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Carcinogen Aflatoxin B_1 Is Located Preferentially in Internucleosomal Deoxyribonucleic Acid following Exposure in Vivo in Rainbow Trout†

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ABSTRACT: The purpose of this work was to investigate the distribution in chromatin of deoxyribonucleic acid (DNA) adducts of aflatoxin B_1, following exposure in vivo. Rainbow trout were injected intraperitoneally with radiolabeled aflatoxin B_1, a potent procarcinogen known to readily induce hepatocellular carcinomas in these fish. After maximum incorporation, liver nuclei were prepared and digested with micrococcal nuclease. Mono-, di-, and trinucleosomal fractions were purified from several stages of nuclease digestion, and the lengths and specific activities of their DNA were determined. The results indicate that aflatoxin B_1 is ~5 times as likely on a per nucleotide basis to localize on internucleosomal (linker) DNA as on nucleosomal core DNA in this system.

Although chemical carcinogens are believed to initiate their action through mutagenic interaction with the genome (Miller & Miller, 1971; Ames, 1979), our knowledge of the actual sites of carcinogen attack in higher organisms is at present very limited. One initial approach to this problem involves examining the influence of those chromatin structural features which one can clearly define on the accessibility of carcinogens to DNA in vivo. The genome of all eukaryotes is now recognized to be organized into a repeating subunit structure [for reviews, see Kornberg (1977); Garret (1979)]. This structure, called the nucleosome, consists of about 165-245 base pairs of DNA[1] together with all of the histones. The nucleosome can in turn be subdivided into a ubiquitous "core particle" containing 146 base pairs of DNA wound one and three-fourths turns around a core of eight histone molecules (two each of histones H_2A, H_2B, H_3, and H_4) and a variable region of 20-70 base pairs of "linker" DNA. The linker provides additional binding sites for histones H1 and other nonhistone proteins of suspected regulatory or structural function (Noll & Kornberg, 1977; Lohr et al., 1977a; Levy-Wilson et al., 1979; Weisbrod et al., 1980). These elements, perhaps in combination with local variations in histone sequence (Spinelli et al., 1979) or modification (Davie & Candido, 1978) are believed to control chromatin configuration in some manner which reflects, and perhaps mediates, gene expression and differentiation patterns. For example, regions of the genome enriched in transcribing sequences appear to be preferentially digested by brief treatment of chromatin with pancreatic DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1976; Weisbrod et al., 1980) though the structural basis for this is incompletely defined at present.

Several groups have recently begun to investigate the chromosomal distribution of carcinogen-DNA adducts formed in vivo in animal systems by using DNase I or micrococcal nuclease (MNase), both of which can selectively degrade linker DNA under appropriate conditions, as probes of chromatin structure. Adducts of the bulky procarcinogen *N*-hydroxy-2-acetylaminofluorene were reported to be preferentially distributed in the acid-soluble fractions upon exhaustic digestion of rat liver nuclei with MNase (Metzger et al., 1977), suggesting a preferred linker distribution for this carcinogen. However, the same adducts were also found to be enriched in the fractions resistant to exhaustive DNase I digestion (Ramanathan et al., 1976a; Metzger et al., 1977) which implies a preferred core DNA distribution. By comparison,

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[1] Abbreviations used: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; dpm, disintegrations per minute; $NaDodSO_4$, sodium dodecyl sulfate.

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methylating carcinogens were observed to produce adducts which were not only sensitive to release from chromatin by exhaustive MNase digestion (Ramanathan et al., 1976b) but were also preferentially liberated by DNase I (Ramanathan et al., 1976b; Cox, 1979). Sudhakar et al. (1979), who examined the kinetics of release of adducts of the carcinogens 1-methyl-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, concluded that these compounds preferentially alkylated linker and core DNA, respectively. Contrary to all these reports, Pegg & Hui (1978) could find no evidence for preferential release of methylated adducts from chromatin of treated animals either by DNase I or by MNase.

No consistent pattern of effects of chromatin structure, procarcinogen size, or adduct size on adduct distribution emerges from these studies. A major difficulty in the interpretation of these studies concerns the use of kinetics or extent of nuclease solubilization of label as the sole criterion for core vs. linker location of adducts. Such studies assume that the adducts being released all reside in native regions having normal nuclease susceptibility. It is possible, however, that an unknown but significant proportion of adducts formed may actually induce localized chromatin structural perturbations. Adducts in these regions would then show enhanced susceptibility to MNase or DNase I quite apart from linker DNA location. Unfortunately, such perturbations would be difficult or impossible to detect in these experiments, where only ~0.1% or fewer of nucleosomes are carcinogen modified. A further complication is that DNase I can also selectively hydrolyze transcriptionally active chromatin (which may show overall enhanced alkylation), so that the resultant kinetic label release curve will be a complicated mixture of adducts released from core and linker DNA, possibly perturbed regions, as well as active chromatin.

