, I was able to obtain a similar "frequency of cutting" near sites 10 and 20, with longer DNase I digestion times.

Interestingly, one can observe multiple cuts in the particle up to position 32, W strand, or position 49, C strand, with parallel cleavage sites in the naked DNA lane. Yet the frequencies of cutting are clearly altered in the nucleosomal vs. free DNA (e.g., W strand sites 20-30, esp. sites 27 and 29; Fig. III.B.3), indicative of some influence of DNA-histone contacts. This is also the strand end (W strand, 5' end) that displays 2.4-fold global suppression throughout its length in AFB<sub>1</sub>-Cl<sub>2</sub> interaction (see below), unlike the other 3 strand ends, which is another factor suggestive of DNA histone contacts, at least at bases 2, 3, 4, 16 and 22 (Fig. III.D.2).

For a more in depth analysis of DNase I cleavage patterns of this reconstituted particle one may consult Simpson and Stafford (1983), Yager (1984), or Richmond, Searles, and Simpson (1988). The latter have reconstituted milligram quantities of this particle with the central 146 bp for X-ray diffraction studies. They also observe multiple DNase I cleavage sites in both strands that are inconsistent with the approx. 10 bp phase in cutting for the rest of the particle. This is described as a possible "flexibility or loosening of the DNA in this region promoted by 300 mM-NaCl present in the digestion mixture". I observe similar multiple, out of phase cuts at only 50 mM NaCl, suggesting a phenomenon



intrinsic to this region of the particle. Richmond, et al. (1988) report twice as much measurable data from their defined sequence crystals over earlier random sequence crystals (Richmond, et al. 1984), so they may be able to determine the DNA path in this multiply cleaved region. Yet, interpretation of the data is complicated by a two-fold heterogeneity due to the asymmetric DNA sequence about the nucleosomal dyad at position 73.

#### B. DIMETHYL SULFATE TREATMENT OF FREE DNA VS. NUCLEOSOMAL DNA

DMS, a relatively small methylating agent (Fig. III.C.1), binds equally to the 5S DNA fragment, whether in a reconstituted nucleosome particle or free in solution. My findings of DMS-nucleosomal DNA binding are generally consistent with the more qualitative results obtained by McGhee and Felsenfeld (1979), who used random sequence particles, though two differences were found. First, McGhee and Felsenfeld observed a clear increase in nucleosomal DNA-DMS binding at or near position 62 on both strands, although they did not estimate its magnitude. I find DMS-naked DNA vs. DMS-nucleosomal DNA binding ratios from 1.05 to 0.96 on both strands between sites 59 and 69 (Figs. III.C.3 and III.C.4). At site 62

on the C strand, the DMS binding ratio is 0.98. I did find larger differences at other sites, from 0.86 to 1.16. These differences are likely due to data scatter rather than actual modulation of DMS binding, as individual replicates occasionally varied slightly more about the mean of 1.0. Second, McGhee and Felsenfeld reported 20-30% faster reaction with nucleosomal DNA. Though I did not conduct kinetic studies, the total amount of methylation I observe by comparing uncut (195 bp) DNA is roughly equal for nucleosomal vs. naked DNA.

Of course, it is possible that in bulk chicken nucleosomes, a significant portion of the particles do have a site of enhanced DMS binding. We may therefore be looking at a subset (one particular particle) that does not display enhanced binding at site 62. Yet, I do not observe enhanced DMS binding anywhere else in this particle. Additional information, gained by repeating their experiments with bulk nucleosomes, or even by examining DMS binding to other sequence-positioned particles, would further a general view of DMS accessibility to nucleosome core particles. In any event, no sites of significantly altered DMS binding are observed in the precise analysis allowed with this sequence-positioned nucleosome core particle.

### C. AFLATOXIN B<sub>1</sub>-Cl<sub>2</sub> BINDING TO FREE DNA VS. NUCLEOSOMAL DNA

#### 1. Relation to DNase I Cutting.

AFB<sub>1</sub>-Cl<sub>2</sub>, a large molecule compared to DMS (Fig.III.C.1), is hindered in its binding to nucleosomal DNA, by a factor of about 2.4 over naked DNA, throughout most, but not all of the nucleosome. Surprisingly, DNA-histone contacts or DNA helical periodicity do not seem to modulate the marked sequence-specificity of AFB<sub>1</sub>-Cl<sub>2</sub> adduct formation observed by (Misra, et al., 1983; Muench, et al. 1983; Marien, et al., 1986, Benasutti, et al., 1988).

