INTERACTION OF AFLATOXIN B₁ WITH THE RIBOSOMAL RNA GENES OF RAT LIVER

by

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B.S., University of Georgia
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ABSTRACT

This thesis reports on studies to describe, in quantitative and qualitative terms, the relationships which exist between the formation of aflatoxin B₁ adducts in the ribosomal RNA genes of rat liver DNA and the inhibition of ribosomal RNA synthesis which results. The information is presented in three parts.

Part I presents a general statement of the thesis problem and a discussion of the toxicity, carcinogenicity, and metabolism of aflatoxin B₁ with special reference to the effects observed on DNA transcription.

Part II describes studies on the time course of AFB₁ adduction within deoxyribonuclease II-sensitive and -insensitive regions of nuclear and nucleolar chromatin DNA. The information on nuclear studies is presented in section A of part II, while the information on nucleolar studies is presented in section B of part II.
1) Methods have been developed for the isolation of DNase II-sensitive and -insensitive fractions of nuclear chromatin DNA from the liver chromatin of aflatoxin B$_1$-treated animals. The DNase II-sensitive chromatin DNA fraction, which accounted for 11.9% of liver nuclear DNA, was shown to be enriched in transcribed gene sequences as compared to DNA from DNase II-insensitive chromatin: DNase II-sensitive chromatin DNA was 5.1-fold enriched in ribosomal RNA gene sequences and 4.2-fold enriched in transfer RNA gene sequences as determined by hybridization to rRNA and tRNA probes.

2) The time course of aflatoxin B$_1$ adduction within DNase II-sensitive, DNase II-insensitive, and unfractonated chromatin DNA indicated that DNA from nuclease-sensitive chromatin is preferentially susceptible to AF$_B_1$ adduction. Over a 24 hour period post-administration of a 1 mg/kg $[^3]$HAF$_B_1$ dose, nuclease-sensitive chromatin DNA bound 2.3-to 3-fold more aflatoxin B$_1$ residues than DNA from the nuclease-insensitive fraction. Aflatoxin B$_1$ residues were also found to be removed at a 3-fold greater rate from DNase II-sensitive versus -insensitive chromatin DNA.

Section B

1) Methods have similarly been developed for the isolation of nucleolar chromatin fractions from aflatoxin B$_1$-treated animals that are sensitive and insensitive to DNase II digestion. The DNase II-sensitive nucleolar chromatin fraction, which accounted for 14% of nucleolar DNA, was 5.6-fold enriched in ribosomal RNA sequences as compared to DNA from the nuclease-insensitive nucleolar fraction. As in the nuclear chromatin studies, aflatoxin B$_1$ residues were bound preferentially to DNase II-sensitive chromatin DNA: two hours post-dosing, DNA from the nuclease-sensitive nucleolar fraction was adducted to a 4.3-fold greater level than DNA from the nuclease-insensitive nucleolar fraction.
These results from both nuclear and nucleolar systems suggested that putative transcribed regions of liver chromatin, distinguished by their sensitivity to DNase II attack, are preferentially susceptible to covalent adduction and damage \textit{in vivo} by aflatoxin B$_1$.

Part III discusses the development of an experimental system to investigate the structural and functional impairment of transcribed DNA regions by chemical carcinogens in which:

(a) carcinogen-DNA adducts can be measured within specific transcribed gene sequences, and

(b) the qualitative and quantitative relationships between carcinogen-DNA adduction and functional endpoints can be investigated in specific gene sequences.

1) Methods have been developed to isolate ribosomal RNA gene sequences from the total liver nuclear DNA of AFB$_1$-treated animals. Liver nuclear DNA, enriched in rDNA sequences by one round of cesium salt density centrifugation, was treated under buffered alkaline conditions to stabilize AFB$_1$-bound radioactivity. The alkali-treated DNA was hybridized to 18S and 28S ribosomal RNA in 70% formamide to form rRNA:rDNA hybrids. The hybrids were purified by two rounds of cesium chloride centrifugation, and the level of AFB$_1$ adduction was determined as functions of time and dose.

2) Determination of the time course of AFB$_1$ adduction to ribosomal DNA confirmed rDNA as a preferential target within nuclear DNA for aflatoxin B$_1$ adduction. Over a 12 hour period post-administration of a 1 mg/kg $[^3]$HAFB$_1$ dose, ribosomal DNA bound 4 to 5 fold more AFB$_1$ residues than total nuclear DNA. The aflatoxin B$_1$ residues bound to ribosomal DNA were also found to be preferentially removed: over the 12 hour period post-dosing, aflatoxin B$_1$ adducts were removed at a 5.7-fold greater rate than aflatoxin B$_1$ adducts in nuclear DNA as a whole.
3) The time course of the three predominant aflatoxin B₁ adducts in nuclear DNA was determined in ribosomal DNA: AFB₁-N⁷-guanine, AFB₁-FAPyr, and AFB₁-Peak F. Nuclear DNA isolates were heated in neutral buffer to selectively liberate the unstable AFB₁-N⁷-guanine moiety. The difference in adduct levels between alkali-treated and hot buffer-treated DNA samples was taken as an approximation of the levels of AFB₁-N⁷-guanine in ribosomal DNA isolates. No differences in the proportions of these three adduct species were found in rDNA as compared to total nuclear DNA.

4) A mathematical representation of the transcriptional processes perturbed by aflatoxin B₁-DNA modifications has been constructed which accurately relates the observed inhibition of ribosomal RNA synthesis to the adduction of ribosomal DNA sequences.

5) The mechanisms underlying the inhibition of ribosomal RNA synthesis by aflatoxin B₁, as modeled by the mathematical representation, were investigated by two approaches. First, the newly synthesized rRNA transcripts in nucleoli of aflatoxin B₁-treated animals were characterized by sedimentation in sucrose gradients. The average size of the rRNA transcripts isolated from animals sacrificed 2 to 12 hours after aflatoxin B₁ administration were found to compare favorably with the transcript size predicted by the model of transcriptional inhibition. Second, an in vitro transcription assay was developed employing nucleolar homogenates from AFB₁-treated animals. The amount of in vitro RNA synthesis supported by these homogenates was measured in the presence and absence of sarkosyl, a detergent which effectively prevents reinitiation of RNA polymerases. It was found that in vitro aflatoxin B₁-induced inhibition of ribosomal RNA synthesis is due exclusively to the template-dependent impairment of RNA polymerase elongation of nascent transcripts. The data from both these studies were shown to support the validity of the model equation to relate rDNA adduction and rRNA synthesis in aflatoxin B₁-treated animals.

Thesis Supervisor: Dr. Gerald N. Wogan
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To

My

Family
Part I

Chapter 1: Introduction - General Statement of the Thesis Problem

Chapter 2: Literature Review and Perspective
Chapter 1

Introduction - General Statement of the Thesis Problem

The irreversibility of tumor initiation, as well as the heritability of the tumor phenotype, have focused attention on DNA as the critical macromolecular target for chemical carcinogens (1,2). Changes in DNA sequences, as products of carcinogen-DNA interactions, provide a molecular basis for the observed alterations in gene expression which accompany neoplastic transformation by chemicals (3,4). Emphasis on carcinogen-induced DNA sequence changes in the etiology of tumor development has been further strengthened by the qualitative, and sometimes quantitative, correlations between compounds which exhibit both carcinogenic and mutagenic properties (5-9).

Identification of the reaction products of carcinogen-DNA interactions has fostered not only kinetic studies of DNA adduct appearance and disappearance within treated cell populations, but also mechanistic investigations relating specific adduct patterns with the ultimate development of tumors in target organs of susceptible species (10-13). These investigations in whole animal tissues have been extended to more recent studies in cell culture. Employing eucaryotic mutation assay systems,
quantitative relationships have been established between the *in vivo* binding of carcinogens to DNA and the induction of gene locus mutation (14).

DNA is not uniformly susceptible to carcinogen modification, however. The organization and structure of chromatin influence the accessibility of regions of DNA to this modification *in vivo*. Differentiated eucaryotic cells transcribe only a limited and tissue-specific portion of their nuclear DNA sequences (18). This DNA fraction, termed transcribed DNA in the literature, constitutes approximately 10% of total nuclear DNA (19-21). Morphological and biochemical evidence suggests that these gene sequences are maintained in a discrete active conformation distinguishable from the majority of nontranscribed DNA. Electron micrographic observations have revealed sites of RNA synthesis within the chromosome to have a more diffuse and extended conformation (26-28). Biochemical investigations have shown transcribed DNA sequences to be distinguishable by their sensitivity to attack by deoxyribonucleases. The selective digestion of DNA within transcribed genes by DNase I (18,29,30) or DNase II (31-33) supports the proposal that the chromatin conformation of expressed genes is different from that of a DNA segment within untranscribed genes.

The preferential accessibility of transcribed DNA to
deoxyribonuclease digestion has been shown also to be associated with an increased susceptibility of these DNA regions to carcinogen modification. When chromatin from carcinogen-treated animals is digested by DNase I or II, the levels of adduction within nuclease-sensitive chromatin DNA are found to exceed the levels of binding to nuclease-insensitive chromatin DNA. Studies employing small (38) as well as bulky (39,40) alkylating compounds have found greater levels of adduction to DNA within nuclease-sensitive chromatin DNA regions.

To adequately investigate the structural and functional impairment of transcribed DNA regions by chemical carcinogens requires experimental systems in which:

1) carcinogen-DNA adducts can be measured within specific transcribed gene sequences and
2) the qualitative and quantitative relationships between carcinogen-DNA adduction and functional endpoints can be investigated in specific transcribed gene sequences.

This thesis describes the development of such a system, schematically summarized in Figure 1-1, which permits the adduction of the ribosomal RNA gene sequences in rat liver by the hepatocarcinogen aflatoxin B₁ to be related experimentally to the impairment of ribosomal DNA transcription in aflatoxin B₁-treated animals. This system
Figure 1-1  Experimental system designed to relate experimentally the adduction of ribosomal RNA gene sequences in rat liver to the impairment of ribosomal DNA transcription in AFB₁-treated animals.
Treat Male Fischer Rat with $^{3}$H-\textit{AFB$_1$} (i.p.)