A more direct approach to assessing the effect of chromatin structure on carcinogen distribution relies on the actual isolation of nucleosomal monomers and oligomers containing varying ratios of linker to core DNA and the determination of adduct distribution among these fragments. Any severely damaged regions should show enhanced nuclease susceptibility, would be degraded early during digestion, and would not be isolatable as labeled nucleosomal material. Jahn & Litman (1979) have recently used this approach to examine the distribution of benzo[*a*]pyrene adducts formed in vitro upon incubation of rat liver nuclei with a microsomal activating system. Qualitative results were reported which indicated that on a per nucleotide basis, benzo[*a*]pyrene adducts formed in vitro were not distributed randomly in chromatin but were preferentially located in linker DNA. Kootstra et al. (1980) used a similar approach to show that adducts of benzo[*a*]-pyrenediol epoxide exhibited a 3–4-fold preference for linker DNA in isolated chick nuclei.

It is important to extend these results to in vivo systems and to provide a quantitative estimate of the distribution asymmetry for a range of carcinogens. We report here the results of studies using tritiated aflatoxin B_1 (AFB_1), perhaps the most potent naturally occurring procarcinogen, administered in vivo to rainbow trout by a route known to produce hepatocellular carcinomas in these sensitive fish (Scarpelli, 1976; Sinnhuber et al., 1977; Wales et al., 1978). The results suggest that aflatoxin adducts in this system distribute preferentially in linker DNA in vivo, with an ~5-fold preference per nucleotide over core DNA.

Experimental Procedures

Chemicals. Tritiated aflatoxin B_1 (15 Ci/mmol) was purchased from Moravek Biochemicals. The purity of each lot was checked by radioscanning of silica gel chromatograms developed in chloroform–acetone (9:1).

Carcinogen Binding in Vivo. Trout (0.6–1 kg) were injected intraperitoneally with [^{3}H]AFB_1 (50 μCi, 25 μg, in 0.5 mL of ethanol). Binding of AFB_1 was observed to reach a broad plateau from 4 to 24 h in this system. Fish were sacrificed after 24 h, and livers were immediately excised and perfused with ice cold Hewish & Burgoyne (1973) buffer containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and 50 mM sodium bisulfite, pH 7.2, followed by mincing and homogenization in a glass–teflon homogenizer.

Nuclei Preparation, Nuclease Digestion, and Nucleosome Preparation. Liver homogenates were filtered through cheesecloth and Miracloth, and nuclei were sedimented at 1900*g* for 5 min. The pelleted nuclei were resuspended and washed 3 times in homogenization buffer, once in the same buffer containing 0.15% Non-idet NP40 (Shell Chemicals) and twice in nuclease digestion buffer (0.3 M sucrose, 10 mM Tris-HCl, pH 7.2, 0.7 mM calcium chloride, and 0.2 mM PMSF).

Nuclei were incubated at 25 °C with micrococcal nuclease (MNase) (Worthington Biochemical Corp.) at 1.5–2 mg of DNA and 120 units of MNase per mL. Reactions were stopped at various times by adding EDTA to 5 mM and chilling on ice. The percent digestion to acid soluble nucleotides was determined as described (Jahn & Litman, 1979) except by using the diaminobenzoic acid assay for DNA (see below). Nuclei were lysed by dialysis against 10 mM Tris-HCl, pH 7.2, 0.7 mM EDTA, and 0.2 mM PMSF. Nucleosome monomers and oligomers were separated preparatively by layering clarified lysates onto 5–20% linear sucrose gradients and centrifuging at 113000*g* for 24 h at 4 °C.

DNA Extraction and Determination. Samples were treated with ribonuclease and Pronase, followed by phenol extraction and extensive isoamyl alcohol–chloroform extraction as described elsewhere (Lohr et al., 1977b). Alcohol–chloroform extractions beyond five cycles did not result in further changes in DNA specific activity. Traces of phenol were removed by extraction with ether. The DNA content of these samples was determined by an adaption (Lohr et al., 1977b) of the diaminobenzoic acid assay (Kissane & Robins, 1958). All samples were counted in Aquasol (New England Nuclear) on a Beckman 7500 Scintillation Counter with internal correction for quenching and efficiency variation.

Covalent and Noncovalent Aflatoxin Binding. Controls were carried out to determine the efficacy of the above procedures for removing AFB_1 noncovalently bound to DNA. Calf thymus DNA (150 μg) and [^{3}H]AFB_1 (0.15 μCi, 0.25 μg) were incubated in homogenization buffer for 4 h at 25 °C and 18 h at 4 °C, and the DNA was extracted as above. Three alcohol–chloroform extractions removed 96% of the tritium counts from the DNA fraction. In several experiments ~4% of the counts remain unextractable even from heat-denatured DNA, perhaps due to tritium exchange.

