Quantitative and Qualitative Characterization of Aflatoxin B1 Adducts Formed in Vivo within the Ribosomal RNA Genes of Rat Liver DNA

T. Rick Irvin and Gerald N. Wogan

Laboratory of Toxicology, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

We have examined the time course and patterns of covalent aflatoxin B1-DNA adducts produced within the ribosomal RNA gene sequences isolated from the liver nuclear DNA of aflatoxin B1-treated animals. Liver nuclear DNA was initially enriched in ribosomal DNA by cesium salt density centrifugation, and incubated under alkaline conditions to stabilize bound aflatoxin B1-DNA moieties. Alkalii-treated DNA was hybridized to 18S and 28S rRNA, and the RNA-DNA hybrids were recovered by cesium chloride centrifugation. Ribosomal DNA sequences within nuclear DNA were found to be preferential targets for aflatoxin B1 modification. Over a 12-h period after administration of 1-mg [3H]aflatoxin B1/kg dose ribosomal DNA contained 4 to 5 times more aflatoxin B1 residues per mg DNA than did total nuclear DNA. Aflatoxin B1 residues bound to ribosomal DNA were also found to be removed more rapidly than from total nuclear DNA by a factor of 5.7 over the 12-h period postdosing. Levels of the principal aflatoxin B1 adduct, 2,3-dihydro-3-hydroxy(N7-guanyl)aflatoxin B1, as well as the stable formamidopyrimidine derivatives of the parent adduct were also determined. Nuclear DNA isolates were heated to induce depurination of the principal N7-guamine adduct, and differences in adduct levels between alkali-treated (stabilized) and depurinated DNA samples were taken as an approximation of initial levels of this aflatoxin B1-DNA moiety in ribosomal isolates. No differences in the proportions of these aflatoxin B1-DNA adduct species were found in ribosomal DNA as compared to total nuclear DNA, and we conclude that the preferential formation and removal of aflatoxin B1-DNA moieties within ribosomal DNA is not associated with a pattern of adducts qualitatively different from that in total nuclear DNA.

INTRODUCTION

Investigations into mechanisms underlying chemically induced carcinogenesis and mutation have focused attention on alterations of DNA structure and gene expression elicited by carcinogenic and mutagenic compounds. Identification of adduct moieties resulting from carcinogen-DNA interactions has permitted not only kinetic studies of DNA adduct appearance and removal within cells, but also mechanistic studies seeking to relate specific DNA adduct patterns with tumor development in target tissues. Relationships have been established between target organ specificity and concomitant sensitivity of target organ DNA to carcinogen modification and damage (1, 2), and also between persistence of specific DNA adduct lesions in target organs and the ultimate appearance of tumors in these organ sites (3).

Many factors are known to affect the kinds and amounts of DNA adducts formed when cells are exposed to carcinogens. For example, enzymatic competence for carcinogen activation is an important determinant of adduct localization within specific tissues and cell types under in vivo conditions (4). Further, adduct persistence can be strongly affected by DNA repair capacities of specific cell types (5). Under in vitro conditions, neighboring base composition can influence the reactivity of specific nucleophilic centers with respect to distribution of carcinogen adducts within defined nucleotide sequences (6, 7).

The organization and structure of chromatin also modulates the susceptibility of DNA to carcinogen modification and damage. Morphological studies suggest that transcribed gene sequences are maintained in a discrete, open conformation distinguishable from nontranscribed DNA (8, 9). Biochemical investigations, in which transcribed regions undergo selective digestion by DNase I or II, further support the ultrastructural observations (10, 11).

We previously reported preferential susceptibility of specific gene sequences, the RNA genes of rat liver, to modification by AFB1 (12). Over an 8-fold dose range, (0.25 to 2.0 mg AFB1/kg), rDNA contained 4 to 5 times more AFB1 residues than did nuclear DNA, indicating that rDNA is preferentially accessible to carcinogen modification in vivo. This preferential binding was not attributable to enrichment of these sequences in guanine, the specific site of AFB1 addition, suggesting that rDNA regions have increased accessibility to carcinogen modification, possibly due to the diffuse conformation maintained within transcribed DNA regions.