Isolate Liver Nuclear DNA

Isolate Ribosomal DNA Sequences

- Determine Levels of Bound AFB$_1$ Residues
- Determine Identity of Major AFB$_1$ Adduct Species

COMPARE

Relate the AFB$_1$ Adduction of Ribosomal DNA to Perturbations in rRNA Synthesis

COMPARE

Determine the Size Distribution of Nascent Ribosomal RNA Macromolecules

Isolate Nucleolar RNA (rRNA)

Isolate Liver Nucleoli

Treat Male Fischer Rat with AFB$_1$ (i.p.)
permits the first opportunity to study in vivo both chemical damage and repair within a specific transcribed gene sequence as well as the functional impairment in those same gene sequences which result.

Using this model system, ribosomal RNA gene sequences from $[^3H]-AFB_1$-treated rats are selectively isolated from the total liver DNA via cesium salt density gradient centrifugation and hybridized to complementary ribosomal RNA. Total AFB$_1$ modification and adduct identification are determined as functions of time and dose after toxin administration. Concurrently, nucleolar RNA is isolated from liver nuclei of AFB$_1$-treated animals. Nucleolar RNA synthesis inhibition is measured and the nascent nucleolar RNA transcripts are characterized with respect to size distribution. Comparisons of the time-course of ribosomal RNA gene adduction with total nuclear DNA permit accurate investigations of the preferential susceptibility of transcribed DNA regions to carcinogen adduction; in addition, the removal of carcinogen adducts can be investigated within expressed regions of the genome versus the cellular genome as a whole. Finally comparisons of the time-course of rDNA adduction and ribosomal RNA synthesis inhibition can be made and integrated into a mathematical representation which relates aflatoxin B$_1$ adduction to DNA
template inactivation.

Aflatoxin \( B_1 \) was chosen as the model carcinogen for these studies because aflatoxin \( B_1 \)-DNA reaction products have been isolated and chemically well characterized. The only known site of reaction of aflatoxin \( B_1 \) is at the \( N^7 \) position of guanine forming the \( \text{AFB}_1-N^7 \)-guanine adduct as the major DNA adduct (Figure 1-2). Further, virtually all of the DNA bound radioactivity derived from aflatoxin \( B_1 \) can be accounted for by adduct species at the \( N^7 \)-position from 2 to 36 hours post-dosing (41). The predominance of aflatoxin \( B_1 \) adduction at this one site, as well as the availability of data concerning adduct stability and removal, proves crucial to the development of hybridization technology permitting isolation of \( \text{AFB}_1 \)-adducted gene sequences and the accurate quantitation of aflatoxin binding and removal.

Ribosomal DNA, the DNA sequences coding for the 45S precursor to 18S and 28S ribosomal RNA, were chosen as the appropriate genes to study for two reasons. Ribosomal RNA genes are present in approximately 300 copies per cell. The presence of multiple copies of this gene makes it technically feasible to isolate sufficient rDNA to determine both adduct levels and adduct identities. In addition, extensive investigations of the specific endpoint to be studied,
Figure 1-2  Structure of \( \text{AFB}_1^{-\text{N}^7}-\text{guanine} \), the principal
AFB\(_1\)-DNA reaction product both \textit{in vivo} and \textit{in vitro}. 
aflatoxin B₁-induced inhibition of ribosomal RNA synthesis, have shown this inhibition is due to impairment of the ribosomal DNA template (42,43). Perturbations in ribosomal RNA synthesis can therefore be related to the adduction of ribosomal DNA sequences in the same experimental system.

Chapter 2 begins with a review of the literature concerning three topics. First, the toxicology and metabolism of aflatoxin B₁ is reviewed briefly with emphasis on the interaction of aflatoxin B₁ metabolites with cellular macromolecules. A critical review of studies investigating the impairment of transcriptional processes by aflatoxin B₁ is also included to place the objectives of this thesis project in perspective.

Chapter 3 details preliminary investigations on the distribution of AFB₁ residues within DNase II-sensitive and -insensitive fractions of nuclear and nucleolar chromatin DNA enriched in transcribed gene sequences. The data presented as well as the literature cited indicate that, as has been shown with other chemical carcinogens, AFB₁ is preferentially bound within DNase II-sensitive DNA regions enriched in transcribed DNA sequences.

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Chapter 4 presents a new model system and technology examining the time course of carcinogen binding and adduct identification within ribosomal DNA gene sequences. A detailed explanation is first given for isolating the
ribosomal RNA gene sequences of whole rat liver DNA from aflatoxin B$_1$-treated animals; protocols for stabilizing AFB$_1$-bound moieties are given as well as evidence on the purity of the ribosomal DNA isolate based on high pressure liquid chromatography analysis of the constituent nucleotide bases and gel electrophoretic analysis of Eco RI-digested samples. Data is further presented on the time-course and dose-response of AFB$_1$ adduction within the ribosomal DNA isolate as well as the time course of AFB$_1$ adduction populations within these gene sequences.

Chapter 5 analyzes the effects of acute AFB$_1$ administration on ribosomal RNA synthesis in the nucleolus. Both the qualitative and quantitative effects of AFB$_1$ on ribosomal RNA synthesis are investigated along with the effects of AFB$_1$, measured in vitro, on the initiation versus elongation of RNA transcripts by nucleolar homogenates. A mathematical representation of the nucleolar transcriptional processes perturbed after AFB$_1$ administration is presented which relates the adduction of ribosomal DNA to the inhibition of ribosomal RNA synthesis which results.
Chapter II. Literature Review and Perspective

A. General Aspects of Aflatoxin Toxicity and Carcinogenicity

The aflatoxins are a family of heterocyclic secondary metabolites produced by several strains of Aspergillus that have been of great interest to the agricultural and toxicological communities for over 20 years. Their initial discovery came as a consequence of the death of a large number of farm animals, including turkeys in Britain and breeding trout in America, that were fed aflatoxin-contaminated feedstock (44-46). Identification of the aflatoxins as the responsible toxic agents in these cases and their subsequent testing in other species has shown that a large number of laboratory and domestic animals are susceptible to their toxic activity (47).

The wide geographical distribution of Aspergillus species and the recognition that aflatoxin could be produced under most conditions supporting fungal growth have stimulated research into the occurrence and distribution of these compounds (47). The geographical dimensions of this problem were recognized when aflatoxins were identified in peanut meals originating in many parts of the world. Subsequently, a wide variety of foodstuffs including corn, cottonseed, and soybeans were found to commonly be contaminated with one or more of these toxins (48-51).
Structural identification of the constituents of food-borne aflatoxin mixtures found aflatoxin B₁ to be the major component of toxin mixtures produced by the great majority of aflatoxin-secreting fungi. The structures of aflatoxin B₁ and some of its naturally-occurring derivatives are shown in figure 2-1.

In addition to being the predominant component of aflatoxin mixtures, aflatoxin B₁ is also the most toxic (45). Chronic feeding studies employing purified aflatoxin B₁ or aflatoxin B₁-contaminated foods have further demonstrated this compound to have carcinogenic activity in a wide range of species, including rodents, birds, fish, and primates (47, 52-54). For both aflatoxin B₁ toxicity and carcinogenicity, the liver is the main target organ in most species; other organs, however, including the kidney and the glandular stomach, are targets for aflatoxin B₁ toxicity and carcinogenicity in particular animal species or under specific nutritional conditions (55-57).

Aflatoxin B₁ is one of the most potent hepatocarcinogens known; liver tumors are induced in the rat or rainbow trout at feed levels of 1 or 15 parts per billion, and in rats given a single sublethal dose (7.65 mg/kg) (58-61). The induction of hepatic tumors by aflatoxin B₁ has been found to be strongly influenced by the dosage regimen. Further, male rats are more susceptible to tumor induction than female rats. In a study of male and female rats fed 1 ppm
Figure 2-1 The structures of aflatoxin \( B_1 \) and some of its naturally-occurring derivatives.
AFB₁, 82% of the males developed carcinomas by 41 weeks of administration; no malignant tumors were observed in females by 41 weeks and no evidence of carcinoma was observed until 64 weeks after initial aflatoxin administration. Moreover, the average AFB₁ intake by an animal prior to initial tumor appearance was 2.9 mg for males compared to 5.9 mg for females. In contrast to this delayed appearance of tumors, female rats developed precancerous lesions within about the same treatment intervals as male rats. The delayed tumor appearance was thus attributed to a slower progression from the preneoplastic to the neoplastic state rather than the slower development of the early carcinogenic processes.

The aflatoxins have also been implicated as tumorogenic agents in man. For many years, these compounds have been suspected as the responsible agents for acute toxicosis in human populations that consume mold-contaminated grain (62-64), and they are under suspicion as contributing factors to Reye's Syndrome (55,66).

Epidemiological studies, conducted predominately in Southeast Asia and Africa, have shown a strong correlation between the extent of aflatoxin ingestion and primary liver cell cancer (67-70). Hepatitis B virus is concurrent in these regions, however, and may also figure, along with aflatoxin B₁, in the human hepatic carcinogenesis observed (71). Nonetheless, on the basis of observations in man and results in laboratory animals, the International Agency for
Research on Cancer (IARC) has found the aflatoxins to be "probably carcinogenic for humans" (72).

