Quantitation of aflatoxin B₁ adduction within the ribosomal RNA gene sequences of rat liver DNA
(carcinogen–nucleic acid interactions/DNA-RNA hybridization)

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ABSTRACT The in vivo formation of covalent aflatoxin B₁ (AFB₁)-DNA adducts within the rRNA gene sequences of nuclear DNA has been studied in AFB₁-treated rats. Liver nuclear DNA, enriched in ribosomal DNA (rDNA) by one round of cesium salt density gradient centrifugation, was treated under buffered alkaline conditions to convert unstable AFB₁-N₂-guanine adducts to stable AFB₁-formamidopyrimidine derivatives. The alkali-treated DNA was hybridized to 18S and 28S rRNA in 70% formamide buffer to form rRNA-DNA hybrids. These hybrids were separated from the bulk of nuclear DNA by two rounds of centrifugation in CsCl, and the level of AFB₁ adduction to rDNA versus total nuclear DNA was compared as a function of dose 2 hr after AFB₁ administration. Over an 8-fold dose range (0.25–2.0 mg of AFB₁ per kg of body weight), rDNA contained 4- to 5-fold more AFB₁ residues than nuclear DNA, indicating that rDNA is preferentially accessible to carcinogen modification in vivo. While aflatoxin B₁ forms adducts with DNA principally at guanine residues, the guanine enrichment of rDNA was insufficient to explain the magnitude of observed preferential AFB₁ modification of rDNA. These results support the hypothesis that rDNA regions are preferentially accessible to carcinogen modification because of the diffuse conformation maintained within transcribed genes. This experimental approach permits the quantitative description of carcinogen modification within a defined gene sequence; further refinement of this approach may be useful in defining the precise relationships between covalent chemical–DNA interactions and the alterations in gene expression that result.

The irreversibility of tumor initiation, as well as the heritability of the tumor phenotype, have drawn attention to DNA as the critical macromolecular target in chemical carcinogenesis (1, 2). Changes in DNA sequences, brought about by carcinogen–DNA interactions, could constitute molecular bases for observed alterations in gene expression that accompany neoplastic transformation by chemicals (3, 4). Evidence supporting a putative role of carcinogen-induced DNA sequence change in initiating tumor development has been provided by both qualitative and quantitative correlations between the carcinogenic and mutagenic potency exhibited by many carcinogens and the potency of chemicals as carcinogens compared to the total levels of covalent DNA modification observed in treated animals (5–9).

Elucidation of the molecular bases for mutation and tumor initiation by carcinogens will require detailed knowledge of DNA modification within specific base sequences that can be related to alterations in gene function. Earlier experiments with this objective have included attempts to relate mutation frequency with DNA adduct formation by mutagens/carcinogens such as benzo[a]pyrene (10) and aflatoxin B₁ (AFB₁) (11, 12). While this approach has provided quantitative data on apparent mutagenic efficiency of various DNA adducts, structural modifications could not be localized within the marker genes in which mutation was scored. Furthermore, this quantitative approach assumes that DNA–carcinogen adducts are randomly distributed. Recent studies have indicated the susceptibility to carcinogen modification is not random but is affected by factors such as transcriptional activity, specific neighboring base composition, and nucleotide sequence (13).

We have conducted experiments to investigate the structural and functional impairment of a specific gene sequence, the rDNA gene, by the hepatocarcinogen AFB₁. Our experimental design permits simultaneous investigation of chemical damage and repair within an expressed gene as well as the resulting functional impairment in those same gene sequences. AFB₁ was used in these studies because products of its reaction with DNA have been thoroughly characterized, and the only known site of reaction is at the N₂ position of guanine (14–16). Furthermore, extensive information about AFB₁-DNA adduct stability and removal has facilitated development of hybridization technology permitting isolation of AFB₁-adducted gene sequences and quantitation of aflatoxin residues within them.

rDNA, the DNA sequences coding for the 45S precursor to 18S and 28S rRNA, was investigated for two reasons. First, these genes are present in approximately 300 copies per cell in the rat, and the presence of multiple copies makes it feasible to isolate sufficient rDNA for quantitative adduct analysis. Second, rRNA synthesis is preferentially inhibited (as compared to the synthesis of mRNA and tRNA) in AFB₁-treated animals, exclusively to the impairment of rDNA template function (17). These biochemical findings suggest that the rDNA genes are preferential targets for AFB₁ modification and thus appropriate model gene sequences with which to study localized carcinogen modification.