With the development of this experimental system, which permits simultaneous investigation of AFB1-DNA adduct formation within genes, as well as the resulting functional impairment in those same gene sequences, we have addressed further questions concerning molecular events underlying AFB1-rDNA adduct removal and repair. The major AFB1-DNA adduct formed in vivo and in vitro, AFB1-N7-guamine, is chemically unstable, leading not only to spontaneous depurination with the creation of apurinic sites, but also to base-catalyzed hydrolysis of the guanine ring forming 2 pyrimidine derivatives at the N7 adduct (AFB1-FAPyr as well as a second, as yet structurally uncharacterized AFB1-guanine derivative termed AFB1-Peak F). These interactions are shown in Chart 1. Recent studies have demonstrated that the rate of chemical depurination of the N7-guamine adduct moiety is markedly affected by initial binding levels; since depurination rate was increased in heavily adducted DNA, the localization of AFB1 modification within rDNA could permit greater access of DNA repair proc-

1 Financial support for this work was provided by Grants 5-T32-E507020 and 5-P01-ES00597 from NIH.
2 Present address: Laboratory of Toxicology, Department of Veterinary Anatomy, Texas A & M University, College Station, TX 77843.
3 To whom requests for reprints should be addressed. Received 12/26/84; revised 4/19/85; accepted 4/24/85.

The abbreviations used are: AFB, aflatoxin B1; AFB-FAPyr, aflatoxin B1-formamidopyrimidine; AFB-N7-guamine, 2,3-dihydro-3-hydroxy(N7-guanyl)aflatoxin B1; rRNA, ribosomal DNA; HPLC, high-pressure liquid chromatography.
ess to modified gene sequences. Qualitative or quantitative differences might therefore exist in the AFB1:DNA adduct populations of transcribed and nontranscribed DNA. It was the objective of the work reported here to obtain data concerning this.

We studied the time course of AFB1:DNA adduct formation and removal within DNA gene sequences isolated from liver nuclei of AFB1-treated animals. The results show that AFB1:DNA residues are preferentially formed and removed from rDNA as compared to unfractionated nuclear DNA.

### MATERIALS AND METHODS

**Chemicals.** AFB1 was obtained from Makor Biochemicals, Ltd. (Jerusalem, Israel). [3H]AFB1 (14 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Ultrapure sucrose (DNase and RNase free) and cesium chloride were obtained from BRL, Inc. (Gaithersburg, MD). Tris buffer, cesium sulfate (Grade 1), and ribonuclease A (R-4875) were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware used in hybridization steps was washed with 0.01% diethyl pyrocarbonate and autoclaved for 4 h to minimize endogenous nuclease activity.

**Animals.** Male Fischer rats were obtained as weanlings from Charles River Breeding Laboratories (Wilmington, MA). They were housed in pairs in suspended, wire-bottom cages and fed Ziegler rodent chow and water ad libitum.

**Isolation of rDNA.** The experimental procedures used for rDNA isolation have been previously described in detail (12) and can be summarized as follows.

Male Fischer rats (125 to 150 g) were given injections of 1 mg [3H]AFB1/kg in 0.05 ml of dimethyl sulfoxide and were killed 2 to 12 h later. After perfusion in situ with buffer [25 mM Tris-HCl (pH 6.9); 0.25 M sucrose; 3.3 mM CaCl2], the livers were minced and homogenized in the above buffer plus 1% Triton X-100 (v/v). A crude DNA preparation, isolated from the liver nuclear fraction via modification of the phenol extraction method of Marmur (14), was sheared by homogenization and subjected to cesium salt density centrifugation, yielding a final nuclear DNA fraction relatively free of RNA and protein contamination. Each gradient was fractionated while monitoring 254 nm absorbance, and DNA fractions of maximum density were retained. These fractions, which accounted on average for approximately 10% of the total fractionated DNA, were briefly dialyzed, and the DNA after precipitation in cold ethanol was subsequently solubilized in 0.11 M glycine:NaOH (pH 10.5). After incubation for 30 min at 37°C, the alkali-treated DNA was again recovered by ethanol precipitation. Samples of the DNA collected before and after glycine treatment were analyzed to quantify the loss of aflatoxin-bound radioactivity due primarily to tritium exchange during base treatment. AFB1 tritium bound to DNA was determined by liquid scintillation. DNA concentrations were quantified by the diphenylamine colorimetric reaction as modified by Giles and Myers (15).

