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MODIFICATION OF PROTEIN SYNTHETIC COMPONENTS BY AFLATOXIN B_1*

BEVERLY A. LYMAN,† LENKE ERKI, DIANE W. BIEDRZYCKA, THOMAS M. DEVLIN and JOHN J. CH'IH‡

Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, PA 19102-1192, U.S.A.

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Abstract—Molecular sites of perturbation by the hepatocarcinogen aflatoxin B_1 (AFB_1) in the protein synthesis initiation complex were assessed using isolated hepatocytes and a cell-free activating system containing microsomes and cytoplasmic ribonucleoprotein complexes (cRPC). Ribosomal proteins showed no detectable modification by the toxin in either system. With hepatocytes, initiation factors demonstrated only slight modification by AFB_1. RNAs from both hepatocytes and the cell-free system with microsomes and cRPC were modified, with poly(A)-containing RNA exhibiting at least a 5-fold higher modification than poly(A)-lacking RNA. The poly(A)-lacking RNAs were modified in the order 28S rRNA > 18S rRNA > 5–6S rRNA > 4S tRNA. Guanine was the target base of AFB_1, but only 10% of the AFB_1-GMP adducts were on guanines located in a poly(G) region. These results suggest that guanine modification in RNAs may be responsible for the observed inhibition of translational initiation by AFB_1 to a greater extent than modification of either ribosomal intrinsic or associated proteins.

Aflatoxin B_1 (AFB_1§), a product of *Aspergillus flavus*, requires microsomal activation to AFB_1-8,9-epoxide in order to bind cellular macromolecules [1, 2]. Binding of the epoxide to DNA, RNA and protein is known to contribute to the hepatocarcinogenesis caused by ingestion of AFB_1-contaminated compounds [3–5]. Much evidence has been presented that, among other cellular processes, AFB_1 inhibits protein synthesis in both intact animals and cells in culture [6–10]. Using isolated rat hepatocytes, we previously demonstrated that AFB_1 inhibits translational activity in a time- and dose-dependent fashion [11]. Mechanistically, results from this and other laboratories suggest that initiation of protein synthesis is the principal loci affected by AFB_1 in mammalian cells [8–11]. Since control of translation in eukaryotes resides primarily at the level of initiation [12], the subcellular components involved in the formation of the functional 80S monosome that may be perturbed by the toxin warrant investigation. These are the various RNAs, ribosomal proteins, and initiation factors. It was of interest to study the components to which AFB_1 binds in an attempt to elucidate the mechanism by which it alters the translational apparatus and inhibits protein synthesis. Although it is known that the degree of AFB_1 modification is DNA > RNA > protein [2, 13], quantitative studies of its binding to the various species of RNAs, ribosomal proteins, and initiation factors have until now been lacking. We have employed isolated hepatocytes and a cell-free activating system to examine both qualitative and quantitative covalent modification of polysomal components by [^{3}H]AFB_1. Some of the observations described in the present paper have been presented in a preliminary communication [14].

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‡ To whom reprint requests should be addressed.

§ Abbreviations: AFB_1, aflatoxin B_1; cRPC, cytoplasmic ribonucleoprotein complexes; eIF, eukaryotic initiation factor; mRNP, messenger ribonucleoprotein complex.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest purity available. Oligo(dT)-cellulose was from Collaborative Research, Waltham, MA. Phenol (redistilled nucleic acid grade) was from Bethesda Research Laboratories, Bethesda, MD. Collagenase, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol, heparin-Sepharose CL-6B, lithium chloride, QAE-Sephadex A-25, RNase T_1 and T_2, sodium dodecyl sulfate (SDS), Triton X-100, urea, and yeast RNA were from the Sigma Chemical Co., St. Louis, MO. Ultrapure sucrose (RNase-free) was from Schwarz-Mann, Orangeburg, NY. Acrylamide, ammonium persulfate, *N,N'*-methylene-bis-acrylamide, Coomassie brilliant blue, and *N,N,N',N'*-tetramethylethylenediamine were from Bio-Rad Laboratories, Richmond, CA. [^{3}H]AFB_1 (12–30 Ci/mmol) was from Moravek Biochemicals, Brea, CA. OCS and ACS-II scintillants and NCS tissue solubilizer were from the Amersham Corp., Arlington Heights, IL.