A sample of the aflatoxin–DNA incubation mixture was also sedimented on a sucrose gradient as for nucleosome preparation, and the distributions of tritium and of DNA were determined. Again over 95% of the tritium remained at the top of the gradient away from the DNA.

The covalent adducts of AFB_1 formed in trout liver were found to consist mainly of AFB_1–N_7-guanine, traces of the N_7-guanine adduct of aflatoxin P_1 (a metabolite of AFB_1), and significant amounts of at least four other partially characterized adducts. These results will be reported in detail elsewhere (Croy et al., 1980).

Table I: Average DNA Lengths from Chromatin Fragments Purified at Various Stages of Nuclease Digestion

nuclease digestion		nucleotide length of purified fragments					
		monomer		dimer		trimer	
time (min)	%	band width	median	band width	median	band width	median
0.5	2.7[a]	169-234[b]	201[c]	314-479	390	483-741	612
1	5.2	155-224	185	285-479	382	519-684	601
3	13	141-174	157	271-427	346		
6	20	129-177	153	258-386	322	427-596	511
9	26	132-151	142	285-367	326		
12	29	134-157	144	271-386	322	427-556	491

[a] Percent digestion to acid-soluble nucleotides. [b] Band widths here are examples drawn from a single set of gels. [c] Median positions are the means of three determinations. Though band widths were high (a reflection of population heterogeneity), their positions relative to known markers were highly reproducible, and the triplicate determinations agreed within ±3% or less.

Electrophoresis and DNA Length Determination. Purified DNA samples were electrophoresed on mini-slabs (0.5 × 80 × 120 mm) of 4% acrylamide gel at 70 V for 100 min with buffers previously described (Loening, 1967). Gels were stained for 20 min in 0.5 μg/mL ethidium bromide and photographed under ultraviolet light. Fragment lengths were determined by using *Hae*III restriction fragments of PM2 DNA and *Hha*I fragments of pBr 322 DNA as markers (a gift of Dr. Court Saunders). Histones were separated on a mini-slab apparatus as described (Matsudara & Burgess, 1978) and stained with 0.1% amido black in 7% acetic acid–25% ethanol.

Yields of Purified Nucleosomes. Yields of each fraction varied according to digestion time. At 60 s, yields of purified monomers, dimers, and trimers per 4 g of liver were 12.4, 27, and 17 A_{260} units, respectively. At 12 min, monomer yield was 44.3 A_{260} units/4 g of liver.

Results

Preparation of Carcinogen-Labeled Nucleosomes from Trout Liver. Trout were labeled in vivo with [^{3}H]AFB$_1$, liver nuclei were prepared, and oligonucleosome fragments were generated by nuclease digestion as described under Experimental Procedures. Examples of the two-step purification of AFB$_1$-labeled oligonucleosomes from various stages of MNase digestion are illustrated in Figure 1. Peak fractions containing mono-, di-, and trinucleosomes from the first run of each digest (Figure 1A) were pooled individually and rerun on second gradients to reduce cross-contamination (Figure 1B). Similar purifications were carried out for 0.5-, 1-, 3-, 6-, 9-, and 12-min digests. Analysis of samples heavily overloaded on DNA gels (e.g., Figure 1B) indicated negligible cross-contamination for all final preparations except 30-s mononucleosomes, which contained ~10% higher oligomers. [The final specific activity of this fraction, however, would not be significantly affected by such contamination since these monomers have virtually intact spacer plus core DNA lengths (see Table I)].

Determination of Average Repeat Length in Trout Liver. The range and median DNA lengths of the monomer, dimer, and trimer nucleosome populations at various stages of digestion are shown in Table I. The lengths decreased as a result of the known exonucleolytic trimming action of MNase, with a final trimmed length in this determination of 144 nucleotide paris for mononucleosome core DNA. Average core plus linker repeat length in trout liver was determined from these data to be 206 (±6) by the method of Lohr et al. (1977b), compared to the value of 200 (±30) reported from trout tests with earlier methods (Honda et al., 1975). The average internucleosomal (linker) DNA length in trout is therefore ~62 base pairs from these data.

Distribution of Aflatoxin Adducts in Nucleosomal and Internucleosomal DNA. The specific activity of purified mononucleosomal DNA at various stages of digestion is shown in Table II. Fully trimmed core nucleosomes with 144 base pairs of DNA had a measured specific activity in this experiment of ~26,000 dpm/mg (26 200 ± 490, 25 900 ± 700). However, whole repeat DNA[2] or DNA from the 30-s digest (very little linker trimming) had specific activities of ~57 000 dpm/mg (56 500 ± 5100 for 30-s monomers). These results clearly demonstrate that aflatoxin–DNA adducts formed in vivo are found in vitro preferentially distributed in the internucleosomal regions of chromatin; nuclease removal of linker DNA, which comprises 30% of the repeat, reduces the specific activity by over 50%.