B. Aflatoxin B₁ Metabolism: Binding to Macromolecules

Like many xenobiotics, aflatoxin B₁ is principally metabolized by the microsomal mixed-function oxidases, a group of cytochrome-coupled, oxygen- and NADPH-dependent enzymes primarily associated with endoplasmic reticulum of the liver and other organs (73). Microsomal metabolism of aflatoxin B₁ results in direct hydroxylation of the AFB₁ ring carbons as well as oxidative demethylation of AFB₁ to phenolic derivatives; Figure 2-2 summarizes the known, as well as the hypothesized, metabolic routes for AFB₁. The net result after metabolic oxidation of the parent aflatoxin B₁ structure is detoxification by the formation of various hydroxylated derivatives of increased water solubility and, thus, increased ease of removal from the body via excretion in the urine or bile. These hydroxylated derivatives can also be conjugated with sulfate, glucuronic acid, or glutathione, forming water soluble conjugates which also can be readily excreted in the urine or bile (74-75). Enzyme systems, similar to those associated with the endoplasmic reticulum, are also found in the nuclear membrane which are capable of metabolizing aflatoxin B₁ (77,78). While the level of metabolism by
Figure 2-2  Known and hypothesized metabolic routes for $\text{AFB}_1$.  
Taken from reference #73.
preparations of nuclear membranes is only 10-15% of the level attained by isolated microsomes, similar AFB₁ metabolites are formed.

During metabolic transformation, highly reactive, electrophilic, aflatoxin metabolites are generated which can react covalently with nucleophilic centers in cellular macromolecules such as DNA, RNA, and proteins. The first clear indication of the generation of reactive intermediates during the biotransformation of aflatoxin B₁ came from in vitro bacterial mutagenicity studies (79). Aflatoxin B₁ was not mutagenic to tester strains of Salmonella in the absence of added mammalian microsomal fractions, but was a potent mutagen in the presence of a microsomal fraction. None of the hydroxylated or demethylated aflatoxin metabolites however, either with or without metabolic activation, showed the mutagenic potency exhibited by aflatoxin B₁. Further evidence for the generation of highly reactive intermediates during aflatoxin detoxification came in mutagenicity studies employing liver microsomal enzymes from animals pretreated with phenobarbital, an inducer of microsomal oxidase activity (80). Aflatoxin B₁ mutagenicity was increased several fold using microsomal enzymes from phenobarbital-pretreated animals compared to untreated controls.

In vitro incubation of AFB₁, microsomal preparations, and appropriate cofactors with RNA, DNA, or polynucleotides confirmed that during metabolic detoxification reactions,
chemically-reactive electrophilic intermediates of aflatoxin B₁ are generated which can covalently adduct nucleophilic sites within cellular macromolecules. Garner et al., employing in vitro incubation preparations, found AFB₁ binding to nucleic acids at levels as high as 1 aflatoxin B₁ residue per 30 nucleotides (80-82). Mild acid hydrolysis of RNA adducted in vitro with liver microsomes and AFB₁ by Swenson et al. resulted in the liberation of aflatoxin dihydrodiol (2,3-dihydro-2,3-dihydroxy aflatoxin B₁); subsequent incubation of AFB₂, which is saturated at the 2,3-position of the terminal furan ring, resulted in no detectable nucleic acid adduction. The formation of a terminal diol upon hydrolysis of nucleic acid-AFB₁ adducts, coupled with the lack of adduction by an aflatoxin analog devoid of a vinyl bond in the terminal furan ring, led to the following hypothesis: aflatoxin B₁-2,3-oxide is the electrophilic aflatoxin intermediate responsible for nucleophilic adduction and is the probable ultimate carcinogenic metabolite of aflatoxin B₁ (73).

Supporting this hypothesis was, first, the isolation of aflatoxin dihydrodiol after hydrolysis of liver DNA and RNA from rats administered AFB₁ (83-84). Second, an electrophilic analog of the hypothesized epoxide, aflatoxin B₁ 2,3-dichloride, has been shown to possess characteristics of the epoxide in biological systems (85). The dichloride analog of aflatoxin B₁ was more potent than AFB₁ as a
bacterial mutagen and in the induction of tumors in both mice and rats. When incubated with DNA or RNA, the dichloride reacted spontaneously to yield adduct species which produced aflatoxin B$_1$ dihydrodiol upon hydrolysis in aqueous solution.

Confirmation for the 2,3-oxide intermediate in the metabolism of AFB$_1$ came with the isolation and structural identification of the predominant in vitro and in vivo AFB$_1$-DNA adduct (86,87). After incubation in a microsomal preparation with calf thymus DNA, 90% of the covalently bound AFB$_1$ was represented by 2,3-dihydro-2(N$^7$-guany1)-3-hydroxy-AFB$_1$. The structure of this adduct suggested the initial formation of AFB$_1$-2,3-oxide by the microsomal fraction followed by decomposition to form a carbonium ion at the C-2 position on the terminal furan ring (88). Attack on this carbonium ion by nucleophilic sites in DNA, predominately at the N$^7$ position of guanine, would result in the isolated guanine-aflatoxin B$_1$ adduct after hydrolysis.

In vivo studies of the aflatoxin B$_1$-DNA adducts formed during either a toxic or carcinogenic dosage regimen have shown that the initial AFB$_1$-N$^7$-guanine adduct is not stable (89-91). Over a 72 hour period following 0.6 mg/kg AFB$_1$ dose to rats, AFB$_1$-N$^7$-guanine adduct levels decreased from 90% of the bound radioactivity at 2 hours post-dosing to 1% of the bound radioactivity at the 72 hour timepoint.
Figure 2-3 summarizes the time course of AFB₁-N⁷-guanine disappearance from rat liver DNA in vivo. Since the total level of radioactivity decreases with time, some of the AFB₁-N⁷-guanine adduct is removed either by hydrolysis of the aflatoxin moiety to form aflatoxin B₁-dihydrodiol, spontaneous hydrolysis of the N-glycosidic bond yielding an apurinic site, or enzymatic removal by DNA repair enzymes (92). Recent in vitro studies have shown the AFB₁-N⁷-guanine adduct to be relatively chemically stable in DNA with a half-life of approximately 100 hours. In contrast, the half-life of aflatoxin-bound radioactivity in the liver DNA of AFB₁-treated animals has been determined to be 12 hours (93). These data suggest that enzymatic removal plays some role in the liberation of aflatoxin moieties from DNA.

A second mechanism exists for removing AFB₁-N⁷-guanine residues. As shown in Figure 2-4, the binding of AFB₁-2,3-oxide at the N⁷ position of guanine generates a positive charge on the 7,8,9-ring of guanine. This positive charge causes the C-8 carbon of guanine to be susceptible to base attack, breaking the C⁸-N⁹ guanine bond and neutralizing the positive charge. This new adduct moiety, tentatively identified as 2,3-dihydro-2-(N⁵-formyl-2',5',6'-triamino-4'-oxo-N⁵-pyrimidyl)-3-hydroxyaflatoxin B₁, is stable in DNA over a period of 48 hours and represents the predominant aflatoxin B₁-DNA adduct species after twenty-four
Figure 2-3  Time course of the predominant APB₁ adduct species in rat liver nuclear DNA (Ref. # 94).
Figure 2-4  Chemical instability of $\text{AFB}_1-N^7$-guanine DNA adducts.
hours post dosing. A third DNA-\( \text{AFB}_1 \) adduct, tentatively identified as 2,3-dihydro-2(\( \text{N}\)-dihydro-8-hydroxy-N\( ^7 \)-guan yl)-3-hydroxy-aflatoxin B\( _1 \) also results from hydroxyl attack at the C-8 position of guanine and stabilizes the aflatoxin-bound moiety in DNA.

These two stable adducts (known informally in our laboratory as AFB\( _1 \)-FAPyr and AFB\( _1 \)-Peak F respectively) have received much attention with respect to potential mechanisms for the carcinogenic activity of aflatoxin B\( _1 \). The most efficient dosage regimen for aflatoxin carcinogenesis is small, repeated dosages given over a long period of time. Adduct studies in rat liver DNA from animals administered AFB\( _1 \) on a carcinogenic dosage regimen have found that both stable AFB\( _1 \) adducts accumulate during the course of toxin administration (90,91). Thus the molecular events accompanying tumor initiation by aflatoxin B\( _1 \) would be expected to depend either on these two stable adduct species or the apurinic site generated during AFB\( _1 \)-N\( ^7 \)-guanine hydrolysis.

\textit{In vivo} and \textit{in vitro} studies have also indicated that AFB\( _1 \)-2,3-oxide as well as other aflatoxin B\( _1 \) metabolites can bind to various proteins. After a 1 mg/kg AFB\( _1 \) dose, 66\% of nuclear AFB\( _1 \) is bound to DNA two hours post dosing; another 8\% is bound to chromosomal proteins (93). While the structures of the protein adducts have not been elucidated,
two AFB₁ metabolites have specifically been implicated, in addition to AFB₁-2,3-oxide, as possible protein-binding species.

As shown in Figure 2-3, Aflatoxin B₂ₐ, formed by the metabolic hydroxylation of AFB₁, spontaneously binds to proteins under physiological pH conditions (94); aflatoxin B₁ dihydrodiol, formed by hydration of the 2,3-epoxide, also reacts with proteins (95). In both cases, the presumed mechanism of reaction is Schiff base formation between the amino groups of proteins and the dialdehyde derivatives of the AFB₁ metabolites. Because of the low toxicity and biological activity of both aflatoxin dihydrodiol and aflatoxin B₂ₐ, these protein adducts are not likely to be the principal macromolecular lesions responsible for the toxic or tumorigenic effects of aflatoxin B₁; they may, however, contribute to chromosomal alterations induced after aflatoxin B₁ exposure.

C. Perturbation of DNA Transcription by Aflatoxin B₁

Research interest in the mode of action of AFB₁ on DNA transcription and the role of transcriptional perturbations in the liver pathogenesis of AFB₁ has been based on three observations. First, changes in the levels of RNA synthesis on liver cells after AFB₁ treatment are among the earliest
and most dramatic effects of exposure to the compound. Clifford and Rees (96) found that an LD50 dose of AFB1 to rats inhibited incorporation of [14C]-orotate into liver nuclear RNA by 80% within 15 minutes post dosing. Further, this high level of inhibition was found to be maintained up to 17 hours after initial toxin administration (97).