To avoid or destroy RNase activity, the following precautions were taken: all glassware was soaked in 1.0 N NaOH, rinsed with distilled water, and oven

baked. Reagents were autoclaved and gloves were worn at all times. When necessary, heparin (4 units/ml) or SDS (0.1%) was added to solutions.

Animals. Male Wistar rats (300 ± 25 g), maintained on a 12-hr light/dark cycle, were allowed water and Ziegler's rat chow (Gardner's, PA) *ad lib.* Hepatocytes were prepared according to the procedure of Berry and Friend [15] with modifications as previously described [16]. In experiments using livers, animals were anesthetized with Ketaset (16 mg/100 g body weight), and livers were perfused *in situ* with 0.1 M sodium phosphate buffer (pH 7.4), excised, and rinsed in perfusing buffer containing 0.25 M sucrose (SSP buffer).

Incubations. In experiments employing intact hepatocytes, cells (1 to 1.5 × 10^6 cells per ml) were incubated with a catalytic amount of [^{3}H]AFB_1, to achieve a final concentration of 14.5 nM, for 60 min at 37° under 95% O_2/5% CO_2 with gentle shaking. After incubation, aliquots of the cell suspension were removed for determination of cell viability and cellular content (total and bound) of [^{3}H]AFB_1; the remaining suspension was used to isolate various subcellular components.

In experiments investigating microsomally-activated [^{3}H]AFB_1 binding to RNA and protein, liver microsomes (25 mg) were incubated for 10 min with 5 mg cytoplasmic ribonucleoprotein complexes (cRPC) in the presence of $MgCl_2$ (2 mM), heparin (100 units), and an NADPH-generating system (glucose-6-phosphate, 3.4 mM; glucose-6-phosphate dehydrogenase, 50 units; $NADP^+$, 0.5 mM) in a final volume of 25 ml of 0.1 M sodium phosphate buffer (pH 7.4). After 10 min, [^{3}H]AFB_1 (5 μCi) was added and incubation continued for 60 min. Upon completion, 50 μl of incubation mixture was removed for determination of [^{3}H]AFB_1 content as previously described [17]. The remaining incubation mixture was used for RNA isolation.

Isolation of subcellular components. [^{3}H]AFB_1-treated hepatocytes (1 to 1.5 × 10^8 cells) were lysed and fractionated by differential centrifugation to obtain nuclei, microsomes, mitochondria, and cytosol [18]. In experiments utilizing cRPC, cells were lysed and fractionated to obtain nuclei, cRPC and cytosol as previously described [16]. Ribosomal subunits were separated from messenger ribonucleoprotein complex (mRNP) and nascent peptides by incubating cRPC with puromycin (0.5 mM), EDTA (40 mM) and KCl (500 mM) followed by density gradient centrifugation. Ribosomal proteins were isolated by first dissociating cRPC into subunits according to the procedure of Blobel and Sabatini [19]; then fractions corresponding to either 40S or 60S subunits were resuspended in 20 mM Tris–HCl (pH 7.5) containing sucrose (0.2 M), NH_4Cl (0.1 M), Mg^{2+}-acetate (5 mM), and dithiothreitol (1 mM). Ribosomal proteins were then extracted with 3 M LiCl/4 M urea [20], and separated by 10% polyacrylamide gels containing 0.1% SDS [21].