There is also no correlation between AFB<sub>1</sub>-Cl<sub>2</sub> binding to nucleosomal DNA and sites devoid of DNase I cleavage, e.g., positions 77, 114, 116, and 117 on the W strand or 84, 85, 124 and 126 on the C strand (Fig.III.D.2). Neither is AFB<sub>1</sub>-Cl<sub>2</sub> binding altered at preferred DNase I cleavage sites, such as position 111, W strand, or 80, C strand. This seems somewhat surprising, as one might have expected some hindrance of AFB<sub>1</sub>-Cl<sub>2</sub> accessibility to DNA segments facing the histone core. In this context, it must be noted that DNase I binds in the minor groove of B-form DNA (Lutter, 1978, 1979; Suck, et al. 1988), whereas the precovalent binding of AFB<sub>1</sub> is believed to be in the major groove, due to the availability of N7-

guanine there (see below).

In another context, it must be remembered that in this sequence-positioned particle, sites of strong, weak, or no DNase I cleavage occur, overruling the marked DNA sequence-specificity of DNase I. At precisely these same sites, no change is observed in the equally pronounced sequence-specificity of AFB<sub>1</sub>-Cl<sub>2</sub> binding.

Perhaps the simplest explanation for the DNase I vs. AFB<sub>1</sub>-Cl<sub>2</sub>-induced nucleosome cleavage patterns lies in the amount of steric hindrance involved in the respective DNA-ligand interactions. DNase I, at 42 kilodaltons, is not free to conduct a two dimensional search along the DNA strand for preferred binding sites, due to DNA-histone contacts. Yet, AFB<sub>1</sub>-Cl<sub>2</sub>, a largely planar molecule (van Soest and Peerdeman, 1970a,b) with a molecular weight of only 383, appears free to conduct just such a two-dimensional search along the DNA strand, even to a sequence-preferred guanine that fortuitously faces the histone core. Additionally, minor groove width is critical for DNase I binding (Suck, et al. 1988), but binding of planar AFB<sub>1</sub>-Cl<sub>2</sub> seems relatively unaffected by fluctuations of major groove width between the inside and outside of the DNA helix wrapped around the histone core. When considered in the context of macromolecular size, the disparate DNA interactions of these two molecules is understandable.

## 2. AFB<sub>1</sub>-Cl<sub>2</sub> Binding and Local Nucleosomal DNA Bending

X-ray crystallography has revealed sites of sharp DNA bending located +10 and +40 bp from the nucleosomal dyad axis, which correspond approx. to sites 33, 63, 83, and 113 (Richmond, et al., 1984). The C strand has AFB<sub>1</sub>-Cl<sub>2</sub> binding sites at 33, 84, and 114, with binding ratios of 2.09, 2.55 and 2.02, respectively. Similarly, the W strand contains an AFB<sub>1</sub>-Cl<sub>2</sub> binding site at 111 with a binding ratio of 2.64. Hence, if this particle contains sharp DNA bends at or near these sites, such a DNA conformation does not strongly alter AFB<sub>1</sub>-Cl<sub>2</sub> binding.

Once the structure of this 5S particle has been determined by x-ray crystallography, we can conduct a more exacting analysis of the influence of local DNA bending on AFB<sub>1</sub>-Cl<sub>2</sub> adduct formation. Assuming that this particle also contains several sharp DNA bends, the probability is high that at least one of the AFB<sub>1</sub>-Cl<sub>2</sub> binding sites lies within a bent region.

## 3. Rotational Orientation of Nucleosomal DNA and AFB<sub>1</sub>-Cl<sub>2</sub> Binding

As DNA winds around the histone core, its double helical structure periodically places the major groove with its N7-guanine in all possible orientations with the

histone core and solvent. If such a periodicity were reflected in AFB<sub>1</sub>-Cl<sub>2</sub> binding, it should appear in sites on a single strand spaced 5 bp apart, or in adjacent sites on different strands, lying almost across the DNA dyad. (The G-C base-pairing character of DNA prevents AFB<sub>1</sub>-Cl<sub>2</sub> adduct sites directly opposite one another.) The multiplicity of such sites with essentially equal binding ratios strongly argue that DNA orientation with respect to the histone core has no significant effect on AFB<sub>1</sub>-Cl<sub>2</sub> binding. Compare especially binding to sites from 98 to 121 (Fig.III.D.2). Over these 23 base pairs, there are 15 binding sites. Several cases of binding across the dyad axis or binding with a 5 bp stagger within a strand occur, yet no significant pattern of binding modulation is evident.