In this report, we describe the isolation of rDNA from the liver of AFB₁-treated animals and the determination of levels of adduction within rDNA as well as in total nuclear DNA after single doses of AFB₁ (0.25–2.0 mg/kg). The results indicate that rDNA, a region of actively transcribed sequences within the nuclear genome, is preferentially susceptible to carcinogen modification in vivo.

MATERIALS AND METHODS

The experimental strategy employed for rDNA isolation is summarized in Fig. 1. Pertinent details concerning the procedures used can be summarized as follows:

Male Fischer rats (125–160 g), obtained from Charles River Breeding Laboratories, were injected with [³H]AFB₁ at 1

Abbreviations: AFB₁, aflatoxin B₁; AFB₁-N₂-guanine, 2,3-dihydro-2-(N₂-guanyl)-3-hydroxyaflatoxin B₁; AFB₁-PAPyr, 2,3-dihydro-2-(N₂-pyrimidyl)-2',5',6'-trimino-4'-oxo-N₂-pyrimidyl-3-hydroxyaflatoxin B₁; rDNA, ribosomal DNA; TK, thymidine kinase.

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mg/kg (AFB1: Makor, Jerusalem, Israel; [3H]AFB1: Moravek Biochemicals, Brea, CA) in 50 μl of dimethyl sulfoxide and decapitated 2 hr after dosing. Their livers were perfused in situ, and purified nuclei were prepared by a modification of the Blobel-Potter method (18) with all procedures being performed at 0–4°C. Nucleic acids were isolated from the resultant crude nuclear preparation by phenol extraction essentially as described by Marmur (19). The nuclear DNA isolate was resuspended in H buffer (50 mM Hepes, pH 7.0/5 mM Na2EDTA) at a 0.5 mg/ml and sheared by 15 strokes in a Teflon-glass homogenizer. Sodium sarcosinate was added to 1%, and the mixture was again homogenized. To each vol of homogenized nuclei, 7 vol of 6 M CsCl, 0.8 vol of 3 M Cs2SO4, and 0.25 vol of H buffer were added. The samples were transferred to Beckman 38-m1 quick-seal tubes, overlaid with mineral oil, and centrifuged at 167,000 × g in a Beckman VTI 50 rotor for 10.5–12.5 hr at 20°C. Each cesium gradient was fractionated while monitoring absorbance at 254 nm, and the DNA fractions of greatest cesium salt density (accounting for approximately 10% of the total DNA loaded onto each gradient) were retained.

Nuclear DNA fractions enriched in rDNA as described above were briefly dialyzed, precipitated with ethanol, and solubilized in 0.11 M glycine/NaOH, pH 10.5, at a DNA concentration of 0.7–0.9 mg/ml. After incubation for 30 min at 37°C, the solution was made 0.3 M in NaCl, and the DNA was recovered by ethanol precipitation. Samples of the DNA fraction were taken before and after glycine treatment to determine the loss of aflatoxin-bound radioactivity (due mainly to tritium exchange) during base treatment. AFB1-bound tritium was determined by liquid scintillation counting, and DNA was quantified by the diphenylamine colorimetric reaction as modified by Giles and Myers (20).

rDNA sequences were isolated from nuclear DNA via hybridization with rRNA according to procedures adapted from the methods of Wellauer and Dawid (21, 22). The alkali-treated nuclear DNA pellet was washed twice with 70% ethanol/0.3 M NaCl (9:1, vol/vol) and dissolved in formamide hybridization buffer [70% formamide (vol/vol)/0.3 M NaCl/10 mM Tris-HCl, pH 7.2/2.1 mM Na3EDTA] at a DNA concentration of 15 μg/ml. The 18S and 28S rRNA hybridization probes were prepared by layering total liver RNA onto 10–40% detergent sucrose gradients and chromatographing the 18S and 28S peaks on an oligo(dT)-cellulose column as described by Aviv and Leder (23). Purified 18S and 28S rRNA were added to the nuclear DNA solution at a final concentration of 15 μg/ml for each RNA species. The nucleic acid mixture was transferred to ampoules, incubated at 50°C for 3 hr, cooled to 4°C, and finally dialyzed against TE buffer (10 mM Tris-HCl, pH 7.2/2.5 mM Na3EDTA). Cesium chloride was added to a density of 1.71 g/ml, and the DNA–rRNA mixture was centrifuged as described above. DNA peak fractions of maximum density, comprising approximately 20–25% of the DNA band, were readdusted to a density of 1.72 g/ml with CsCl and recentrifuged. The rDNA–rRNA hybrids were located on the gradient by filter hybridization to 32P end-labeled 18S and 28S rRNA as described by Mertz and Gurdon (24).