Second, the extent of AFB1-induced inhibition of transcription was observed to parallel species and tissue susceptibility to AFB1-induced toxic effects. Of the laboratory animals susceptible to the acute and chronic effects of AFB1, the mouse is among the most resistant, with a single dose LD50 ten times higher that that for rats of the same age (98). And unlike most species, the mouse responds with effects in the kidney, where large hemorrhagic lesions are found 48-72 hours after AFB1 administration; damage to liver tissue at these doses is minimal.

After the administration of a single lethal dose of AFB1, no alterations in the level of RNA synthesis within the mouse liver were observed up to 72 hours post administration (98). In the mouse kidney, however, RNA synthesis was inhibited more than 50% within 3 hours post dosing; this decrease was accompanied by a 50% decrease in the levels of kidney RNA which persisted for more than 72 hours. Inhibition of RNA polymerase activities in both the mouse liver and kidney paralleled the observed changes in total RNA synthesis. While no effect on mouse liver
polymerase activity was noted, a 40% decrease in kidney polymerase activity was evident two hours post dosing.

Third, interest in the perturbations of DNA transcription by aflatoxin B₁ was sparked by the dramatic changes in the morphology and ultrastructure of liver cell nucleoli in animals administered the toxin. The nucleolus is the nuclear organelle serving as the site for ribosomal RNA synthesis. Though accounting for 50% of total nuclear RNA synthesis, the nucleolus contains only 5% of total nuclear DNA; of this 5% only 0.5-1% codes for 45S rRNA, the precursor to 18S and 28S ribosomal RNA (99). Normally the nucleolus is composed of two fractions, the fibrillar and the granular. The fibrillar component is composed of RNA and DNA and serves as the main site for ribosomal RNA synthesis; the granular component is predominately composed of RNA and serves as the assembly site for ribosomal proteins with newly synthesized rRNA to form ribosomal subunits.

Within 15 minutes after administration of a 1 mg/kg dose to male rats, perturbations in the structure of liver nucleoli were observed (100). Most prominent of the observed structural alterations was the segregation of the granular and fibrillar components with the formation of nucleolar cap regions. Comparative time-course studies of nucleolar morphology changes with inhibition of nucleolar RNA polymerase activity found a close correlation between
these two parameters. At 1 hour after administration of a 1 mg/kg dose, maximal inhibition of nucleolar polymerase activity was accompanied by complete segregation of the two nucleolar components. By 36 hours post-dosing, liver nucleolar RNA polymerase activity and nucleolar RNA synthesis had returned to near control levels; similarly nucleolar capping was not demonstrable and the fibrillar and granular components were well integrated.

In a very real scientific as well as historical sense, elucidations of the underlying mechanisms by which AFB₁ inhibits DNA transcription have paralleled advances in the fractionation and understanding of nuclear RNA polymerases. Unlike bacterial RNA polymerases, which have been studied in great mechanistic detail, the eucaryotic polymerases are in close association with chromosomal proteins and DNA; thus their separation and characterization have been far more difficult. Windnell and Tata were the first to partially differentiate ribosomal RNA-specific polymerase activity from extranucleolar activity (101). In the presence of magnesium and low salt concentration, rat liver nuclei were observed to synthesize RNA in vitro similar to rRNA, while in the presence of manganese and high salt, "DNA-like RNA" (i.e. mRNA) was predominately synthesized, along with some rRNA. This separation of nuclear RNA polymerase activities by ion and salt concentration maxima was thus incomplete, and would prove to be only a first approximation of the true
disparate activities present. This point is crucial to the critical analysis of later studies on the preferential effects of carcinogens, particularly aflatoxin B₁, on nuclear RNA polymerase activity. Investigators as well as reviewers of the literature on this topic would inaccurately use these criteria to assess impairment of the nuclear and nucleolar polymerases.

Isolation of individual RNA polymerase species was successfully accomplished by the high salt extraction procedure of Roeder and Rutter (102,103). Using rat liver nuclei, RNA polymerases were solubilized by sonication in high ionic strength ammonium sulfate followed by centrifugation of the chromatin in a low ionic strength (NH₄)₂ SO₄ buffer and fractionation of the chromatin-free extract by DEAE-Sephadex chromatography. More recent improvements in these methods by Jacobs (104-106) and Yu (107-109) have permitted greater resolution of these protein species for study. RNA polymerase I, the nucleolar polymerase, is the smallest of the RNA polymerase proteins with a short half-life (t=1.3 hours) (109). Actinomycin D, a large heterocyclic antibiotic which binds adjacent to guanine residues, preferentially inhibits RNA synthesis by this enzyme due to the enrichment of guanine bases in nucleolar ribosomal RNA genes.

Extranucleolar RNA polymerase activity has been resolved into two distinct enzyme species. RNA polymerase II is
responsible for synthesis of heterogeneous nuclear RNA (hnRNA) which further processed into functional mRNA species. RNA polymerase III is the larger extranucleolar polymerase which synthesizes transfer RNA as well as the 5S RNA species associated with the 60S ribosomal subunit. Polymerase II has further proven amenable to study because of its sensitivity to α-amanitin, a toxin isolated from the poisonous mushroom *Amanita phalloides*. In sharp contrast to RNA synthesis inhibitors like actinomycin D which inactivate the DNA template, α-amanitin specifically interacts with the polymerase molecule; biochemical investigations of toxin binding by cosedimentation of the enzyme with $^{14}$C-methyl]-α-amanitin indicate that the stoichiometry of toxin-polymerase binding is 1:1 (105,110).

Investigations into the modes and sites of action underlying DNA transcription inhibition by aflatoxin B$_1$ have centered on the differential effects observed in nucleolar versus nuclear RNA synthesis. Lafarge and Frayssinet (111) first quantitated the incorporation of radioactive RNA precursors into nuclear and nucleolar RNA after toxin exposure. Two hours after a 0.5 mg/kg AFB$_1$ dose to partially hepatectomized male rats, total nuclear RNA synthesis was inhibited 67% while nucleolar synthesis was inhibited 92%. Additional studies using non-hepatectomized animals produced similar results indicating that liver regeneration did not greatly effect the differential
inhibition observed (112).

The preferential inhibition of nucleolar RNA synthesis was first attributed solely to preferential inhibition of nucleolar RNA polymerase activity. Pong and Wogan first attempted to quantitate AFB₁-induced inhibition of nucleolar and extranucleolar RNA polymerase activities by selective ionic strength and metal cofactor preference (100). Magnesium- and manganese-activated RNA polymerases in liver nuclei administered 1 mg/kg AFB₁ were found to be inhibited 60% and 55%, respectively; this inhibition was abolished by 36 hours after dosing and exhibited a dose dependence within the dose range of 0.05 to 0.5 mg AFB₁/kg body weight. Since, however, both nucleolar and extranucleolar RNA polymerases exhibit activity in the presence of manganese, as pointed out earlier, these observations of AFB₁-induced polymerase inhibitions cannot be attributed specifically to individual enzymes.

Saunders et al. first successfully isolated extranucleolar and nucleolar RNA polymerase enzyme fractions from the liver nuclei of AFB₁-treated animals (113). Following solubilization by sonic disruption, nuclear enzyme preparations were fractionated by DEAE-Sephadex chromatography and assayed using an exogenous DNA template (calf-thymus DNA). While nucleolar polymerase activity remained at control levels, extranucleolar RNA polymerase activity was inhibited 60% two hours post dosing; twenty-
four hours after toxin administration, extranucleolar RNA polymerase activity had returned to control levels, with kinetics similar to those observed using an *in vivo*-treated template. Studies by Akrinrimisi *et al.* further supported the conclusion established by Saunders: extranucleolar RNA polymerases are inhibited after exposure to AFB$_1$, but nucleolar RNA polymerase is not. Upon incubation with a rat liver microsomal fraction, aflatoxin B$_1$ inhibited extranucleolar RNA polymerase activity 40% when assayed with calf thymus DNA (114); nucleolar RNA polymerase I activity was not altered from control levels. Since only the polymerases were incubated with the metabolizing system, and not the DNA template, evidence first surfaced in these studies and those of Saunders that AFB$_1$ inhibits RNA synthesis in the nucleus by more than one mechanism. Given the large (90%) decrease in nucleolar RNA synthesis noted by Lafarge and Frayssinet using both an *in vivo*-treated template and polymerase, and the lack of inhibition observed by Akrinimisi employing a nontreated template, the dramatic decrease in RNA synthesis within the nucleolus appears to result from the impairment, by AFB$_1$, of the nucleolar DNA template itself.

Recent investigations by Yu have determined, both qualitatively and quantitatively, the multiple sites of action by which AFB$_1$ impairs nuclear DNA transcription (115). Using an elegant experimental design, nucleolar
and extranucleolar fractions from liver nuclei were isolated from rats 2 hours after a 3 mg/kg AFB₁ dose and assayed for the capacity to synthesize RNA. Actinomycin D was subsequently added to inactivate the endogenous DNA template and a synthetic DNA template, poly(dI-dC), insensitive to the effects of Actinomycin D, was added; this reaction mixture thus measured the effect of in vivo-administered aflatoxin B₁ on the nucleolar and extranucleolar polymerases. In addition, α-aminitin was added to the extranucleolar reaction mixture in order to assay the AFB₁-induced inhibition of RNA polymerase II versus RNA polymerase III.

In the nucleolar fractions isolated two hours after toxin administration, nucleolar RNA synthesis was inhibited 90%, 15% of which was retained by the enzyme (RNA polymerase I) when assayed with an exogenous template; most of the inhibition of nucleolar RNA synthesis was therefore due to the AFB₁-induced impairment of the nucleolar template activity. RNA polymerase II was found to be inhibited 50% compared to controls while RNA polymerase III, first studied a separate extranucleolar enzyme in this investigation, showed no inhibition of enzyme activity after aflatoxin B₁ exposure. These findings thus confirmed the hypothesis supported by the work of Sanders put forth earlier: the preferential inhibition
of nucleolar RNA synthesis by AFB₁ treatment is due to the impairment of nucleolar template function, while the quantitatively less severe inhibition of extranucleolar RNA synthesis is due principally to the inhibition of RNA polymerase activity.