Gale, et al. (1987) recently reported UV photoproduct formation in whole cells and in mononucleosomes which displays a striking 10.3 bp periodicity, presumably corresponding to sites where the DNA phosphate backbone is farthest from core histones. Both a significant change in adjacent pyrimidine orientation and local distortion of the DNA helix are required for pyrimidine dimer formation, and a periodic variation in these parameters may explain their results. As N7-guanine is accessible from the major groove, AFB<sub>1</sub>-Cl<sub>2</sub> precovalent binding and subsequent adduct formation may involve only

minor helix distortion, accounting for the absence of periodicity in binding ratios we observe. In this connection, it should be noted that McGhee and Felsenfeld (1982) used T4 DNA, which is glucosylated in the major groove at roughly every third base pair, to reconstitute nucleosomes. When analyzed by a variety of methods, these nucleosomes behaved identically to native nucleosomes, indicative of no significant DNA major groove-histone interactions. Additionally, Raman spectroscopy of nucleosomes indicates no direct histone-DNA bonding at N7-guanine (Goodwin, et al. 1979). The summation of all the evidence suggests that the whole major groove must be equally accessible in nucleosomal DNA, at least to agents as small as AFB<sub>1</sub>-Cl<sub>2</sub>. This is reflected in equivalent AFB<sub>1</sub>-Cl<sub>2</sub> adduct levels in the central 100 bp of nucleosomal DNA, regardless of the DNA rotational orientation with respect to the histone core.

#### 4. Nucleosomal DNA End Effects on AFB<sub>1</sub>-Cl<sub>2</sub> Binding

The 2.4-fold global protection I do observe decreases toward unity in three of the four DNA strand ends as the particle boundary is approached. The right side of the particle (as diagrammed in Fig.III.D.2) is increasingly accessible to AFB<sub>1</sub>-Cl<sub>2</sub> binding in both strands, beginning about 20 bp from the particle boundary. At the left end of the particle, C strand binding reflects a similar

transition from protected sites 14 and 15 to sites 0 and -1, which bind AFB<sub>1</sub>-Cl<sub>2</sub> as well as does free DNA. In contrast, the W strand, at sites 2, 3, and 4, is protected from AFB<sub>1</sub>-Cl<sub>2</sub> binding to a greater extent than the other three strand ends, at a level consistent with internal nucleosomal DNA. Models of nucleosomal DNA-histone contacts have been developed by Mirzabekov and coworkers by crosslinking DNA to histones, then cleaving the DNA strand at the site of attachment, which leaves DNA strand fragments 5' to the histone still attached. When analyzed by this method, the average nucleosomal core is found to contain 5' strand ends free of histone contacts for about 20 bp, while 3' strand ends are reported to be bound to histone H3 (Shick, et al., 1980). The reconstituted particle used in these experiments displays no such symmetry of AFB<sub>1</sub>-Cl<sub>2</sub> binding to 5' ends, perhaps reflecting DNA sequence-specific effects unobservable in average particles. The 3' ends do reflect symmetrical AFB<sub>1</sub>-Cl<sub>2</sub> binding patterns. In this case, however, histone H3 contact (if it exists in these specific particles) does not confer protection on the 3' ends, but instead the binding affinity approaches that of free DNA.

Several lines of evidence indicate that about 20 bp on each end of the nucleosome are not as tightly associated with the histone core as the central 100 bp of DNA.



Weischet et al., (1978), using a homogenous preparation (in terms of DNA length) of random sequence nucleosome core particles, demonstrated a biphasic DNA melting profile characterized by changes in absorbance at 260 nm and in heat capacity. The increase in 260 nm absorbance during the first melting phase corresponded to about 40 bp, which was postulated to correspond to about 20 bp on each end of the particle. Simpson (1979) prepared synthetic core particles with poly(dA-dT)·poly(dA-dT). The particles were studied at 45°C, where the DNA ends were expected from the above hypothesis to be released and denatured. The 5' DNA ends were <sup>32</sup>P-labeled, then treated with S1 nuclease at this temperature. Loss of both labeled ends, as revealed by autoradiography, and trimming down to approx. 105 bp, as evidenced by ethidium staining, demonstrated release of approx. 20 bp from each end of these particles, thus confirming the postulate of Weischet, et al.