The rDNA hybrid fractions were pooled, dialyzed against TE buffer, and digested for 30 min at 44°C with heat-treated RNase A (Sigma) added to give 20 μg/ml. The RNase digest was extracted with an equal volume of phenol, and the rDNA was recovered from the aqueous layer by ethanol precipitation. The purity of rDNA isolates was tested by digesting samples with EcoRI restriction endonuclease (Bethesda Research Laboratories), electrophoretically separating the digest on a 1% agarose gel stained with ethidium bromide. The gel contents were also transferred to a nitrocellulose filter and hybridized with [32P]rRNA according to the Southern method (25).

RESULTS

Isolation of AFB1-Adducted rDNA. Employing these procedures, we have purified a rDNA from the liver nuclear DNA of animals administered single doses of AFB1. Total nuclear DNA was sheared and banded in cesium chloride/cesium sulfate density gradients, which served two purposes: DNA purification via removal of RNA and protein and enrichment of rDNA genes, which have a higher guanine and cytosine content than does nuclear DNA and therefore band at a higher density on cesium salt gradients. Consistent with previous reports, rDNA sequences were found to band in the lower (greater density) side of the nuclear DNA peak. A fraction enriched in rDNA gene sequences and suitable for hybridization to rRNA could thus be selectively separated from nuclear DNA.

Adduct Stabilization. The predominant adduct formed initially in DNA modified by AFB1, either in vitro or in vivo is 2,3-dihydro-2-(N'-guanyl)-3-hydroxy aflatoxin B1 (AFB1-N'-guanine) (14–16). This adduct is chemically unstable, a characteristic shared by all N'-modified guanines, and readily undergoes spontaneous depurination. To enable quantification of AFB1 adduction to rDNA, it was necessary to convert AFB1-32P-guanine adducts to a stable form that would survive the rDNA hybridization steps. AFB1-modified nuclear DNA was therefore treated with alkali; hydroxide attacks at the C8 of guanine, breaking the C8–N7 bond and neutralizing the destabilizing positive charge generated on the N7→C8=N7 bonds (17, 26). Two new adduct forms are produced. The quantitatively major adduct has been structurally identified as 2,3-dihydro-2-(N'-formyl-2',5',6'-triami-
no-4'-oxo- N⁷-pyrimidyl)-3-hydroxyaflatoxin B₁ (AFB₁-FAPyr). The second adduct, structurally related to AFB₁-FAPyr, has been termed "AFB₁-Peak F.

Fig. 2 illustrates the conversion of AFB₁-N⁷-guanine to these two stable adduct forms. Chromatographic analysis was performed on glycine-treated and untreated DNA adducted in vitro to a level of 1,550 pmol of AFB₁ per mg of DNA (14). As shown in Fig. 2A, in vitro AFB₁-adducted DNA contains predominantly AFB₁-N⁷-guanine; after alkaline glycine treatment, AFB₁-N⁷-guanine is quantitatively converted to AFB₁-FAPyr and AFB₁-Peak F (Fig. 2B). Approximately 1.2% of the radioactivity as AFB₁-N⁷-guanine remains unconverted and is thus lost. In addition, exposure of [⁴H]AFB₁ to alkaline conditions causes loss of AFB₁-bound tritium radioactivity. An average of 18% (14-24%) of the bound radioactivity was lost from nuclear DNA samples during adduct stabilization under the alkaline glycine buffer conditions. Parallel studies in which DNA adducts with [¹⁴C]AFB₁ were similarly treated indicated that, of the average 18% tritium loss, 6% was due to hydrolysis of the bound AFB₁ moiety (either as bound AFB₁-guanine adducts or as the aflatoxin moiety itself); the remaining 12% of tritium radioactivity loss was thus due to tritium exchange. Samples of nuclear DNA were taken before and after treatment to determine the amount of tritium radioactivity retained in each sample set.