D. Mechanisms of Chemical Carcinogenesis: Role of DNA Modification

Current concepts on the mechanisms by which chemical carcinogens induce tumor formation generally fall into one of two groups. The first, termed epigenetic mechanisms, assumes that neoplastic transformation of cells occurs by changes in the expression of the cellular genome without permanent alterations in DNA composition or structure (116). A fundamental basis for various epigenetic theories of cancer comes from observations on the development of organisms from single fertilized ova. During the periods of early cellular proliferation and organogenesis, many cells become committed to developmental programs and divide repeatedly to give rise to additional cells committed to these same developmental programs.

The studies of Jacob and Monod (117) provide bacterial models for mechanisms by which chemicals could induce proliferation of cells via alteration of normal transcriptional control mechanisms. The permanent alteration of genetic expression in bacteria via chemical interaction with regulatory proteins provides potential models of transcriptional controls on the expression and/or repression of information in target cells for carcinogenic
chemicals. If, as has been postulated, cellular differentiations during embryogenesis are the consequence of epigenetic phenomena, similar epigenetic modifications of cellular transcription or translation (or both) may also be operative in the transformation of normal cells to tumor cells of stable phenotype (118).

In contrast to epigenetic hypotheses, genetic mechanisms assume that cellular transformation from a normal to tumor phenotype is dependent on permanent alterations in genomic information (119). The evidence supporting the formulation of various genetic hypotheses for cancer has come from three principal areas. First, transformed cells exhibit a distinctive, heritable phenotype. A common characteristic shared by transformed cell in culture (120–122) as well as metastizing tumor tissues (123–128) is the expression of a dedifferentiated phenotype distinct from untransformed, normal cells. The stability of this dedifferentiated state as well as its inheritance by progeny cell populations suggests that, among the events mediating the action of chemical carcinogens, the permanent changes in DNA expression, as a result of changes in DNA structure, are among the events mediating the action of chemical carcinogens.

A second line of evidence supporting genetic mechanisms of chemical carcinogenesis has come from studies of UV-induced carcinogenesis (119). Patients with the syndrome
**Xeroderma Pigmentosum** are extremely susceptible to the
development of skin cancer as a consequence of external
exposure to sunlight. Using cells in culture from these
patients, it has been found that X.P. cells are greatly
impaired in their capacity for error-free repair of DNA
containing UV-induced or chemically-induced nucleic acid
lesions (129-133). Cells from X.P. patients are also more
sensitive to the toxic and mutagenic effects of carcinogens (132).
Studies by Stich and Lashes (132) have shown that both AFB$_1$
and the structurally related mycotoxin sterigmatocystin
induce mutation in X.P. cells at much lower doses than they do in
normal cells. The preferential susceptibility of X.P. cells
to cell transformation and mutation by carcinogens thus supports
the emphasis on DNA as one of the critical macromolecular
targets for chemical carcinogen action.

Third, evidence in support of a genetic mechanism for
chemical carcinogenesis comes from the correlation between
the carcinogenic and mutagenic properties shared by many chemicals
in bacterial and mammalian systems (6,8,9,). Investigations
employing several systems have shown a positive correlation
between the carcinogenic potency *in vivo* and mutagenic
potency *in vitro* for a series of related carcinogens (133).
Wong and Hsieh have tested various naturally occurring
aflatoxin derivatives and their animal biotransformation
products for bacterial mutagenic potency when coupled with a rat liver microbial enzyme preparation (5). The relative mutagenic potency of these aflatoxin species in this in vitro system qualitatively reflected their potency in vivo as animal carcinogens. Similar results were found by Ong and coworkers using *Neurospora crassa* systems (134-136). Additional support for direct links between mutation and cancer is provided by the formation of stable, but revertable, temperature-sensitive malignant transformants of BHK cells by treatment with N-nitrosomethylurea or 4-nitroquinoline-1-oxide (137).

For many years, the great diversity of structure among the classes of carcinogenic chemicals worked against the formulation of unifying theories to explain how xenobiotics could induce malignant transformation and tumor growth (138-140). With the discovery of DNA as the repository of cellular information and the observations on the heritability of the transformed phenotype, it became clear that DNA could be a target macromolecule for carcinogen action.

Research on the metabolism of chemical carcinogens, chiefly by the team of J. A. and E. C. Miller, has provided critical information needed to construct unifying theories of chemical carcinogen action. It is now recognized that most of the carcinogens to which humans or animals are exposed are really precarcinogens (141-145): these chemicals must be metabolized to ultimate forms that actually induce tumors.
This general pathway for the activation (and deactivation) of chemical carcinogens is given in Figure 2-5.

A common structural feature recognized for the ultimate forms of chemical carcinogens is that they appear to be strong electrophiles. Thus according to this metabolic pathway, proximate carcinogens are ingested and metabolized, either in target organs or at other sites, to electrophilic intermediates which can react with critical macromolecular targets such as DNA. The genetic (and/or epigenetic) consequences of these interactions are presumed to initiate the heritable and essentially irreversible changes that are characteristic of neoplastic transformations and tumors.

This unifying model for the metabolic activation of chemical carcinogens, along with the previously summarized evidence supporting genetic mechanisms of chemical carcinogenesis, has focused research efforts on elucidating the mechanisms by which chemical-DNA interactions produce a heritable, transformed phenotype in treated cells. Particular emphasis has been placed on determining not only the structure of the reaction products resulting from DNA adduction and modification, but also their biological activity as premutagenic and precarcinogenic lesions (146-150). The accessibility of regions of DNA to in vivo chemical modification and damage has in turn been found to be a function partly of
Figure 2-5 Metabolic pathway for the activation of environmental chemical carcinogens to ultimate carcinogenic forms.
Precarcinogen \(\rightarrow\)

Precarcinogen (Electron-deficient), Mutagenic \(\rightarrow\)

Ultimate Carcinogen(s) \(\rightarrow\)

Electrophilic (Electron-rich) Atoms (O, N, S, C) in Cellular Macromolecules \(\rightarrow\)

Carcinogen Residues Bound Covalently to Informational Macromolecules (DNA) \(\rightarrow\)

Specific Alterations in Critical Macromolecules \(\rightarrow\)

Expression of Altered Information \(\rightarrow\)

Growth of Clones into Gross Tumors
the structure and organization of chromatin DNA. As outlined in the subsequent chapters of this thesis, we have determined the levels of adduction within transcribed regions of the liver nuclear genome in animals administered the carcinogen aflatoxin B₁ (AFB₁). The evidence presented suggests that transcribed regions of RNA are preferentially susceptible to adduction and to functional impairment by this carcinogen. With respect to the genetic mechanism of carcinogenesis and the model for chemical carcinogen activation summarized above, these findings indicate that precarcinogenic and premutagenic lesions, as products of chemical-biological interaction, may occur to a greater frequency in these transcribed regions of DNA.
Part II

Chapter 3: Studies on AFB₁ Adduction of Deoxyribonuclease II-Sensitive and -Insensitive Fractions of Nuclear and Nucleolar Chromatin DNA

Section A - Nuclear Studies

Section B - Nucleolar Studies
Chapter 3

Studies on AFb₁ Adduction of DNase II-Sensitive and -Insensitive Fractions of Nuclear and Nucleolar Chromatin DNA

Section A - Nuclear Studies

Introduction - Literature Review and Perspective

Single-celled organisms, such as bacteria, are comprised of a cell within which all the various categories of biochemical activities required for existence must be housed. Multicellular organisms possess the potential for cell specialization and new levels of organization in which particular groups of cells are designated to perform specific physiological or biochemical functions. Cell specialization thus provides living organisms a potential for development within their environment beyond the levels attainable by procaryotes (160).

All the different cell types in an organism arise from one cell, however - the fertilized egg. During development, cells derived from the many divisions of the fertilized egg become committed to increasingly specific developmental programs (161). To accomplish this diversity of potential phenotypes from a cell population sharing a common genotype, eucaryotic cells exercise several mechanisms for controlling gene expression.

Transcriptional control of the expression DNA sequences is one mechanism controlling eucaryotic gene expression. In
differentiated eucaryotic cells, a limited and tissue-specific fraction of nuclear DNA sequences is transcribed (19). This fraction, termed transcribed DNA, comprises approximately 10-15% of total nuclear DNA. Electron micrographic evidence first suggested that these transcribed gene sequences are morphologically distinct within the chromosomal complement of DNA. Transcribed gene sequences are observed to be maintained in a discrete, active conformation distinguishable from nontranscribed DNA. The morphologically distinct loops on chromosomes known as lampbrush structures were among the first such structures demarcating transcribed gene sequences to be described. Sheer (162) has presented conclusive evidence for the close relationship between the development of lampbrush chromosome loops and the transcriptional activity of specific gene sequences. In *Xenopus laevis* oocytes, lampbrush structures are observed in the heavily transcribed ribosomal RNA genes of the nucleolus. In oocytes treated with an inhibitor of transcription, the mycotoxin actinomycin D, reduction in the number of lampbrush chromosome loops was found to parallel the inhibition of 45S ribosomal RNA synthesis.

The polytene chromosome puffs of *Diptera* also have provided evidence for the maintenance of a discrete, active conformation within transcribed DNA sequences. In studies with the Diperan *Chironomus tentans*, Clever observed the patterns of polytene chromosome puffing at various stages of larval development (163,164). It was found that these
puffing patterns followed a fixed sequence with specific bands puffing at one stage and regressing at a later stage. In larvae injected with the hormone ecdysone, which induces molting, chromosome puffing patterns were observed which are known to take place just before molting, a time when the insect spontaneously releases ecdysone into the hemolymph. These observations indicated that the hormone alters the structure and conformation of molting-specific genes which accompany the initiation of transcription of molting-specific DNA sequences.