McMurray and van Holde (1986) studied the interaction of ethidium, an intercalating dye, and nucleosomal core particles. They demonstrated that ethidium binding to core particles, a highly cooperative event, causes DNA dissociation. Use of the analogous intercalator, methylpropidium-EDTA·Fe(II) (MPE) demonstrated that the initial binding was to DNA ends. When 8-9 ethidiums per core particle are bound on a DNA end, the DNA is

stiffened to the point that an H2A-H2B histone dimer is released, followed by complete dissociation of the DNA from the remaining histone core. This amount of ethidium binding was estimated to occupy approx. 17 bp.

These various findings also suggest that the observation of increased AFB<sub>1</sub>-Cl<sub>2</sub> binding in the last 20 bp of each end of the particle is a consequence of "loosening" in the DNA ends. The gradual decrease in accessibility as the central core is approached suggests that the loosening may be gradual.

#### 5. Sequence-specific Binding of AFB<sub>1</sub>-Cl<sub>2</sub>

Mattes, et al. (1986b) has suggested that sequence-specific alkylation patterns on naked DNA may predict DNA damage in isolated chromatin or in vivo. This work has actually tested these ideas at the nucleosomal level, finding that beyond a global suppression, the marked (10-100 fold) sequence-specificity of AFB<sub>1</sub>-Cl<sub>2</sub> is itself unaltered by nucleosomal DNA-histone contacts.

The site of precovalent AFB<sub>1</sub>-Cl<sub>2</sub> binding is believed to be the major groove (Lin, et al., 1977; Benasutti, et al., 1988). Though global AFB<sub>1</sub>-Cl<sub>2</sub> nucleosomal binding is suppressed 2.4-fold, this study has demonstrated the major groove to be equally accessible to AFB<sub>1</sub>-Cl<sub>2</sub> within the central 100 bp of nucleosome core particles. The nucleosomal DNA conformation apparently does not alter

guanine electronegativity, one of the factors in sequence-specific binding (Mattes, et al., 1986; Marien, et al., 1987). It appears that the local macromolecular environment which determines AFB<sub>1</sub>-Cl<sub>2</sub> sequence-specific binding is unaltered in nucleosomal DNA, or altered so slightly that no change is observed in the sequence-specific DNA alkylation of AFB<sub>1</sub>-Cl<sub>2</sub>.

#### 6. The Global Suppression of AFB<sub>1</sub>-Cl<sub>2</sub> Binding to Nucleosomal DNA

AFB<sub>1</sub>-Cl<sub>2</sub> adduct formation is 2.4-fold greater in free DNA than in the central 100 bp of the reconstituted particle. One possible reason for this is that global DNA-histone interactions may suppress pre-covalent AFB<sub>1</sub>-Cl<sub>2</sub> DNA binding. AFB<sub>1</sub> has a pre-covalent association with DNA characterized by strong AFB<sub>1</sub> hypochromism (Clifford and Rees, 1969; our measurements), and the observation that activated AFB<sub>1</sub> covalently binds 4-fold more strongly to double helical rather than single-stranded DNA (K. Marien, in preparation). Intercalation of AFB<sub>1</sub>-Cl<sub>2</sub> between DNA bases was an initially attractive explanation for AFB<sub>1</sub>-Cl<sub>2</sub> pre-covalent binding, as it offered a way for the molecule to be equally exposed to the major groove whether it faced inward or outward relative to the histone core, facilitating N7-guanine binding. This could also explain how the middle of three

guanines would be most often adducted, since intercalation occurs between guanine residues, and as the intercalated AFB<sub>1</sub>-Cl<sub>2</sub> would have a 1:1 probability of attacking the guanine on either side, binding to the middle guanine would be favored.

Subsequent experiments (Sec.III.A.3) determined that even a 10,000:1 molar ratio of AFB<sub>1</sub>:relaxed plasmid would not intercalate to induce DNA supercoiling. (These supercoiling studies were done with AFB<sub>1</sub> and not AFB<sub>1</sub>-Cl<sub>2</sub>, as AFB<sub>1</sub>-Cl<sub>2</sub> is almost instantaneously activated in aqueous buffer, upon which it covalently binds to DNA or is hydrated. Hence we have not eliminated, in the strictest sense, intercalation as the precovalent mode of AFB<sub>1</sub>-Cl<sub>2</sub> binding.)