Table II: Specific Activity of DNA from Mononucleosomes Isolated at Different Stages of Nuclease Digestion

% digestion	length (base pairs)	sp act.	
		obsd[a]	expected[b]
2.7	201	56.5 (5.1)	55.9
5.2	185	44.9 (10.8)	48.6
13	157	32.7 (1.6)	34.3
20	153	31.6 (0.95)	32.2
26	142	26.2 (0.49)	25.9
29	144	25.9 (0.7)	25.9

[a] Values are means (±range) from duplicate DNA assays and counts, expressed as disintegrations per minute per milligram of DNA × 10^{-3}. [b] Based on a 5:1 specific activity of linker vs. core DNA by using 57 × 10^3 dpm/mg as the defined specific activity of the 206 base pair repeat DNA and 144 base pairs as the core DNA length; see text.

The relative distribution of adducts in core and spacer DNA can be calculated as follows. At 57 000 dpm in 1 mg of core plus linker repeat, 18 000 dpm derive from 0.7 mg of core DNA (25 900 × 0.7). The remaining 39 000 dpm must derive from 0.3 mg of linker DNA, which therefore has a specific activity of ~130 000 dpm/mg. Hence the observed linker to core distribution of aflatoxin–DNA adducts per milligram is 130 000/25 900 = 5.0.

Aflatoxin Distribution in Particles from Intermediate Digests. Support for the accuracy of the 5:1 estimate was gained by examining the specific activity of nucleosome DNA preparations at intermediate stages of nuclease digestion and trimming (as opposed to the initial and final monomer states used above). An expected specific activity of DNA at any stage of linker trimming can be formally derived in terms of relative core and linker DNA lengths[3] and carcinogen binding

[2] The specific activity required here is for the intact core and linker repeat, not for whole chromatin DNA which includes an unknown proportion of nonnucleosomal repeat material. An estimate for intact repeat DNA of ~57 000 dpm/mg is given by extrapolation of the particle specific activities in Table II and Figure 2 to zero digestion.

ratios as follows: Let n_L and n_C be the probability of AFB_1 binding per base pair of linker and core DNA, respectively (n_C/n_L = relative specific activity ratio; 0.2 for our estimate). Let $F_L = 1 - F_C$ = the fraction of an intact repeat which is linker (62/206 = 0.3 for trout). Then the total binding probability per base pair in an intact repeat (specific activity) is

$$A^0 = n_L F_L + n_C F_C \quad (1)$$

For oligomers with m cores, the number of linkers will be $m - q$, where q is the fraction of the end linker DNA progressively trimmed after initial MNase cleavage ($0 \leq q \leq 1$). If the number of base pairs in a core = N_C and the number of base pairs per linker = N_L, then the total number of AFB_1 binding sites in an m-mer at any stage (q) of trimming is

$$N_m{}^q = mN_C n_C + (m - q)N_L n_L \quad (2)$$

and the specific activity is

$$A_m{}^q = \frac{mN_C n_C + (m - q)N_L n_L}{mN_C + (m - q)N_L} \quad (3)$$

Noting that $F_C = N_C/(N_C + N_L)$ and $F_L = N_L/(N_C + N_L)$, this reduces algebraically to eq 4. Since $F_C n_C + F_L n_L$ is

$$A_m{}^q = \frac{F_C n_C + F_L n_L - \dfrac{q n_L F_L}{m}}{1 - q\dfrac{F_L}{m}} \quad (4)$$

defined by eq 1 as repeat specific activity and $A^0 = A_m{}^q$ where $q = 0$, this reduces and rearranges to eq 5 which provides the

$$\frac{A_m{}^q}{A_m{}^0} = \frac{1 - \dfrac{qF_L}{m}\left(\dfrac{1}{F_L + F_C n_C/n_L}\right)}{1 - q\dfrac{F_L}{m}} \quad (5)$$

ratio of trimmed to starting specific activity at stage q.

The validity of eq 5 is checked simply. For a monomer preparation ($m = 1$) where $F_L = 62/206 = 0.3$, $n_C/n_L = {}^1/_5$, and at $q = 1$ (full trimming) eq 5 predicts $A_1{}^1/A_1{}^0 = 0.456$, which is identical with our finding of 26 000/57 000 = 0.456.

To verify the accuracy of our determination of $n_C/n_L = {}^1/_5$, we used eq 5 to calculate "expected" specific activities for monomer preparations of known length (L) at various stages of MNase trimming [$q = (206 - L)/62$]. As shown in Table II, these values generally agree closely with actual specific activity determinations from the various monomer preparations.