The need to investigate the structural and functional differences between transcribed and nontranscribed regions of chromatin has spurred the development of strategies for chromatin fractionation. Two principal methodological problems are encountered. To avoid cross-contamination of transcribed and nontranscribed DNA sequences, nuclear chromatin must first be sheared into small fragments (19,33); this shearing method should not lead to appreciable protein denaturation, rearrangement, or dissociation. Second, a method is required to permit physical separation of transcribed chromatin from nontranscribed chromatin. In addition, one basic criterion must be met by the transcribed fractions separated from total nuclear chromatin: the transcribed fractions must be enriched in DNA sequences complementary to cellular RNA species.
The first fractionation techniques to be developed for selectively separating transcribed chromatin were based on mechanical shearing of nuclear chromatin preparations followed by continuous gradient or step gradient centrifugation (165-167). Rodriguez and Becker (168) separated sheared rat liver chromatin by glycerol gradient centrifugation into fractions termed euchromatic and heterochromatic, based on their capacity to support \textit{in vitro} RNA synthesis by \textit{E. coli} RNA polymerase. Separation in these glycerol gradients was based on the slow sedimentation of template-active (euchromatic) fractions and the rapid sedimentation of the template-inactive (heterochromatic) fractions. This separation strategy was founded on the tight, denser coiling of heterochromatin compared to the open, less-dense coiling of euchromatin that had been observed in electron micrographs.

A second characteristic used to identify the template-active chromatin fractions in these glycerol gradients was the cosedimentation of nascent RNA. In sheared chromatin preparations from animals administered the radioactive RNA precursor $^3$H-orotic acid, more than 90% of the nascent RNA formed \textit{in vitro} cosedimented with the template-active fraction. Anderson et al. similarly separated a template-active fraction of sheared rat ventral prostate chromatin (169). L (light) chromatin, a quantitatively minor, structurally less compact chromatin fraction which remained near the top
of sucrose density gradients, was classified as a transcriptionally-enhanced fraction of the isolated chromatin preparations. This classification was based on: (a) the association with the L chromatin fraction of trichloroacetic acid-insoluble radioactivity after in vivo administration of 14C-uridine or orotic acid or after in vitro incubation with E. coli RNA polymerase and radioactive precursors; and (b) a 10-20 fold greater template activity of L chromatin compared to whole nuclear chromatin when assayed with the bacterial polymerase.

Bonner et al. pioneered the use of deoxyribonucleases for selectively liberating transcribed portions of nuclear chromatin (19,170-172). Deoxyribonuclease II was found to attack preferentially a minor portion of chromatin DNA which was enhanced in its ability to serve as a template for exogenous RNA polymerase compared to the bulk of nuclease-resistant chromatin. Furthermore, DNase II-fractionation experiments using chromatin prepared from hepatoma cells that had been pulse-labeled with 3H-uridine revealed that over 60% of the label fractionated with the 10% of DNase II-sensitive chromatin (19).

Digestion of rat liver chromatin preparations with DNase II followed by selective treatment of the DNase-sensitive fraction with divalent cations such as Mg²⁺ was found to yield a chromatin fraction that differed in
three ways from unfractionated chromatin, thus indicating enrichment in transcribed sequences. First as compared to unfractionated chromatin (19), the nuclease-sensitive fraction was enriched in nonhistone proteins but depleted of histone proteins (Table 3-1). This is consistent with the previously described ultrastructural studies by Sheer, in which transcribed DNA was observed to be deficient in histone proteins but enriched in nonhistone proteins, including RNA polymerase. Second, the DNase-sensitive fraction, as summarized in Table 3-1, was 7-fold superior to nuclease-insensitive chromatin as a template for RNA synthesis.

Third, hybridization of nuclease-sensitive and -insensitive chromatin DNA to sheared, total liver RNA under conditions of vast RNA excess indicated that the nuclease-sensitive DNA was enriched in expressed gene sequences. Total cell RNA from rat liver was sheared to an average size of 1000 nucleotides, incubated with DNA from either chromatin fraction, and the RNA:DNA hybrids isolated by hydroxyapatite chromatography. The DNase-sensitive fraction was found to be enriched approximately 4-fold in sequences that code for cellular RNA. From the evidence above, it was concluded that this fractionation procedure produces a chromatin fraction enriched in a subset of the transcribed genomic sequences.

The fundamental biological properties which cause
Table 3-1 Properties of Nuclease-Sensitive and Nuclease-Resistant Chromatin Fractions Isolated by Selective Deoxyribonuclease II Treatment of Rat Liver Chromatin. Taken from the report of Gottesfeld and Coworkers (19).

<table>
<thead>
<tr>
<th>Chromatin Sample</th>
<th>% Chromatin* DNA</th>
<th>Template Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Nuclease-Sensitive</td>
<td>11.3 ± 3.9</td>
<td>65</td>
</tr>
<tr>
<td>Nuclease-Insensitive</td>
<td>84.6 ± 4.8</td>
<td>9</td>
</tr>
</tbody>
</table>

*Mean of 11 determinations ± standard deviation as estimated by absorbance at 260 nm.

**Percent template activity as assayed according to McConnell and Bonner (173).
transcribed chromatin to be preferentially recognized by deoxyribonucleases has yet to be determined. Analysis of the chromatin digestion products after DNase I treatment has revealed the presence of periodic cleavage sites in DNA that liberate nucleosomes, the basic chromosomal subunits that consist of DNA periodically folded around a histone core, creating a string of DNA-protein particles; these core particles are connected by lengths of DNA, termed linker DNA (174-177). Analysis of the DNA cleavage products in molecular hybridization reactions with complementary DNA transcripts of specific genes has demonstrated that this subunit periodicity is maintained over transcribed segments of the genome (178-181). Since the interaction of histones with DNA is responsible for the generation of the nucleosomal repeat, these studies have suggested that some histones reside along transcribed DNA and that the mere presence of histone proteins is not sufficient to restrict transcription.

Chemical modifications of the amino acid backbone of histones, however, have been shown to correlate with the deoxyribonuclease sensitivity of gene sequences. Considerable post-synthetic modification occurs with histone proteins: they occur at specific times in the cell cycle and at specific sites on histone macromolecules as a consequence of the action of specific enzymes (182). The types
of *in vivo* histone modifications include acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination. In no case are all histone molecules modified in the same way at the same time; the amount of histone modified can range from a few percent to almost 100%.

A rôle for histone modification in controlling gene activity is suggested by the contrast between the extremely conservative amino acid sequence of the histones as a group and the variability of histone post synthetic modifications (182). Clearly if a conservative amino acid substitution, e.g. from leucine to isoleucine, is a forbidden one, then the introduction of a methyl group or the change of charge within the histone amino acid sequence could have profound effects on some aspects of histone-DNA interactions. Both observations combine to strongly implicate these post-synthetic modifications as important in determining the biological activities of histones.

Of the various chemical modifications of histones, acetylation of histone-contained lysine residues has been clearly shown to correlate with the deoxyribonuclease sensitivity of chromatin regions. Davie and Candido have presented evidence for the hyperacetylation of histone H4 in nuclease-sensitive genes (183). Chromatin from trout testis at an early stage of development were digested with DNase II and the solubilized products fractionated into Mg2+-soluble
and -insoluble components according to the methods of Gottesfeld et al. described earlier (19,184). An examination of the histones from these fractions showed that highly acetylated species of histone H4 (di-, tri-, and tetra-acetylated) were associated principally with the DNase II-soluble chromatin fraction. These results are consistent with those of Levy-Wilson and coworkers, who applied this procedure to Drosophila cells and observed a 40% increase in the incorporation of labeled acetate into the histone fraction of nuclease-sensitive chromatin (185).

Specific types of non-histone proteins have also been shown to be associated preferentially with transcribed gene sequences. The HMG (high mobility group) proteins are a group of non-histone chromosomal proteins present in high concentrations, $10^5 - 10^6$ molecules per nucleus, in a variety of eucaryotic cells (186). Of the major HMG proteins purified to homogeneity, HMG 6 in trout testes and HMG 14 and 17 in the calf thymus and chicken erythrocyte have been shown to be major stoichiometric structural components of nucleosome cores which are highly enriched in transcribed DNA sequences (187-188). HMG's 14 and 17 also have been shown to be responsible for conferring DNase sensitivity to transcribed gene sequences (189). The globin gene, a gene transcribed only in the erythrocyte, was found to be sensitive to digestion by DNase I in erythrocyte chro-
matin but not in brain, fibroblast, or oviduct chromatin. Removal of HMG 14 and 17 from these four chromatin preparations by moderate salt extraction, which maintains normal nucleosomal structure, was found to destroy the selective DNase-sensitivity of globin genes; reconstitution of the depleted chromatin fractions with HMG 14 and 17 resulted in successful reconstitution of the selective DNase sensitivity of the globin gene.

As with histone proteins, nonhistone HMG proteins have been shown to undergo post-synthetic chemical modification that correlates with the deoxribonuclease sensitivity of gene sequences. Upon fractionation of chromatin from butyrate-treated cells, Levy-Wilson found that most of the HMG proteins associated with nucleosomes preferentially sensitive to micrococcal nuclease were enriched in transcribed gene sequences (190); electrophoresis of HMG's 14 and 17 from these nucleosomes resolved them into a series of bands resulting from hyperphosphorylation of the HMG amino acids. Levinger and Varshavsky have also shown selective association of a second non-histone protein, ubiquitin, within transcribed regions of the Drosophila genome (190). Two-dimensional hybridization mapping was performed on nucleosomes from the transcribed copia and heat shock 70 genes as well as nontranscribed satellite DNA. While 50% of the nucleosomes from the transcribed genes contained ubiquitin
-H2a (a histone–ubiquitin conjugate), less than 4% of the nucleosomes of tandemly repeated, nontranscribed satellite DNA contained U-H2A. These results suggested the preferential localization of ubiquitin within transcribed gene sequences.