The precovalent association of AFB<sub>1</sub>-Cl<sub>2</sub> is apparently major groove binding (Lin, et al., 1977; and Benasutti, et al., 1988). The exact nature of the global change in nucleosomal DNA which effects AFB<sub>1</sub>-Cl<sub>2</sub> adduct formation is unknown. A greater degree of freedom in the DNA ends or "naked" DNA than in the central 100 bp of the particle may explain the global suppression of AFB<sub>1</sub>-Cl<sub>2</sub>-DNA binding.

## 7. Relation to Other AFB<sub>1</sub>-Chromatin Binding Studies

Earlier work (Bailey, et al. 1980) found a 5:1 internucleosomal (linker) DNA to core DNA ratio of

adduction in vivo in liver DNA when rainbow trout were injected intraperitoneally with  $^3\text{H}$ -AFB<sub>1</sub>. The specific activity of nucleosome dimers and trimers, connected with linker DNA, was compared to the specific activity of mononucleosomes trimmed to 145 bp, to deduce this linker to core binding ratio. A similar ratio was observed in AFB<sub>1</sub> adduction to ribosomal RNA genes, which were 5 fold more accessible than the remaining bulk nuclear DNA. Ribosomal RNA genes appear to be non-nucleosomal when examined by electron microscopy (Scheer, 1980; Labhart and Koller, 1982) and may behave similarly to linker DNA. In the reconstituted 5S particle, the free DNA ends or "linker" DNA are only adducted 2.4-fold more than core DNA. This discrepancy may be due to an additional level of core DNA protection conferred by chromatin condensation. The system used for this work only assesses differences of AFB<sub>1</sub>-Cl<sub>2</sub> adduction due to the first level of chromatin packaging, the nucleosomal core particle.

It must be remembered that homogenous sequence-positioned particles are necessarily a subset of the "average" nucleosome core particle. Further, the sequence used here does not place guanyl sites in every possible nucleosomal position, hence data are not available on all nucleosomal locations. Though other nucleosome conformations where local DNA-histone contacts

further modulate AFB<sub>1</sub>-Cl<sub>2</sub> or DMS binding cannot be ruled out, the major conclusions from this study are: (i). individual, nucleosomal guanine bases are neither markedly exposed to or shielded from alkylation by these two agents; (ii). consequently, the marked sequence-specificity of AFB<sub>1</sub>-Cl<sub>2</sub> is not altered by the nucleosomal DNA conformation. Based on the data available, the covalent and/or precovalent binding of AFB<sub>1</sub>-Cl<sub>2</sub> to nucleosomal DNA is suppressed by a global DNA conformation, but is not further modulated by local histone interactions, DNA rotational orientation, or localized DNA bending.

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**APPENDIX**

## APPENDIX A: AFLATOXIN SAFETY PROCEDURES

## AFLATOXIN SAFETY PROCEDURES

(In use at the Department of Food Science and Technology,  
Oregon State University.)

## GENERAL PRECAUTIONS

1. Personal Protection: Rubber gloves (preferably a heavy type like Edmont-Wilson) and lab coats should be worn whenever handling aflatoxins. Lab coats should be changed at least weekly. If a lab coat or piece of clothing is contaminated, it should be decontaminated before leaving the laboratory. Avoid using gloves to open doors, drawers, operate instruments, etc. It is advisable to wash hands with soap at conclusion of all operations with aflatoxins.
2. Cover bench tops with absorbent protective paper (the type used in isotope work). The paper should be changed regularly and immediately in the case of overt spills or contamination.
3. Immediately after use, place contaminated items in Oakite/bleach solution as directed in Dishwash or Disposal Procedures (see below). Do not leave contaminated items on benchtops!
4. Eating, drinking, or smoking are not allowed in rooms where aflatoxins are being used.
5. Pipetting by mouth is not allowed.
6. No unauthorized persons are allowed in the laboratory.
7. Aflatoxins are to be handled only as solutions and are to be quantitated by chromatographic, radiometric or spectrophotometric techniques, rather than by direct weighing. (Exceptions are preparation of stock solutions from manufacturers' shipping containers, and chromatographic treatment, especially thin-layer work.)
8. If solutions are spilled, decontaminate area immediately.
9. Equipment from other laboratories should not be used in any laboratory where work with mycotoxins is being conducted. Also, once an item is used in a mycotoxin laboratory, it should not be used in another laboratory.