To isolate selective cellular fractions, liver homogenate was centrifuged twice at 9770 *g* for 15 min at 4°, discarding pellets each time. The resulting supernatant fraction was centrifuged at 105,000 *g* for 60 min at 4°; the pellet was resuspended in SSP buffer and then recentrifuged at 105,000 *g* for 60 min at 4°. The final microsomal pellet was resuspended in SSP buffer. Protein was determined by the procedure of Lowry *et al.* [22]. The cRPC from liver was isolated as previously described [16], and the concentration was estimated using the equivalence of 10 A_{260} = 1 mg/ml.

Isolation of protein factors. Eukaryotic initiation factors (eIFs) were isolated by modified procedures of Schreier and Staehelin [23] and Waldman *et al.* [24]. The modification included use of post-ribosomal supernatant as well as ribosomes as starting fractions for the extraction, since eIFs are found associated with ribosomes during active translation and free in the cytosol at all other times. Another modification was the inclusion of a heparin-Sepharose affinity column to selectively isolate initiation versus elongation or release factors. Crude ribosomal salt wash and salt-treated post-ribosomal supernatant were adjusted to 0.04 M Tris–HCl (pH 7.6) and then to 60% saturation with ammonium sulfate. The precipitates were resuspended in buffer A (10% glycerol, 0.02 M Tris–HCl, pH 7.6, 1.0 mM dithiothreitol, 0.2 mM EDTA) containing 0.12 M KCl, dialyzed, and applied to a heparin-Sepharose column (4 ml bed volume). The eIFs were eluted by buffer A containing 0.5 M KCl, dialyzed against buffer A containing 0.12 M KCl, and adjusted to 45% saturation with ammonium sulfate. The precipitated proteins, lacking some eIFs, were resuspended in buffer A containing 0.12 M KCl and dialyzed. The supernatant fraction was adjusted to 60% saturation, and precipitated proteins, comprising all the eIFs, were resuspended and dialyzed.

Isolation and separation of cytoplasmic RNA. Polysomal and cytosolic RNAs were extracted from cRPC and cytosol of [^{3}H]AFB_1-treated hepatocytes by phenol/chloroform. Total RNA from the cell-free activating system (containing [^{3}H]AFB_1, microsomes, and cRPC) was similarly extracted. Poly(A)-containing RNA, taken as mRNA, was obtained by oligo(dT)-cellulose chromatography [25]. Poly(A)-lacking RNAs were separated by centrifugation on a 5–20% sucrose gradient.

Hydrolysis of AFB_1-RNA adducts. RNA isolated from either hepatocytes or the cell-free microsomally-activated cRPC was digested with RNase T_1 or T_2 as follows. For RNase T_1 digestion, RNA was dissolved in 25 mM Tris–HCl (pH 8.0) and incubated with 200 units enzyme at 37° for 24 hr. For RNase T_2 digestion, RNA was dissolved in 50 mM potassium acetate (pH 4.5), incubated with 10 units enzyme at 37° for 24 hr, and then adjusted to neutrality with 20 mM sodium carbonate buffer (pH 9.7). The mononucleotides, 3′-GMP (T_1 digestion) or 3′-CMP, 3′-AMP, 3′-UMP, and 3′-GMP (T_2 digestion), were separated by QAE-Sephadex chromatography [26].

Radioactivity determination. Counts per min (cpm) were corrected for background and then converted to disintegrations per min (dpm). Samples containing TCA-insoluble radioactivity were solubilized in NCS before addition of OCS scintillant. ACS-II was added directly to aqueous samples. Gels containing the [^{3}H]AFB_1-protein adduct were sliced into 1.2 mm slices; each slice was solubilized with NCS:water (9:1) for 2 hr at 50° before addition of OCS.