Histoné and nonhistone proteins are modified in a variety of ways and to varying extents within transcribed regions of chromatin: these differences in modifications among transcribed genes, however, do not result in differing sensitivities to DNase activity. Using the enzyme DNase I, Garel and coworkers have compared the nuclease digestion of three genes in chick oviduct nuclei transcribed at different rates (191). Genes for ovalbumin, the major protein synthesized in oviduct cells, were very sensitive to DNase I digestion in oviduct chromatin, while the nontranscribed globin genes were digested at a 5-fold lower rate in this tissue. Another set of transcribed genes, however, which is transcribed at frequencies orders of magnitude lower than that of the ovalbumin genes, and are rarely represented in oviduct mRNA, were as sensitive to DNase I treatment as were the ovalbumin genes. These results suggested that the maintainence of an active conformation about transcribed genes is not correlated with the polymerase distribution within a particular gene. The active conformation is therefore not confined to sequences actively engaged in
transcription, but rather reflects structural properties about a subpopulation within a cells' chromatin complement that represents the transcriptional potential of a particular cell type.

Transcribed regions of nuclear chromatin, biochemically distinguishable by their preferential susceptibility to deoxyribonuclease attack and digestion, are also distinguishable by a second criterion: nuclease-sensitive chromatin DNA is preferentially susceptible to covalent modification by chemical carcinogens. When chromatin preparations from carcinogen-treated animals are deoxyribonuclease digested, the levels of adduction within the liberated fractions, enriched in transcribed DNA sequences, are found to exceed the levels of binding to the remaining nuclease-insensitive chromatin fraction. Moyer et al. isolated liver chromatin from rats administered the hepatocarcinogen N-hydroxy-2-fluorenylacetamide (N-OH-2-FAA) and separated euchromatic (transcriptionally-active) and heterochromatic (transcriptionally-inactive) chromatin fractions via sedimentation through sucrose gradients as discussed earlier in the studies of Anderson et al. (192,169). The greater transcriptional capacity of the slowly sedimenting (euchromatic) fractions was confirmed in this report by enhanced incorporation of radioactive precursors into RNA sedimenting with this fraction and by the increased template
activity of the slowly sedimenting chromatin fraction for *in vitro* RNA synthesis by purified RNA polymerase. N-OH-FAA bound 3.3-fold more to euchromatic DNA than in heterochromatic DNA 2 hours after a single i.p. injection of the compound. The bound carcinogen was also more rapidly removed from the euchromatic DNA fraction: over a period of 48 hours post dosing, bound radioactivity was removed twice as fast from the euchromatic DNA fraction as from the heterochromatic DNA fractions.

As described in this first section of chapter 3, we have investigated the kinetics of carcinogen binding within regions of deoxyribonuclease II-sensitive chromatin DNA which are enriched in transcribed gene sequences. We have developed protocols which permit the isolation of a DNase II-sensitive chromatin fraction from the liver of aflatoxin B₁-treated animals enriched in transcribed gene sequences as shown by hybridization with two classes of RNA: 18S and 28S ribosomal RNA and 4S transfer RNA. As described below, the time-course of AFB₁ adduction to liver DNA from DNase II-sensitive and DNase II-insensitive chromatin has been determined. Preferential adduction to nuclease-sensitive chromatin DNA has been found. Furthermore, evidence is presented indicating that, within these DNase II-sensitive fractions of chromatin DNA, bound aflatoxin residues are removed at a faster rate than from nuclear DNA as a whole.
Methods and Materials

Chemicals

AFB₁ was purchased from Makor, Inc. (Jerusalem, Israel). [³H] AFB₁ (specific activity, 20 Curies/millimole) and [¹⁴C] AFB₁ (specific activity, 40 millicuries/millimole), both with radiochemical purities of greater than 98%, were purchased from Moravek Biochemicals (City of Industry, California). Ultra-pure sucrose (DNase and RNase free) and cesium chloride were obtained from BRL, Inc. (Gaithersburg, MD). Tris buffer, cesium sulfate (grade 1), deoxyribonuclease II (DN-II-HP) from porcine spleen, and Ribonuclease A (R-4875) were purchased from Sigma Chemical Co. (St. Louis, MO). [³H] Thymidine (specific activity 20 Curies/millimole), [³H] Orotic Acid (specific activity 20 Curies/millimole), [³²P]-gamma-ATP (specific activity 1000-3000 Curies/millimole), and T₄ polynucleotide kinase were purchased from New England Nuclear Corp. (Boston, Mass.). Nitrocellulose filters (Type HA, 13 mm diameter, 0.45 micron pore size) were from Millipore Corp. (Bedford, Mass.).
Animals

Male CDF Fischer rats (Charles River Breeding Labs, Wilmington, Mass.) were housed in pairs in suspension wire cages and allowed Zeigler Bros. rat and mouse ration (Gardner, PA) and water ad libitum. Carcinogen dosing of rats (125-150 grams) was by intraperitoneal injections of [3H]-AFB1 dissolved in 0.05 ml of glass-distilled dimethyl sulfoxide (Burdick and Jackson Labs, Muskegon, Mich.).

Preparation of Nuclei

Purified nuclei were prepared by a modification of the Blobel-Potter method (197). All procedures were performed at 0-4°C. Animals were stunned and decapitated, and their livers were perfused in situ via the portal vein with 10 ml of ice cold TSKM buffer [0.025 M Tris-HCl, pH 7.0 : 0.25 M sucrose : 5.0 mM KCl : 3.3 mM MgCl₂]. The excised livers were blotted, weighed, and minced into TSKM buffer + 1.0% Triton X-100 (v/v) (7.5 ml buffer per gram of liver) with a tissue press (Harvard Apparatus Co., Inc., Harvard, Mass.). The suspension was homogenized by hand with 12 strokes of a glass dounce homogenizer (tight pestle), filtered through 2 layers of cheesecloth, and finally filtered through a layer of 260 gauge nylon mesh. The filtrate was centrifuged for 10 minutes at 650 x g at 4°C, and the resulting pellet was washed twice in TSKM buffer alone.
The crude nuclear pellet was resuspended in TSKM buffer, and the molarity adjusted to 1.5 molal using 0.025 M Tris-HCl : 2.2 M Sucrose and centrifuged for 30 minutes at 172,000 x g in a SW-40 Ti Beckman rotor to yield a clear nuclear pellet as judged by phase microscopy of a nuclear sample stained with 0.1% toluidine blue : 0.25 M sucrose. The yield of nuclei, measured by the quantitative recovery of DNA from the original liver homogenate, was 55-60%.

Isolation and Digestion of Nuclear Chromatin

All glassware used in chromatin isolation steps was washed with 0.01% diethyl pyrocarbonate (DEP) (Sigma Chemical), autoclaved for four hours, and finally silanized to minimize nonspecific protein adsorption.

Nuclear chromatin was isolated by the method of Rodriguez and Becker (198) as modified by Schwartz and Goodman (199). The nuclear pellet was resuspended in TS buffer (0.025 M Tris-HCl pH 7.0 : 0.25 M Sucrose) at 1 ml/gram liver, pelleted by centrifugation and washed briefly in 20 mM Tris-HCl, pH 7.0 (Tris Lysis Buffer – TLB) at 1.5 ml/gram liver. The washed nuclei were resuspended in TLB and homogenized by five to ten strokes of a close-fitting dounce homogenizer; disruption of nuclei was checked as described previously by phase microscopy. The nuclear
lysate (4.5 ml) was layered over sucrose step gradients consisting of 1.6 M Sucrose - 0.025 M Tris-HCl, pH 7.0 (bottom layer, 4.5 ml) and 1.3 M Sucrose - 0.025 Tris-HCl, pH 7.0 composed in a chilled SW40 Beckman polyallomor centrifuge tube. The step gradients were subsequently centrifuged at 40,000 rpm for 90 minutes in a SW40 Ti rotor at 4°C.

The resulting chromatin pellet was washed with 25 mM sodium acetate, pH 6.6, with complete resuspension between each of the two washings. After the final wash, the chromatin solution was adjusted to give an A_{260nm} of approximately 10 (measured in 0.9 N NaOH). The solution was brought to 24°C and DNase II was added from a stock solution of 1000 units/ml to yield a final concentration of 100 units/ml. The reaction was allowed to proceed for 5 minutes, after which the reaction mixture was cooled in ice slush and adjusted to pH 7.2 by the addition of 200 mM Tris-HCl, pH 11.

Nuclease-resistant chromatin (labeled P1 fraction) was removed by centrifugation in a SW40 Beckman rotor for 20 minutes at 14,500 rpm, 4°C. The nuclease-sensitive supernatant was transferred to 30 ml beakers, cooled to 4°C, with a small stirring bar in the bottom; one ninety-ninth volume of 200 mM MgCl₂ was added dropwise (final concentration 2 mM) while the chromatin sample was slowly stirred. After 30 minutes of stirring, the chromatin suspension was centri-
fuged again in SW 40 tubes as described above yielding a pellet (labelled P$_2$) and a supernatant fraction (labelled S$_1$).

**Isolation of DNA from Chromatin Fractions**

To the nuclease-sensitive supernatant fractions (S$_1$ fraction), one 200th volume of sodium sarkosinate (ICN, Inc.) was added and the sample homogenized with 6 to 8 strokes of a tight-fitting teflon pestle in a glass homogenizer. To the nuclease-resistant pellet (P$_1$ fraction), 1.25 ml sarkosyl buffer (50 mM HEPES, pH 7.0: 0.5% sodium sarkosinate: 5 mM EDTA pH 7.0) was added per gram of rat liver and the mixture was homogenized. To each 1.25 ml of homogenized S$_1$ or P$_1$ fraction, 6.98 ml of 6M CsCl and 0.77 ml of 3 M Cs$_2$SO$_4$ were added and the mixture homogenized with 6 to 8 homogenizer strokes.