rDNA sequences were selectively isolated from these nuclear DNA fractions by hybridization with rRNA using methods adapted from those of Wellauer and Dawid (16, 17). DNA isolates were incubated in 70% formamide buffer at a concentration of 0.015 mg/ml; 18S and 28S rRNA were added to a final concentration of 0.015 mg/ml for each RNA species, and the nucleic acid mixture was incubated at 50°C for 3 h. The hybridization mixture was chilled, dialyzed, and subjected to 2 successive CsCl gradient centrifugations. The rDNA fractions, identified by filter hybridization of cesium chloride aliquots with [3H]RNA, were pooled and digested with ribonuclease A to remove DNA-bound rRNA. Samples of the rDNA isolate were taken for DNA quantitation (via UV absorbance at 280 nm and by colorimetry) and DNA-bound radioactivity determination (as described above).

Analysis of rDNA Adduct Populations. rDNA isolates were made 0.1 N in HCl by the addition of 1 N HCl, heated at 90°C for 15 min, and cooled on ice. Potassium acetate and ZnCl2 were subsequently added to final concentrations of 50 mM and 0.1 mM, respectively, and the deproteinized, denatured DNA was digested for 3 h at 37°C with nuclease P1 (Sigma Chemical Co.: 1 mg enzyme protein/50 mg DNA). The DNA hydrolysate was made 5% in ethanol and injected onto a C18/Bondapak column (Waters Associates) eluted at ambient temperature with a 45-min linear gradient (flow rate, 1.0 ml/min) 13 to 18% ethanol adjusted to a pH of 5.0 by the addition of formic acid.

As summarized in Chart 2, a second set of nuclear DNA preparations, enriched in rDNA sequences by one round of cesium salt density centrifugation, was incubated in 20 mM sodium phosphate:1 mM Na2 EDTA (pH 6.8) at 85-88°C for 20 min at a DNA concentration of 0.6 to 0.7 mg/ml. As originally described by Lawley and Brooks (18), heat treatment of N7-substituted guanines causes depurination under near neutral conditions; after heat treatment of AFB1-adducted DNA, only AFB1-FAPY adduct derivatives (formed by scission of the N7, C8, N9 ring of AFB1-substituted guanines) should remain. The neutral buffer-treated DNA preparations were subsequently hybridized to rRNA; the resulting DNA isolates were depurinated via mild acid treatment, hydrolyzed with nuclease P1, and analyzed by HPLC, as described above.

### RESULTS

Chart 3 compares the levels of adduction within ribosomal DNA and unfractionated nuclear DNA over a 12-h period after treatment with AFB1. rDNA contained more AFB1 residues per mg DNA than did total nuclear DNA over the entire period; 2 h after toxin administration, 4.8-fold more AFB1 residues were present per mg DNA than did total nuclear DNA over the entire period; 2 h after toxin administration, 4.8-fold more AFB1 residues were bound per mg DNA to rDNA than to nuclear DNA, and this ratio was similar (4.1) at 12 h postdosing.

As is evident from the slopes of the 2 curves in Chart 3, the rates of AFB1 adduct removal differ between ribosomal and...
AFB2-DNA ADDUCTS IN RAT LIVER rDNA

[1H]-AFB2 – Rot

Isolate Liver Nuclear DNA
Enriched in Ribosomal DNA by Cesium Salt Density

Heat DNA isolate in 20 mM Sodium Phosphate,
1 mM EDTA, pH 6.8
For 20 Minutes at 85–88°C.