RESULTS

Cytoplasmic ribonucleoprotein complex modification. To investigate the degree to which activated AFB_1 binds the cRPC, hepatocyte cRPC was isolated in the presence of Triton X-100. Out of a total of 2.7 pmol AFB_1 detectable, 2.2 pmol, or 81%, was covalently bound. To study where this association occurs, the cRPC were dissociated into nascent peptides and associated proteins, mRNP, and ribosomal subunits, and were separated by density gradient centrifugation (Fig. 1). The first absorbance peak at the top of the gradient containing peptidyl puromycin and other proteins, and the middle absorbance peak, containing mRNP, showed relatively low AFB_1 binding (<400 dpm/fraction respectively). High AFB_1 binding (>1200 dpm/fraction) was found in the third broad absorbance peak containing combined 40S and 60S ribosomal subunits. These results indicated that a component(s) of isolated ribosomes was modified by the toxin.

Association of aflatoxin B_1 with initiation factors. Whether ribosomes or post-ribosomal supernatant were used for the isolation of the initiation factors, the specific radioactivity of [^{3}H]AFB_1-protein at each stage of the isolation was similar (Table 1), which validates using either cellular fraction to obtain eIFs. The final purified fraction (45–60% ammonium sulfate) known to contain all eIFs had a specific radioactivity of 600 dpm/mg protein when obtained from ribosomes, and 663 dpm/mg protein when obtained from post-ribosomal supernatant. The decrease in specific activity as the purification of initiation factors progressed suggests that these proteins are not prime candidates for aflatoxin modification.

Lack of aflatoxin B_1 binding to ribosomal proteins. Because the highest level of [^{3}H]AFB_1 was detected in ribosomal subunits (Fig. 1), ribosomal proteins were investigated. Fractions corresponding to ribosomal subunits (Fig. 1) were pooled and dialyzed, and then proteins were separated by electrophoresis in the presence of SDS. Although at least twelve proteins of different M_r were distinguished, none contained appreciable levels of [^{3}H]AFB_1. A large amount of radioactivity was found at the top of the gel, suggesting that the toxin associated with a species greater than 220 kD that did not enter the gel (data not shown) and which could, because of its size and presence in ribosomes, be RNA. Furthermore, identical results were obtained with ribosomal subunits (40 and 60S) isolated from the cell-free activating system. Therefore, an alternate procedure

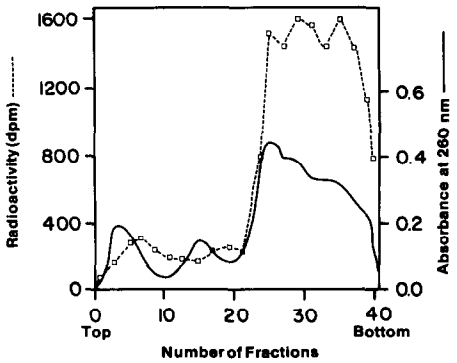

Fig. 1. Aflatoxin B_1 binding to ribosomal subunits. Ribosomal subunits were prepared by treatment of 10 A_{260} units of cRPC with 40 mM EDTA, 0.5 mM puromycin, and 500 mM KCl, separated on 10–30% sucrose gradients made in 50 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 500 mM KCl, and centrifuged at 79,000 *g* for 19.8 hr at 4°. Key: Absorbance at 260 nm (——); and TCA-insoluble radioactivity (—□—).

involving repeated extraction with lithium chloride and urea was performed, and the resulting ribosomal proteins from each subunit were separated by electrophoresis in the presence of SDS. As before, none of these ribosomal proteins contained radioactively labeled AFB_1 (data not shown).

The identical results obtained with various ribosomal protein isolation procedures suggested that ribosomal proteins are not covalently modified by AFB_1 in either hepatocytes or the cell-free system. Since RNAs were suspected to contain sites of AFB_1 modification, a thorough quantitation of AFB_1-RNA adduct was undertaken.

Aflatoxin B_1 modification of RNAs. When specific radioactivities of various species of RNAs were compared, poly(A)-containing RNA, taken as mRNA, was more highly modified than poly(A)-lacking RNA (Table 2). When extracted from hepatocytes, polysomal mRNA had a 5-fold higher specific activity compared to poly(A)-lacking RNA (predominately rRNA), and cytosolic mRNA had a 17-fold higher specific activity compared to poly(A)-lacking RNA (predominately tRNA). When RNAs were extracted from the cell-free activating system, mRNA had a specific activity (dpm/A_{260}) of 14,100 compared to 2,100 for poly(A)-lacking RNA, a 6-fold higher level (Table 2).