rDNA-rRNA Hybridization. The glycine-treated DNA isolates were incubated with rRNA to form rRNA-rDNA R-loop hybrids in formamide buffer as described by Wellauer and Dawid (21, 22). R-loop hybrids are more dense than double-stranded DNA and can thus be separated from single- and double-stranded DNA in cesium chloride gradients, the extent of the shift in density being dependent upon the ratio of RNA to DNA within the hybrid molecules. Fig. 3A illustrates the gradient profile of nuclear DNA, enriched in rDNA by one round of cesium salt centrifugation, hybridized with 18S and 28S rRNA under these conditions. While rRNA-rDNA hybrids banded at a higher density than did the bulk of nuclear DNA, nonribosomal DNA was largely denatured and formed a broad band that obscured the rDNA hybrid peak. A fraction comprising 20-25% of each DNA gradient peak was collected and recentrifuged after adjustment of the solution density to 1.72 g/ml. Fig. 3B illustrates a typical gradient profile after the second centrifugation; here, the rDNA-rRNA hybrid was more completely separated from the broad peak of single-stranded DNA. Over the course of these two cesium salt density gradient steps, an average of 15% of the AFB₁-bound tritium was lost from the rDNA fractions, and levels of bound AFB₁ radioactivity measured in rDNA were corrected to reflect this loss. Yields of the rDNA fraction were 1.7-3.2 g from 25-30 mg of rat liver DNA.

Analysis of the Purified rDNA. The purity of the rDNA obtained from the radioactive peak of Fig. 3B was assessed by digesting the DNA isolate with EcoRI restriction endonuclease, separating the hydrolysates via electrophoresis on a 1% agarose gel, and comparing the digestion pattern with published restriction maps of the rRNA gene region (Fig. 4A) as determined by Tantravahi and co-workers (27). As shown in Fig. 4B, EcoRI digestion of the rDNA isolate produced a digestion pattern comparable to the previously reported rDNA restriction map. Three major bands are seen with sizes of 11.4, 6.7, and 4.9 kb; each band is clear and distinct with little ethidium bromide-staining DNA observable in the background of the gel.

To obtain higher sensitivity, electrophoretically separated EcoRI digests of rDNA were transferred to a nitrocellulose filter by the Southern method, and the filter was then hybridized with purified ³²P-end-labeled 18S and 28S rRNA. As shown in Fig. 4C, the [³²P]rRNA probes bound only to DNA in the principal ethidium bromide-staining bands. No other rRNA-hybrid bands were evident within the background.

AFB₁ Adduction to Ribosomal Versus Nuclear DNA. We compared the levels of AFB₁ adduction within rDNA versus total nuclear DNA, both isolated from the liver tissue of AFB₁-treated animals.Fig. 5 summarizes the levels of AFB₁-
modification within these two DNA populations from animals injected with 0.25–2.0 mg of [3H]AFB1 per kg of body weight and sacrificed after 2 hr. Binding levels extended from 90 to 640 pmol of AFB1 per mg of DNA (30 to 240 AFB1 adducts per 10^7 nucleotides) in nuclear DNA and from 420 to 3,240 (150 to 1,200) in rDNA. AFB1 residues were found to occur preferentially within rDNA at each dose level studied; on a pmol/mg basis, 4.2- to 4.6-fold more AFB1 residues were bound to rDNA than to nuclear DNA at these doses. The increased guanine content characteristic of rDNA sequences could lead to a greater level of binding of carcinogens such as AFB1 that specifically modify guanine bases. High-pressure liquid chromatographic analysis of the base content of rDNA isolates showed them to be enriched by 25% in guanine compared to nuclear DNA (unpublished data). Thus the 4- to 5-fold greater adduction of rDNA versus nuclear DNA cannot be attributed simply to guanine enrichment.

**DISCUSSION**

We have established a dose–response relationship with respect to AFB1 adducts within rRNA genes isolated from the liver tissue of AFB1-treated animals. Further, the data presented in this report, in which AFB1 was shown to modify rRNA gene sequences preferentially, demonstrate the localization of carcinogen modification within specific, expressed gene sequences.

Recent investigations probing the molecular basis of tumor initiation by chemicals have focused attention on the qualitative and quantitative relationships between carcinogen-DNA adduction and carcinogen-induced mutation. Knowledge of the mutagenic efficiency of specific DNA adduct lesions would permit the construction of more accurate models relating structural DNA damage and altered gene expression. Bacterial assay systems have provided useful tools with which to approximate the mutagenic potency of carcinogen-DNA moieties. Stark and co-workers, measuring forward mutation to 8-azaguanine resistance in AFB1-treated Salmonella typhimurium cultures, found approximately 20 AFB1-DNA adducts in the bacterial genome per observed mutation (11), while Beranek and co-workers, measuring reverse mutation to histidine prototrophy in Salmonella, found approximately 6 aminofluorene DNA adducts in the bacterial genome per observed mutation (28). Similar methods using reverse mutation assay systems in Salmonella have been used to evaluate the mutagenic potential of DNA adducts produced by various carcinogens.