Isolate Ribosomal DNA
Determine Amount of Bound AFB2, FAPyr and AFB2, Peak F

\[
\begin{align*}
\text{Quantity of AFB2,–FAPyr} & \quad \text{Quantity of AFB2,–Peak F} \\
\text{AFB2, in Alkali-Treated Ribosomal DNA} & \quad \text{AFB2, in Hot Buffer-Treated Ribosomal DNA}
\end{align*}
\]

= Quantity of AFB2,–N7–Guanine

Chart 2. Protocol to quantify AFB2,–N7–guanine adducts in rRNA gene sequences isolated from the liver nuclear DNA of AFB2,–treated animals.

nuclear DNA fractions. Over the 12-h period of observation, the half-life of bound AFB2 residues within nuclear DNA was calculated to be 11 h, while that of adducts within rDNA was 6 h. The AFB2,–N7–guanine moiety is quite stable in vitro with a half-life of approximately 100 h (13); this observed decrease in the in vivo as compared to the in vitro half-life of AFB2, adducts (11 as compared to 100 h, respectively) suggests an involvement of enzymatic activity in the removal of these adduct species from DNA.

Further examination of the data in Chart 3, in terms of the rate of adduct removal, reveals marked preferential removal of AFB2,–DNA moieties from within rRNA gene sequences. Table 1 summarizes the rates of removal of bound AFB2, moieties from ribosomal and unfractionated liver nuclear DNA during the time intervals between points of observation. From 2 to 12 h after AFB2, administration, the rate of removal of AFB2, adducts from rDNA was 2 to 8 times greater than the comparable value for unfractionated nuclear DNA. These findings indicate that rDNA sequences are not only preferentially susceptible to structural modification and damage by AFB2, but also show enhanced response to those processes leading to removal of AFB2, adduct moieties, as compared to the nuclear genome as a whole.

The preceding experiments were designed to provide quantitative data on total AFB2,–binding levels. It was also of interest to determine the qualitative character of the adduct profiles present in each of the DNA fractions for which quantitative data were collected. However, a different experimental strategy was required for this purpose. In the quantitation experiments, total AFB2, residues were initially stabilized by base-catalyzed conversion to formamidopyrimidine derivatives (Chart 1). This treatment, however, precluded detection of the parent N7–guanine adduct. The procedure used to estimate the amounts of the 3 major AFB2, adduct species present in ribosomal DNA fractions was in principle to quantify in each sample the total AFB2,–bound radioactivity that could be liberated by heat-induced depurination under neutral pH conditions as an index of the amount of the AFB2,–N7–guanine adduct present. The residual, stable radioactivity would thus represent AFB2,–FAPyr and AFB2,–Peak F adducts (see Chart 2).

In order to validate this approach, rat liver DNA was adducted with AFB2, to a level to 1550 pmol AFB2,–/mg DNA (equivalent to 530 AFB2, adducts/106 nucleotides) by incubation in vitro with phenobarbital-induced rat liver microsomes (19). Aliquots of this adducted DNA were heat treated, and these as well as untreated samples were hydrolyzed and subjected to HPLC analysis. In addition, to facilitate quantification of the minor AFB2,–FAPyr and AFB2,–Peak F adducts, base-treated AFB2,–adducted DNA was added to untreated DNA similarly adducted to a level of 50% prior to hydrolysis. The HPLC chromatogram of the hydrolysis products is shown in Chart 4 (left). Heating aliquots of this DNA mixture under the neutral pH conditions described earlier resulted in disappearance of more than 99% of the AFB2,–N7–guanine moieties through depurination, while levels of the stable AFB2,–DNA adducts were unaffected (Chart 4, right).

## Table 1

<table>
<thead>
<tr>
<th>Time interval (h)</th>
<th>rDNA</th>
<th>Nuclear DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–6</td>
<td>118</td>
<td>14</td>
</tr>
<tr>
<td>6–9</td>
<td>98</td>
<td>12</td>
</tr>
<tr>
<td>9–12</td>
<td>43</td>
<td>20</td>
</tr>
<tr>
<td>Mean (2–12 h)</td>
<td>90</td>
<td>15</td>
</tr>
</tbody>
</table>

Chart 3. AFB2, adduction of ribosomal DNA and total nuclear DNA isolated from animals administered 1 mg [3H]AFB2/kg body weight and sacrificed 2 to 12 h after dosing. Points, average of 3 experiments; bars, SE.
AFB₁-DNA ADDUCTS IN RAT LIVER rDNA

Chart 4. Chromatographic analysis of AFB₁-adducted DNA. Rat liver DNA, adducted in vitro with AFB₁, was treated with alkaline glycine buffer, as described in the text. A 50:50 mixture of base-treated and untreated DNA was made and subsequently treated with neutral phosphate buffer as outlined in Chart 2. Left, chromatogram of 50:50 mixture of untreated and base-treated AFB₁-adducted DNA. Right, chromatogram of the same 50:50 AFB₁-adducted DNA mixture subsequently heated to 85–88°C in 20 mM sodium phosphate:1 mM EDTA (pH 6.8) for 20 min.