The poly(A)-lacking RNAs were investigated further to determine the relative extent of their modification by the toxin. Using sucrose density gradient

Table 1. Aflatoxin B_1 association with initiation factors

	[^{3}H]Aflatoxin B_1-protein adduct (dpm/mg protein)	
Isolation step	Ribosomes	Post-ribosomal supernatant
Salt treatment	3520	4880
60% $(NH_4)_2SO_4$ Precipitate	2270	2280
Heparin-Sepharose eluate	1230	1890
45–60% $(NH_4)_2SO_4$ Precipitate	600	663

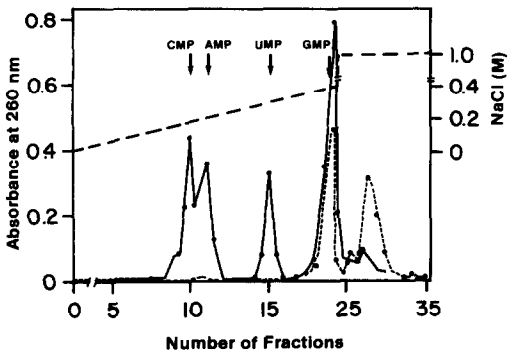

Fig. 2. Ion exchange chromatography of aflatoxin B_1 modified RNA hydrolyzed by RNase T_1 and T_2. Twenty A_{260} units of RNA were hydrolyzed for 24 hr, at 37°, with RNase T_1 or T_2. After incubation, the sample was adjusted to pH 9.7 by adding 20 mM carbonate buffer, pH 9.7. This was applied to a QAE-Sephadex A-25 column, with a bed volume of 13.4 cm^3 and washed for 8 hr at room temperature at a flow rate of 30 ml/hr. Mononucleotides were eluted by a 0.0–0.4 M NaCl gradient and 1.0 M NaCl (——) made in 20 mM carbonate buffer, pH 9.7. Fractions (5 ml) were collected and absorbance was monitored at 260 nm on a Hewlett–Packard 8451A Spectrophotometer. Commercially obtained mononucleotide 3′-phosphates, AMP, CMP, GMP, and UMP, were eluted from a separate column under identical conditions as the sample, as indicated on the figure. Key: RNase T_1 digestion in 25 mM Tris–HCl buffer (pH 8.0) (– – – –); RNase T_2 digestion in 50 mM potassium acetate buffer (pH 4.5) (——).

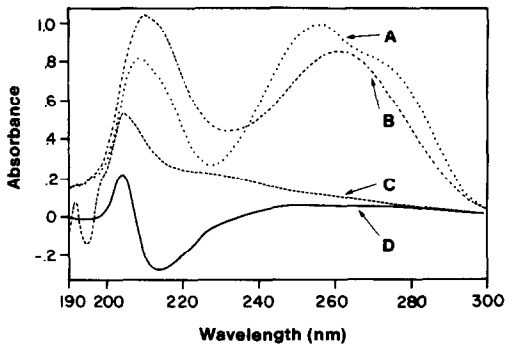

Fig. 3. Absorbance spectra of selected fractions eluted from a QAE-Sephadex column. Full spectral scan was obtained using the Hewlett–Packard 8451 Diode Array Spectrophotometer. Key: curve A, GMP fraction (· · · · ·); curve B, oligonucleotide fraction (· – –); curve C, AFB_1 (– – – –); and curve D, pre-GMP fraction (——).

centrifugation to separate these RNAs that were isolated from either soluble cytosol (predominately 4S RNA) or cRPC (predominately 18S and 28S RNAs), the following specific activities (dpm/A_{260}) were obtained: 4S, 192; 5–6S, 270; 18S, 757; and 28S, 1014. Thus, among poly(A)-lacking RNAs, the rRNAs (28S > 18S > 5–6S) exhibit greater modification than tRNAs (4S).