Further support was provided by analysis of oligonucleosome ($m > 1$) specific activities. For dinucleosomes ($m = 2$) the specific activity would be predicted by eq 5 at $n_C/n_L = 0.2$ to fall during trimming to 44 200 dpm/mg for a perfectly trimmed population. Similarly for trimers, $A_3{}^1 = 48\,900$ by eq 5. The actual specific activities of dimers and trimers prepared at various stages of nuclease digestion are shown in Figure 2. While populations of nucleosome oligomers can never achieve the ideal trimming homogeneity or precision assumed in eq 5 [e.g., band widths are high, oligomers with shorter internal linkers may accumulate, and overtrimming of end cores may occur before cleavage (Table I)] our prep-

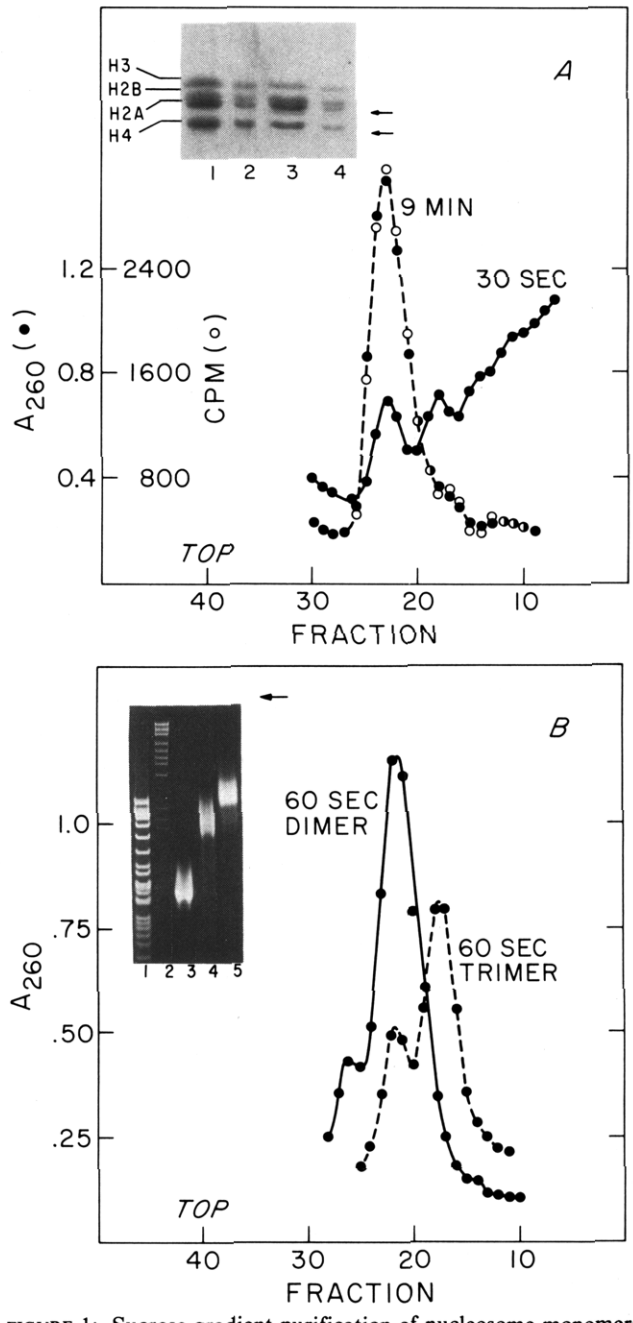

FIGURE 1: Sucrose gradient purification of nucleosome monomers and oligomers from liver nuclei of AFB_1-treated trout. (A) First gradient purification of 30-s and 9-min MNase digests. Fractions of 1 mL each were taken from 40-mL gradients by reverse pumping, and equal aliquots were taken for tritium counting (○) and A_{260} (●) reading. For clarity, the cpm profile of the 30-s digest is omitted but also coincides very closely with the A_{260} profile. (Histone gel insert) (1) Trout red blood cell core histone control (5 μg); (2) same (1 μg); (3) 9-min digest monomer pool (fractions 21–25) histones (4 μg); (4) same (0.5 μg). The arrows indicate faint bands of large acid-soluble fragments presumably of core histone origin. (B) Gradient reruns of dimer and trimer pools from first gradient of 60-s digest. Conditions same as (A) (see Experimental Procedures). The final purified 60-s dimer pool was taken as fractions 21–24 from the main dimer rerun; the purified 60-s trimer pool was taken as fractions 15–17 of the main trimer rerun shown here. (DNA gel insert) (1) pBr 322 fragments; (2) PM2 fragments; (3–5) DNA from purified monomers, dimers, and trimers, respectively, from 6-min digest. The arrow indicates the origin.

[3] This derivation assumes that linker DNA is equally susceptible to AFB_1 attack throughout its length and, for simplicity, that nuclease clevage is equally probable among linkers of varying length in chromatin. In reality, dimers and trimers with shorter than average internal linker may escape cleavage until later in digestion. This, along with excessive end trimming, appears to result in shorter than average oligomers (Table I) with lower than "expected" specific activities (Figure 2, trimers) late in digestion.