Table 2
Quantitation of acid hydrolysis products of AFB₁-modified ribosomal and nuclear DNA at various times after dosing

Concentrations of aflatoxin derivatives hydrolyzed from AFB₁-modified DNA fractions isolated from rat liver 2 to 12 h after administration of AFB₁ (1 mg/kg). In parallel studies using tritium and carbon-14 labeled AFB₁-adducted DNA, it was found that 12% of the AFB₁-bound tritium was liberated from AFB₁:FAPyr and AFB₁:Peak F under these conditions. The levels of bound AFB₁ radioactivity in rDNA prepared from nuclear DNA were thus adjusted to reflect this heat-induced tritium loss.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>AFB₁:N²-guanine</th>
<th>AFB₁:FAPyr</th>
<th>AFB₁:Peak F</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nDNA</td>
<td>rDNA</td>
<td>nDNA</td>
<td>rDNA</td>
</tr>
<tr>
<td>2</td>
<td>251 (87)</td>
<td>1030 (85)</td>
<td>28 (10)</td>
<td>110 (9)</td>
</tr>
<tr>
<td>6</td>
<td>169 (74)</td>
<td>550 (69)</td>
<td>44 (19)</td>
<td>190 (23)</td>
</tr>
<tr>
<td>12</td>
<td>88 (56)</td>
<td>330 (55)</td>
<td>55 (35)</td>
<td>200 (34)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentages of the hydrolysis products represented by each adduct peak.

This heat-induced depurination protocol was used to estimate levels of the 3 principal AFB₁-DNA adducts in ribosomal and nuclear DNA samples isolated at intervals during a 12-h period following aflatoxin dosing as described earlier. The results are presented in Table 2. After 2 h, AFB₁:N²-guanine was the major adduct in nuclear DNA, comprising 87% of the bound AFB₁ radioactivity. The relative proportion of this adduct decreased with time, and after 12 h represented only 56% of the total. Over the same period, the stable AFB₁:FAPyr derivatives increased proportionally, as the total bound adduct levels decreased from 288 to 158 pmol/mg DNA. Although the total adduct levels in rDNA were elevated by a factor of 3 or 4 over those in nuclear DNA, there was no difference in the distribution of individual adducts or in the rate at which they were removed.

DISCUSSION

Differential rates of carcinogen-DNA adduct formation and removal have been described within a specific, expressed region of the nuclear genome. The data presented in this report demonstrate preferential formation and removal of AFB₁-DNA residues within rRNA gene sequences, in the absence of qualitative differences in AFB₁:rDNA adduct populations, as compared to nuclear DNA.
These findings emphasize the importance of chromatin structure and conformation as a determinant in the localization of putative precarcinogenic and premutagenic lesions within carcinogen-treated cell populations. Recent investigations probing structural bases for gene activation and expression have established clear relationships between the transcriptional activity within expressed gene sequences and both the presence of specific chromatin proteins and DNase sensitivity of the respective DNA regions (20-22). Extension of these observations in describing structural associations with localized chemical-DNA damage further suggests important roles for chromatin ultrastructure and morphology in determining carcinogen-DNA adduct formation and repair. The eucaryotic genome is organized into basic subunits consisting of DNA periodically folded around a histone core, creating series of DNA-protein particles (nucleosomes) connected by lengths of DNase-sensitive DNA (linker DNA). The relative accessibility of nucleosome core versus linker DNA to DNase digestion has been shown to correlate well with the susceptibility of these DNA regions to carcinogen modifications. Metzger et al. (23) monitoring the release of micrococcal nuclease-soluble radioactivity from the liver nuclei of [3H]acetaminofluorene-treated animals, found that more than 1.5-fold greater levels of adduct residues within linker than in core DNA; Ramanathan et al. (24), using a similar approach studying the release of radioactivity from the liver nuclei of [3H]dimethylnitrosamine-treated animals by DNase I, found 2.6-fold greater levels of methylation in linker as compared to core DNA. Similar studies monitoring [3H]AFB1 distribution within core and linker DNA isolated from hepatic tissue have shown preferential linker DNA modification in the rainbow trout (25) and the rat.6