Identification of aflatoxin B_1-nucleotide adducts. AFB_1-RNA adducts isolated from both hepatocytes and the cell-free microsomal activating system were digested exhaustively with RNase T_2. The resulting

Table 2. Specific radioactivity of [^{3}H]aflatoxin B_1-RNA adducts

	Specific radioactivity (dpm/A_{260})	
Cellular fraction	Poly(A)-lacking	Poly(A)-containing
Polysomes	4,120 ± 911	21,900 ± 2,800
Cytosol	1,460 ± 650	25,800 ± 4,730
Microsomes*	2,100 ± 100	14,100 ± 850

* RNAs were phenol extracted from the cell-free microsomal activating system and separated on oligo(dT)-cellulose columns. Data are mean ± SE from four experiments.

mononucleoside 3′-monophosphates were separated by QAE-Sephadex A-25 anion exchange column chromatography (Fig. 2). When AFB_1-RNA adducts were digested with RNase T_1, 3′-GMP was the only material eluted from the column with 0.0–0.4 M NaCl gradient. The undigested oligonucleotides were removed from the column using 1.0 M NaCl. To identify the nucleotide where covalent modification by AFB_1 occurs, the radioactivity associated with the various fractions was determined and results are presented in Table 3. When AFB_1-RNA adducts isolated from hepatocytes were hydrolyzed by RNase T_1, 80% of the radioactivity was present in the post-GMP fraction; these undigested oligonucleotides (Fig. 3, curve B) remained on the column and required high salt for elution. Eleven percent of the radioactivity was present where 3′-GMP fractions were identified (Fig. 3, curve A). When the adduct was treated with RNase T_2, however, 28% of the radioactivity was found associated with 3′-GMP, and no radioactivity was detected in either the undigested oligonucleotide fraction or fractions containing CMP, AMP, and UMP. Due to a greater than expected cumulative variation, the fractions that eluted prior to 3′-GMP were examined and 72% of the added radioactivity was recovered. Further comparison of the spectrum of the fraction (Fig. 3, curve D) with that of AFB_1 (Fig. 3, curve C) suggested that the pre-GMP fraction contained an acid-labile AFB_1-GMP metabolite. Taken together, these results indicate that GMP is the sole site of AFB_1 modification as revealed by RNase T_2 digestion, and that 11% of the guanine nucleotides was located in poly(G) tracts as revealed by RNase T_1 digestion.

To further verify the specificity of the guanine nucleotide as the site of AFB_1 modification, AFB_1-RNA adducts isolated from the cell-free microsomal system were hydrolyzed by RNase T_1 and T_2. Results presented in Table 3 indicate that 3′-GMP was the site of modification, with 9% of the nucleotide located in poly(G) tracts.

DISCUSSION

Previous work from this laboratory demonstrated that AFB_1 affects the joining of 60S ribosomal subunits to form 80S monosomes to a greater extent than it affects formation of the functional 40S subunits [11]. To study the structural aspects of AFB_1 modification, a tracer amount of toxin was used,

which allowed binding to macromolecules without causing deleterious cellular ultrastructural changes seen with higher concentrations [27, 28]. Two systems were used in this study: an isolated hepatocyte suspension to closely approximate *in vivo* conditions, and a cell-free activating system in which the cRPC were modified with AFB_1 activated via microsomes fortified with an NADPH-generating system. The use of both systems permitted qualitative and quantitative comparisons of covalent AFB_1 binding.