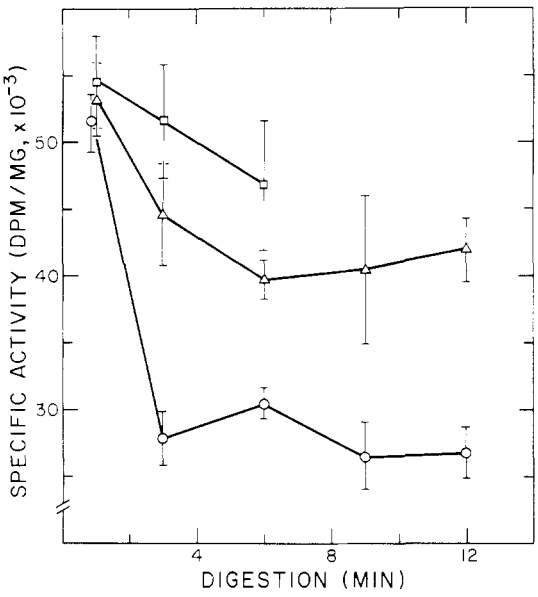

FIGURE 2: Specific activities of the DNA extracted from mono-, di-, and trinucleosomes at various stages of MNase digestion. DNA was extracted from each purified fraction and the specific activity determined as Experimental Procedures. The points represent means of three or more determinations; the error bars are standard deviations. The quantity of trinucleocomes recovered from late digests was too low for adequate specific activity determination; duplicate determination of 12-min trimers gave a value of 44 300 (±7600) dpm/mg. These are separate determinations from those shown in Table II. Monomers (○); dimers (△); trimers (□).

arations from late in digestion do appear to fall within the ranges predicted.

The strength of support for the 1:5 estimate which these results provide depends on the sensitivity of the results to variations in the parameter n_C/n_L. Suppose we take a value of 0.25 or 0.15 for n_C/n_L (instead of the 0.2 found above) and calculate the expected value for A_1^1 (by using A_1^0 = 57 000 dpm/mg as before). We then find that A_1^1 (predicted) = 30 000 dpm/mg for $n_C n_L$ = 0.25 or 21 100 dpm/mg for $n_C n_L$ = 0.15. Both results differ significantly ($P < 0.05$) from our determinations [25 900 ± 700, 26 200 ± 190 (Table I); 26 500 ± 550, 26 700 ± 320 (Figure 2)] which suggests that our 1:5 estimate reliably indicates the specific activity ratio of AFB_1 adducts in core and linker DNA.

Histones. AFB_1 could conceivably induce substantial histone proteolysis involving a major portion of chromatin as part of its highly toxic behavior, thereby influencing adduct distribution in vivo. Analysis of adduct distribution under these conditions would of course provide little information on the nonacute carcinogenic response. To examine this question, we injected trout with varying doses of AFB_1 up to 250 μg/kg (LD_{50} is 300–500 μg/kg in trout), held them for 24 h, and removed their livers. Histones and other acid-soluble proteins were extracted immediately by homogenization in ice-cold 0.4 N HCl to avoid in vitro proteolysis and were examined for integrity by electrophoresis and densitometry scanning of $NaDodSO_4$–acrylamide slab gels. The patterns gave no indication for gross degradation of H1 or core histones (or other proteins) at low to moderate doses of AFB_1, including the level used here for label distribution studies (Table III). Of course, such bulk analysis cannot rule out *localized* histone loss near AFB_1 binding sites, since the proportion of AFB_1-modified "nucleosomes" is too small (10^{-3}–10^{-5}) to examine directly.

Discussion

Ratio of Aflatoxin Adducts in Core and Linker DNA.

Table III: Effect of Aflatoxin Exposure on Histone Integrity in Vivo in Trout Liver

AFB_1 dose (μg/kg)	relative staining[a] of extracted histones			
	H1	H3	H2A + H2B	H4
0	54 (±4)	56 (±2)	100	67 (±3)
0.25	59 (±8)	55 (±3)	100	72 (±7)
2.5	55 (±1)	56 (±3)	100	69 (±3)
25	57 (±3)	59 (±1)	100	75 (±1)
250	36 (±6)	37 (±5)	100	58 (±5)

[a] Histones stained with amido black and measured by direct densitometry of gel. Data normalized to H2A + H2B peak, with no correction for trout histone molar staining ratios. Data are averages (±range) of two or more gels.

Qualitative evidence presented here shows clearly that following in vivo activation and binding, adducts of aflatoxin are distributed preferentially in internucleosomal DNA. Quantitatively, our results indicate a preference of linker over core DNA of 5:1 on a per nucleotide basis. We wish to stress that, because of limitations inherent in the nuclease method (e.g., core preparations are never monodisperse and perfectly trimmed), this ratio can be estimated with only limited certainty. However, future *pairwise* comparisons between carcinogens using this approach in conjunction with double labels can be expected to provide internally consistent and hence useful quantitative information on the influence of carcinogen size (or other characteristics) on genome accessibility.