Investigations examining the importance of chromatin structure in the localization of carcinogen adduct removal have additionally provided direct evidence for the role of DNA-protein interactions in the distribution of DNA repair. Lieberman and coworkers have evaluated the relationship between the formation of carcinogen-DNA moieties and the subsequent distribution of DNA repair-incorporated nucleotides within DNase-sensitive (linker) and -resistant (core) regions of chromatin. Pulse chase and continuous label experiments indicated that, in cells treated with acetaminofluorene (26) or 7-bromomethylbenz(a)anthracene (27), a rapid and extensive redistribution of repair-incorporated nucleotides occurs creating with time a more uniform nucleotide distribution with respect to DNase sensitivity. This time-dependent redistribution of DNA repair products within chromatin was found to be associated with the rearrangement of histones along DNA. These observations concerning chromatin ultrastructure suggest that following the onset of carcinogen-induced DNA repair, nontranscribed regions of chromatin would become increasingly susceptible with time to DNA repair processes. Examination of the rates of AFB1-DNA adduct removal from rDNA versus nuclear DNA given in Table 1 support this hypothesis. During the first 6 h subsequent to AFB1 administration, AFB1-DNA residues were found to be removed at an 8-fold greater rate from rDNA as compared to unfractonated nuclear DNA, however, 9 to 12 h postdosing, the ratio of AFB1-DNA adduct removal from rDNA versus nuclear DNA decreased to 2. Thus AFB1-induced DNA repair synthesis, initially localized within transcribed DNA regions, might extend to non-transcribed regions as repair-induced chromatin conformational changes increase the accessibility of AFB1-DNA adducts within these regions to enzymatic repair activity.

Left unresolved in these and other studies of AFB1-DNA interactions in vivo is the quantitation of specific adduct removal and repair processes operative in AFB1-treated cell populations. Spontaneous removal of AFB1-N7-guanine adducts should give rise to apurinic sites at a high frequency; the dose-responsive relationship established between liver DNA adduction and the excretion of AFB1-N7-guanine in the urine of AFB1-treated animals further supports this mechanism of AFB1-DNA adduct loss (28). Separation of AFB1-modified DNA through sucrose density gradient centrifugation under alkaline conditions, however, did not reveal the presence of persistent alkali-labile apurinic sites or single-strand breaks (29). Thus the extensive loss of AFB1-DNA lesions through depurination appears to be accompanied by cellular processes which efficiently repair AFB1-induced apurinic lesions.

Also left unresolved is a quantitative assessment of the role of enriched guanine content in both the preferential formation and removal of bound AFB1 residues from within rDNA. rDNA isolates prepared as described are 25% enriched in guanine residues compared to unfractionated nuclear DNA (12). While the more open, diffuse conformation of transcribed genes may be an important determinant in the preferential modification of rDNA by AFB1, the relative contributions of chromatin conformation and guanine content remain undefined. In addition, given the observed dependence of in vitro rates of spontaneous depurination and subsequent loss of AFB1-N7-guanine upon initial levels of AFB1 adduction, the preferential loss of AFB1-DNA adducts could result not only from preferential enzymatic removal of these adduct moieties, but also from increased AFB1 adduct destabilization as a result of the localized high concentration of adducts within the guanine-enriched areas of rDNA. We are therefore attempting to define AFB1-DNA interactions within DNA modified in vitro to assess more accurately the role of guanine enrichment as a determinant in the localized formation and loss of AFB1 adducts within these specific, expressed gene regions.

REFERENCES


Quantitative and Qualitative Characterization of Aflatoxin B₁ Adducts Formed in Vivo within the Ribosomal RNA Genes of Rat Liver DNA

T. Rick Irvin and Gerald N. Wogan