When ribosomal subunits were freed from components normally bound in active translation, most of the radioactivity was associated with the subunits (Fig. 1). Since ribosomal proteins were shown not to be modified by AFB_1, ribosomal RNA modification most likely accounts for the observed radioactivity. Considering the relative abundance of poly(A)-lacking RNAs compared to poly(A)-containing RNAs, the amount of radioactivity in the fraction containing mRNPs is consistent with high modification of mRNA. The lack of binding of the toxin to ribosomal proteins was surprising, since some of these proteins are able to be covalently modified under a variety of conditions (reviewed in Ref. 29). It is unlikely that an AFB_1 protein adduct did form, but was subsequently destroyed by the rigorous extractions used to obtain ribosomal proteins, since proteins purified from puromycin-EDTA dissociated 40S and 60S subunits also failed to demonstrate AFB_1 modification.

To assess modification of protein factors by AFB_1, unfractionated initiation factors were investigated rather than individual factors. The main emphasis was to optimize the recovery of factors while eliminating inhibitory components, which was achieved in the 45–60% ammonium sulfate precipitate. The decrease in specific radioactivity throughout the procedure (Table 1) implied removal of a highly modified non-initiation factor component, since purification of initiation factors increases as the isolation progresses [23, 24]. Because the fraction containing initiation factors did show slight modification, it was interesting to speculate which factor(s) was modified. Among the three initiation factors (eIF_2, eIF_3 and eIF_5) shown to be absolutely required for translation [30], eIF_5, a factor known to participate in the joining of the 60S complex to form the 80S monosome, may partially impair its normal function and results in the decreased formation of the 80S initiation complex as previously observed [11].

Carcinogens binding to cellular mRNAs can result in impaired translation [31, 32], particularly by altering interaction of mRNA with ribosomes [33]. It has been shown that AFB_1 also inhibits the synthesis of poly(A)-containing RNA [34]; a combination of decreased synthesis of a specific mRNA along with modification of the existing mRNA by AFB_1 could culminate in cellular insults that may alter function. Such a modification may well suffice to produce toxic and carcinogenic effects, particularly in view of the fact that AFB_1 is one of the most potent hepatotoxins and hepatocarcinogens known. The significance of the selective modification of RNA versus protein in the initiation complex as it relates to AFB_1-induced toxicity is speculative. Because of the preference of the toxin for guanine, modification of the 3′ poly(A) region of the mRNA is unlikely. If one considers binding of AFB_1 to the 5′ cap structure, which is a guanosine residue, this too is unlikely due to methylation of guanine at its N-7 position, the optimal site to which AFB_1 binds [3]. The region in the mRNA that, if modified by AFB_1, would likely result in functional perturbation would be a 5′ region containing the initiator AUG codon. Were this the case, the ability of the mRNA to be translated would be affected, but not its ability to bind in the formation of the preinitiation complex. Perhaps the orientation of the AFB_1 moiety in the mRNA is such that the mRNA may accommodate 40S subunit binding, but binding becomes restrictive as the 60S subunit approaches, hence 60S subunit binding is perturbed. Thus, the molecular mechanisms of AFB_1 action on protein initiation are by no means clearly defined as yet; investigations are currently being undertaken to further elucidate the interaction of these macromolecules.

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Table 3. Radioactivity of selected fractions eluted from a QAE-Sephadex column

Source of AFB_1-RNA adduct	RNase	[^{3}H]AFB_1 radioactivity (dpm × 10^{-4})			
		Pre-GMP	3′-GMP	Oligonucleotide	Total
Hepatocytes	T_1	0.6 ± 0.5 (9%)	0.7 ± 0.4 (11%)	5.1 ± 0.8 (80%)	6.4 ± 0.6 (100%)
	T_2	4.5 ± 1.4 (72%)	1.8 ± 1.4 (28%)	ND	6.5 ± 0.1 (100%)
Microsomal incubation mixture	T_1	0.1 ± 0.1 (3%)	0.3 ± 0.1 (9%)	2.8 ± 0.4 (88%)	3.2 ± 0.3 (100%)
	T_2	0.4 (13%)	2.7 (87%)	ND	3.1 (100%)

Data are mean ± SD from three experiments. Pre-GMP: material eluted between UMP and GMP. ND: not detectable.

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