Adduct Distribution and Histone Integrity in Vivo. We have found no evidence that extensive histone breakdown throughout chromatin in vivo is a mechanism underlying the nonrandom distribution of carcinogen–DNA adducts at the AFB_1 doses used here, though localized or short-term degradation is not ruled out. It was interesting to note, however, that at the dose of AFB_1 approaching LD_{50}, considerable alteration in the staining ratios of extracted histone occurred, perhaps reflecting degradation. Though unlikely to explain the nonrandom distribution of adducts reported here (e.g., H1 was not selectively reduced), this degree of gross proteolysis at high dose may warrant further study for possible involvement in acute toxicity for this highly potent compound. For example it would be important to know whether histone breakdown precedes death of individual cells at acutely toxic doses of AFB_1 or instead follows as a consequence.

In contrast to the in vivo situation, we routinely observe some PMSF- and bisulfite-insensitive histone proteolysis which occurs in vitro during our preparation of nuclei from trout liver homogenates[4] (see insert of Figure 1). Since this occurs in vitro well after any adduct formation is completed, it would be important here only if extensive enough to disrupt nucleosomal integrity or operationally obscure the ability of MNase to differentiate adduct-labeled core and linker DNA. In this regard, it is especially important to note the correspondence between the AFB_1 label profiles and the DNA profiles in Figure 1, which shows that the radiolabeled fragments which we have isolated are not incidental background degradation products from unstructured regions but behave as a bona fide nucleosomal material during successive sucrose gradients. Of similar importance is the correlation between

[4] The proteolytic activity is also present in control fish unexposed to aflatoxion and appears to be a trout liver lysosomal activity released on homogenization which is unusually resistant to inhibitors. Similar protocols have been used in our laboratory to prepare nuclei with fully intact histones from trout erythrocytes, calf thymus, rat liver, and calf thymus, but not from trout liver.

increasing nucleosome number, increasing relative linker DNA content, and increasing specific activity of AFB_1 labeling, which would not be seen if the AFB_1 in the particles which we isolated were randomly distributed or were localized in unstructured DNA.

Carcinogen Adducts and Nuclease Cleavage Properties. The apparent preference of aflatoxin for linker DNA as determined here is unlikely to be influenced by any effect of adducts themselves on MNase cleavage properties. MNase appears to randomly hydrolyze purified alkylated DNA after exposure to a range of carcinogens including dimethylnitrosamine (Ramanathan et al., 1976b), benzo[*a*]pyrene (Jahn & Litman, 1979), methylnitrosourea, and (chloroethyl)cyclohexylnitrosourea (Sudhakar et al., 1979). Although recent evidence shows that DNA regions cross-linked by 4,5′,8-trimethylpsoralin resist liberation by MNase (Wiesehahn et al., 1977), this is readily understood in terms of the cleavage mechanism of MNase. This enzyme cleaves opposite strands of DNA with a two-base stagger (Sollner-Webb et al., 1978); any cross-link in this region would prohibit cleavage of the double helix as such, even though normal MNase phosphodiester hydrolysis had occurred. Indeed the observation that regions of DNA monofunctionally modified (but not cross-linked) by this reagent are readily and randomly excised by MNase supports the above evidence that this enzyme is unaffected by the presence of alkylation adducts on DNA.

Adduct Distribution and DNA Repair. We have examined only one time point, 24 h, after carcinogen exposure. It is important to ask what effect time-dependent processes may have on the apparent distribution ratio. The most significant of these might be DNA repair; if repair occurred preferentially in linker DNA, then the observed ratio would decrease with time after exposure. Indeed, this possibility receives apparent support from recent reports claiming that UV-induced DNA repair synthesis in other systems occurs preferentially in linker DNA (Cleaver, 1977; Smerdon et al., 1978). However, in these studies the linker location of recently inserted nucleotides was defined only by nuclease sensitivity, yet this sensitivity decreased with time. More recent reports suggest that repair of UV dimers and of bulky carcinogen adducts is, in fact, randomly distributed with respect to nucleosomal structure and that the transitory MNase sensitivity of nucleotides inserted during repair is due to localized unfolding–refolding events induced by the repair process itself (Smerdon & Lieberman, 1978; Lieberman et al., 1979) and not to any preferential location of repair in linker DNA.

There was no specific indication in these studies that the lesions themselves (e.g., carcinogen–DNA adducts) undergo any linker–core DNA transitions in vivo prior to repair, though this possibility was not rigorously excluded by those studies or the results presented here. That adducts should become nonrandomly distributed in vivo by rearrangement rather than from initial attack would, of course, be no less an interesting phenomenon.