10. Monitor work area with UV lamps to detect aflatoxins. Low concentrations may not be detectable.
11. Use fume hoods for all operations where contaminated dusts, particles, or mists may be formed (e.g. thin layer spotting, developing and scraping, roto-vapor evaporation, etc.).
12. Use gas tight syringes when measuring small volumes or for HPLC injections to prevent aerosol formation.
13. Place "Cancer Hazard" or other similar labels on carcinogen containers and areas where aflatoxins are being used.
14. Rooms or areas where aflatoxins are being used must be identified by a "Cancer Hazard" or other appropriate sign.
15. If you are in doubt about how to safely deal with a new situation, or are unfamiliar with routine operations, it is your responsibility to ask your supervisor or someone who knows.

#### THIN LAYER CHROMATOGRAPHY

(A potential hazard exists due to airborne silica particles containing aflatoxin.)

1. When possible, use commercially poured TLC plates. Silica particles are strongly bound to the plate and hence there is minimal risk of aflatoxin-borne particles becoming dislodged.
2. Conduct all TLC spotting and developing in the fume hood.
3. When developed spots must be scraped from TLC plates, this operation must be performed in an efficient fume hood and the operator should wear a dust respirator. All loose silica gel and TLC plates must be decontaminated in this fume hood (Oakite/bleach solution - see below).
4. All TLC plates used for aflatoxins must be decontaminated prior to being discarded.
5. Similar precautions must be followed when disposing of packings from liquid chromatography columns.

## DISHWASH PROCEDURE FOR AFLATOXIN CONTAMINATED GLASSWARE

CAUTION: Rubber gloves and laboratory coat or apron must be worn during all dishwash operations. Washing hands at conclusion of job is recommended.

1. FIRST WASH: Glassware must soak at least 12 hr in a solution of Oakite 67 detergent plus laundry bleach (sodium hypochlorite).

a) dish pans: approximately 50 cc (1/8 - 1/4 scoop) detergent plus 1/8 gal. bleach.

b) sink: approximately 200 cc (1/2 - 1 scoop) detergent plus 1/3 - 1/2 gal. bleach.

2. SECOND WASH: Soak at least 12 hr in Oakite solution only. If necessary, use test tube brush to clean debris from glassware. If glassware originally contained radioactivity, detergents designed to remove radioisotopes may be substituted for Oakite.

3. RINSE

a) Drain second wash and rinse each piece thoroughly (3 times) under tap water.

b) Soak piece at least 12 hours in tap water.

c) Rinse each piece thoroughly under tap water.

4. DRY: Hang most glassware on drying rack; small or hard-to-dry items should be dried in an oven.

5. Put away in proper location, making sure glassware is dry and clean.

## SPECIAL NOTES:

1. Small, easily lost or breakable items should be processed separately from the remaining items (e.g., in a beaker).

2. Certain materials such as rubber, cork and metal will corrode or decompose in the wash solutions - they should be processed by dunking them for a few minutes, rather than by soaking.



3. Once a week, wipe the drainboards with detergent-bleach solution.

4. Be sure glassware is completely submerged at each stage, with no air pockets.

#### PROCEDURES FOR DISPOSAL OF AFLATOXIN-CONTAMINATED ITEMS

##### GENERAL:

(Items include paper, disposable pipettes, TLC plates, etc.) Place items in a dishpan containing Oakite/bleach solution (see dishwash procedure) and allow to stand at least overnight. Pour liquid down drain through metal strainer. Transfer solids to a plastic bag and double bag if necessary. Place in waste basket.

##### DISPOSAL OF CONTAMINATED SYRINGE NEEDLES

Draw Oakite/chlorox solution through needle with a syringe. Remove needle and replace plastic cap which came with needle. Discard in disposal dishpan.

##### DISPOSAL OF UNWANTED AFLATOXIN SOLUTIONS

If aflatoxins are in water or water-soluble organic solvents, pour solution into Oakite/bleach. If aflatoxins are in organic solvents which are not soluble in water, pour solutions into ethanol and then dump into Oakite/bleach. It is best to evaporate the organic solvent to a small volume in a fume hood as needed before dumping into ethanol. Glassware which originally contained the aflatoxin should be rinsed with ethanol before being placed in the dishwash solution.