**Fig. 4.** Analysis of purity of rDNA. (Upper) EcoRI restriction endonuclease map for the rat rRNA gene region, taken from ref. 27. NTS, nontranscribed spacer; ETS, external transcribed spacer. (Lower) Electrophoretic separation of isolated rDNA digested with EcoRI. Lane 1, staining with ethidium bromide. Lane 2, autoradiograph of DNA, separated as shown in lane 1, transferred to a nitrocellulose filter by the Southern procedure (25), and hybridized with [32P]rRNA. Sizes of the EcoRI restriction fragments are given in kilobases (kb) as calculated from the parallel separation of an EcoRI digest of λ phage DNA.

**Fig. 5.** AFB1 adduction of rDNA and total nuclear DNA isolated from animals administered 0.25–2.0 mg of [3H]AFB1 per kg of body weight and sacrificed 2 hr after dosing. Each bar represents the mean ± SEM of three experiments.
used to determine the mutagenic efficiency of benzo(a)pyrene (29) and arylamines (13).

The approaches have inherent limitations because they have not localized DNA modification within the specific gene loci at which mutation is simultaneously scored. Recent findings by Kaden and co-workers on the relationship between AFB1-induced mutation and AFB1-DNA adduction in hypermutable human lymphoblast cell lines provide some preliminary data with which to assess, in eukaryotic cells, the importance of gene hypermodification in calculations of the mutagenic efficiency of carcinogen adducts (30). In these experiments, the efficiency of AFB1-DNA adducts giving rise to trifluorothyminidine resistance, due to mutation within the thymidine kinase (TK) gene, was calculated to be about 20%. Assuming the maintenance of a transcribed chromatin conformation within the TK gene similar to the conformation found within rDNA, it can be postulated that a 4- to 5-fold increase in AFB1 modification of the TK gene sequences would be observed; thus the actual mutagenic efficiency of an AFB1 adduct in this hypermutable cell population could be as high as 100%—i.e., one mutation produced, on average—for each AFB1 adduct within a given TK gene sequence. An observed ratio of 1:1 between carcino genesis modification and induced mutation within a specific gene target would have important implications in the interpretation of carcinogen and mutagen bioassay data on chemicals to which humans are exposed.

Left unresolved, however, in these and other mechanistic studies of AFB1-induced mutation is the identification of the specific premutagenic lesion(s) formed in AFB1-modified DNA. Spontaneous or enzymatic removal of the AFB1-N7-guanine adduct should give rise to apurinic sites at a high frequency. Loeb and co-workers have demonstrated that apurinic lesions in DNA result in mutations in defined prokaryotic systems (31). The coexistence of apurinic sites together with stable AFB1-FAPyr adducts represent two types of potentially premutagenic lesions in the DNA of AFB1-treated cell populations. The possibility thus exists that multiple mechanisms may thus account for the mutagenic potency of N2-substituted alkylating agents such as AFB1.

Other parameters of chromatin composition and structure may also modulate the levels of chemical modification within mutable gene loci. Humayun and co-workers have adapted the Maxam-Gilbert procedure for DNA sequence analysis to probe the influence of flanking nucleotide sequences on AFB1-DNA modification (32, 33). Within double-stranded DNA, identifiable guanine residues were found to be more efficiently modified by AFB1, according to rules determined from a knowledge of vicinal nucleotide sequences. While the transcribed conformation of an expressed gene sequence may thus direct preferential modification of these DNA regions upon carcinogen exposure, the nucleotide composition and sequence within a gene may further influence the observed relationships between carcinogen–DNA interactions and biological end points such as mutation.

Our findings justify further refinement of this experimental strategy to define more accurately relationships between carcinogen-DNA modification and the changes in gene expression accompanying chemically induced mutation and tumorigenesis. Preferential localization of carcinogen adducts within expressed gene sequences suggests that DNA perturbations of etiological importance to cell transformation and tumorigenesis may also occur to a greater extent in expressed gene loci. It may be possible to refine the approaches outlined in this report to examine the chemical modification of DNA regions within mutational targets and to describe the underlying mechanisms mediating premutagenic and premutagenic events induced by chemical–DNA interactions.

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