Nonrandom Adduct Distribution in Core DNA. Finally the question arises as to whether those adducts which infrequently occur in core DNA are themselves nonrandomly distributed within the core. Simpson & Kunzler (1979) have recently demonstrated that the central 100 bases of core DNA in poly[d(A–T)] nucleosome reconstitutes are tightly associated with histones but that the 23 or so base pairs on either end are less tightly bound. Van Holde et al. (1980) have provided further evidence by using native core particles; the central 100 base pairs of core DNA are resistant to thermal denaturation, whereas the 23 base pairs at either end, along with linker DNA, are much more easily denatured. It is probable that ease of thermal denaturation and accessibility to carcinogen binding are alternate expressions of the same phenomenon, reflecting the tightness of association of core and linker DNA with chromatin structural proteins. Hence the DNA–carcinogen adducts which do form in core DNA might be expected to concentrate in the terminal regions of the core, owing to a looser histone–DNA interaction in this region. Previous studies on the specific activities of adduct-labeled sub-146 base pair DNase I fragments have been interpreted to suggest nonrandom intracore adduct distribution (Jahn & Litman, 1979). However, the precise origins of these fragments were not clearly defined, and further experiments are needed to clarify this point.

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Structure, Translation, and Metabolism of the Cytoplasmic Copia Ribonucleic Acid of *Drosophila melanogaster*†

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ABSTRACT: We have characterized the copia RNA in the cytoplasm of cultured *Drosophila* cells. Copia RNA was detected and purified by hybridization to DNA of the plasmid cDm1142, which contains the copia sequence. A large fraction (2.2%) of the total cytoplasmic poly(A)$^+$ RNA was found to be copia RNA. Cytoplasmic copia RNA displays all the characteristics expected for a messenger RNA. It possesses a poly(A) tract identical in length with that of total poly(A)$^+$ cytoplasmic RNA. It is associated with polysomes and can be released from this association by treatment with EDTA. When purified copia RNA is added to an mRNA-dependent rabbit reticulocyte lysate, three polypeptides of 51 000, 33 000, and 21 000 daltons are seen. We have not determined if these are different polypeptides or if the two smaller polypeptides are fragments of the 51 000-dalton polypeptide. The half-life of copia cytoplasmic RNA was determined in pulse–chase experiments to be 9.5 h; this is 1.6 times longer than the half-life of the intermediate decay class of total poly(A)$^+$ cytoplasmic RNA. These properties provide strong evidence that copia RNA functions in vivo as a messenger RNA.

A significant fraction of the DNA of metazoans consists of intermediate repeat sequences which are repeated 100–100 000 times in the genome. Although some intermediate repeat DNA codes for structural RNA such as rRNA and tRNA, or messenger RNA such as histone mRNA, the function of most intermediate repeat DNA is unknown.

Three families of repeated DNA with unusual properties have been described in *Drosophila melanogaster*. These families, termed copia, 412, and 297, are dispersed in the *Drosophila* genome. A remarkable feature of the copia, 412, and 297 sequences is that they appear to have the ability to change location in the genome, possibly by recombination between the 0.3-kb[1] repeats located at the 3′ and 5′ ends of the RNA coding regions (Finnegan et al., 1977; Potter et al., 1979; Strobel et al., 1979). The movement of discrete units of DNA within the genome has been postulated to explain a number of genetic phenomena in *Drosophila*, such as high rates of mutation, deletion, and reversion at specific loci (Golubovsky et al., 1977; Green, 1977). The small circular DNAs of *Drosophila*, which are homologous to intermediate repeat DNA, might constitute intermediates in this movement (Stanfield & Lengyel, 1979, 1980).

The copia, 412, and 297 sequences are transcribed in *Drosophila* cultured cells into RNAs of unknown function. By virtue of their binding to oligo(dT)–cellulose, these RNAs are considered to be polyadenylated (Finnegan et al., 1977; Carlson & Brutlag, 1978); whether they are actually messenger RNAs has not been established. Copia RNA is the most abundant and hence the most interesting of these transcripts; it constitutes 3–5% of the cytoplasmic RNA of Echalier Kc_0 cells which binds to oligo(dT)–cellulose (Finnegan et al., 1977).

In order to gain insight into the function of the copia DNA sequences and of mobile DNA sequences in general, it is necessary to characterize the copia cytoplasmic RNA and particularly to determine whether it possesses the properties of a messenger RNA. Using recombinant DNA which contains the copia sequence as a probe, we have examined the cytoplasmic stability, structure, and coding capacity of copia RNA. We demonstrate here that copia cytoplasmic RNA possesses a poly(A) tract, has a cytoplasmic decay rate similar to that of other "middle-abundant" poly(A)$^+$ RNAs, is associated with polysomes, and is released from polysomes by treatment with EDTA. The definitive demonstration that

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kb, kilobase (1000 nucleotides); Na-DodSO$_4$, sodium dodecyl sulfate.

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