The cesium salt-DNA mixture was loaded into quick-seal polyallomar tubes (18 or 27 ml each), capped with mineral oil, and centrifuged in a VTi50 Beckman rotor, 45,000 rpm, 20°C, for 10-12 hours. Special precautions were employed when using this rotor: slow acceleration must be used up to 5000 rpm to permit the cesium salt gradients to form. Similarly, a slow brake must be applied during decelleration from 5000 rpm. DNA was collected by fractionating the gra-
dient with a density gradient fractionator (Instrumentation Specialties Corp., Lincoln, Nebr.) monitoring the gradient contents for absorbance at 254 nanometers. Binding levels of AFB₁ to DNA were calculated by sampling the DNA peak for [³H]AFB₁ by liquid scintillation counting and for DNA content by absorbance at 260 nanometers and by the colcrometric reaction with diphenylamine as described by Giles and Myers (200).
Filter Hybridization of Nuclease-sensitive and -insensitive Chromatin DNA Fractions with Ribosomal and Transfer RNA

To determine the enrichment of the DNase II-sensitive DNA fractions in transcribed gene sequences, samples of nuclease-sensitive and -insensitive liver DNA were bound to nitrocellulose filters and hybridized to saturation with liver ribosomal RNA or transfer RNA, as described by Mertz and Gurdon (201,202). DNA from these two DNase fractions was isolated from AFB$_1$-treated animals via cesium salt centrifugation and precipitated after dialysis against 10 mM Tris-HCl, pH 7.0, 2 mM Na$_2$EDTA, with $-70^\circ$C ethanol. DNA aliquots were denatured in 0.15 M NaOH for 10 minutes, transferred to an ice slush, neutralized with 1 N HCl, and diluted 0.1 mg/ml with SSC buffer (0.15 M NaCl, 0.015M sodium citrate).

DNA was bound to nitrocellulose filters as described by Gillespie and Spiegelman (201). 1.5 micrograms of DNA from each denatured DNA fraction was diluted with 1.0 ml of 2XSSC (0.30 M NaCl, 0.030M sodium citrate). 13mm nitrocellulose filters (0.45 micron pore size) were cut in half, presoaked in 2XSSC for 1 minute and washed with 10 milliliters of the same using a Millipore
filter manifold. After filter washing, the denatured DNA was passed through a filter, then washed twice with 10 ml of 2x SSC. The DNA filters were subsequently dried at room temperature for 4 hours and in a vacuum oven at 80°C for exactly two hours.

DNA filters were incubated in 0.3 ml of 4 x SSC and 0.2% SDS with 0.025 mg/ml of rRNA or tRNA in 5 ml Precision-Reaction vials at 68°C for 24 hours. Filters containing [3H]-labelled rat liver DNA were also incubated to determine the amount of DNA lost during the incubation periods. Ribosomal RNA and transfer RNA were isolated as described in Appendix #2 and [32P]-labeled by incubation with [32P]-gamma-ATP and T₄ polynucleotide Kinase.

After incubation with [32P]-RNA, the DNA filters were washed 4 times with 10 ml of 2XSSC and incubated with Ribonuclease A (heat treated to inactivate DNase activity) at a concentration of 0.02 mg/ml in 2X SSC at 30°C for 30 minutes to remove nonspecifically bound RNA. The filters were subsequently dried at room temperature for 2 hours and the filter-bound [³²P] radioactivity determined by scintillation spectroscopy. Filters containing no DNA were simultaneously incubated in [32P]-labelled rRNA, and the nonspecifically
absorbed RNA background subtracted from each timepoint.
Results and Discussion

Isolation of DNase II-sensitive and -insensitive Chromatin Fractions From AFB₁-Treated Animals.

To examine the distribution of AFB₁ residues within rat liver DNA fractions enriched in transcribed sequences, we have adapted the DNase II - MgCl₂ chromatin fractionation procedure of Gottesfeld et al. for our studies using liver chromatin from AFB₁-treated animals. Chromatin was isolated from a clean liver nuclear preparation as outlined in Figure 3-1 and sheared by incubation with DNase II for 5 minutes. Unsheared chromatin was removed by centrifugation, yielding a pellet termed P₁: as summarized in Table 3-2, this fraction comprised 78% of the input DNA. The resulting supernatant fraction (termed S₁) was subsequently fractionated on the basis of solubility in 2 mM MgCl₂ into a second supernatant fraction (S₂) and a minor insoluble fraction (P₂). The second supernatant fraction S₂ comprises 12% of the input chromatin DNA.
Figure 3-1  Isolation of DNA from nuclease-sensitive and -insensitive chromatin fractions.
Isolation of DNA from DNase II Sensitive and Insensitive Nuclear Chromatin Fractions

$[^3H]-AFB_1$ $\rightarrow$ Rat $\rightarrow$ Liver $\rightarrow$ Nuclei $\rightarrow$ Nuclear Chromatin

DNase II (100 units/ml) $\rightarrow$ 25mM NaAcetate, pH 6.6 $\rightarrow$ Incubate 5 minutes at 24°C $\rightarrow$ Centrifuge (25,000 g, 20 min.)

Pellet (DNase II-Insensitive) Chromatin $\rightarrow$ Supernatant

Supernatant (DNase II-Sensitive) Chromatin $\rightarrow$ Pellet Residue

Solubilize with Sarkosyl Detergent Buffer $\rightarrow$ Add 6M CsCl, 3M Cs$_2$SO$_4$ $\rightarrow$ Centrifuge $\rightarrow$ Isolate DNA $\rightarrow$ Quantitate AFB$_1$ binding
Table 3-2  Distribution of DNA Within Rat Liver Chromatin Fractions Following Partial DNase II Digestion

<table>
<thead>
<tr>
<th>Chromatin Sample</th>
<th>% Total Chromatin DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated Chromatin</td>
<td>100</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
</tr>
<tr>
<td>$P_1$: DNase II-Insensitive</td>
<td>78.0 ± 4.8</td>
</tr>
<tr>
<td>$S_2$: DNase II-Sensitive</td>
<td>11.9 ± 4.8</td>
</tr>
<tr>
<td>$P_2$: DNase II-Insensitive</td>
<td>10.4 ± 3.7</td>
</tr>
<tr>
<td>contamination of the sensitive fraction</td>
<td></td>
</tr>
</tbody>
</table>

* Mean of 9 determinations ± Standard Deviation. DNA was measured by the diphenylamine colorometric reaction according to the procedure of Giles and Myers (198).
DNA was isolated from three chromatin fractions for quantitation of bound AFB₁ residues by cesium chloride/cesium sulfate density gradient centrifugation: the DNase II-sensitive fraction (S₂), the DNase II-insensitive fraction (P₁), and liver chromatin digested with DNase II but not further fractionated (control). The choice of cesium salt density centrifugation to isolate chromatin DNA was based on two factors. First, the procedure permits a relatively rapid isolation of purified DNA, a benefit compared to the lengthy digestion of phenol-extracted DNA preparations with RNase and protease enzyme preparations. Second, the purity of the isolated DNA fraction is superior to that achieved by phenol-extracted DNA preparations. Figure 3-2 illustrates a typical CsCl/Cs₂SO₄ gradient onto which a whole chromatin preparation has been loaded. The solid line represents the absorbance of each fraction at 254 nm, and clearly shows that the DNA fraction is in the center of the gradient. Absorbance at the top of the gradient represents contaminant proteins, while absorbance at the bottom of the gradient represents contaminant nuclear RNA. The chromatin samples illustrated in Figure 3-2 were isolated from rats administered not only 1 mg AFB₁/kg but also either [³H]-orotate (top diagram) or [³H]-leucine (bottom diagram) to quantify the level of RNA and protein contamination, respectively, in the DNA isolate. RNA contamination was found to be less that 1.2% of the DNA fraction (w/w), while protein contamination was less than 0.9% (w/w).
Figure 3-2  Profile of DNA isolated by cesium salt density centrifugation. Liver chromatin samples were prepared from rats administered a 1 mg/kg AFB1 dose and either [3H] orotic acid or [3H] leucine 30 minutes prior to sacrifice. Chromatin samples were prepared as described in the text and fractionated on CsCl/Cs2SO4 density gradients.
The DNase II - Mg\textsuperscript{2+} protocol employed here was adapted from the procedure originally developed by Gottesfeld et al. for isolating fractions of liver chromatin enriched in transcribed gene sequences (19). The theoretical basis of the protocol was built on two facts: a) DNase II preferentially nicks DNA within transcribed genes and b) over short periods of digestion (5-10 minutes), nontranscribed regions of DNA are relatively resistant to nuclease digestion. Time periods within a range of from 2 to 30 minutes have been reported in the literature for DNase II digestion of chromatin to preferentially liberate putative transcribed portions of chromatin.

To determine the optimum time of digestion for liver chromatin fractions isolated from AFB\textsubscript{1}-treated animals by the procedure outlined in Materials and Methods, the kinetics of RNA and DNA release was followed over a 30 minute digestion period (Figures 3-3 and 3-4, respectively). Figure 3-3 summarizes the release of tritium from chromatin fractions isolated from animals administered both AFB\textsubscript{1} and [\textsuperscript{3}H]-orotate as described above. The release of RNA-bound tritium is rapid during the initial four minutes of digestion (phase 1) and subsequently begins to decrease, reaching a second phase by 17 minutes of digestion. Given the predilection of DNase II to cut within transcribed gene sequences, this kinetic profile of RNA release would indi-
Figure 3-3  Kinetics and time course of DNase II digestion of liver nuclear chromatin from animals administered 1 mg/kg AFB₁ and [³H]-orotate 30 minutes prior to sacrifice. Chromatin was prepared as described in the text, and replicate aliquots were digested with DNase II for the times shown. As each timepoint, the undigested chromatin was precipitated by centrifugation and the radioactivity released into the supernatant quantified by liquid scintillation counting.
Figure 3-4 Kinetics and time course of DNase II digestion of liver nuclear chromatin from animals administered a 1 mg/kg AFBl dose and [3H] thymidine 30 minutes prior to sacrifice. Chromatin was isolated as described in the text; DNase II digestion and quantitation of enzyme-solubilized radioactivity were as performed in the description of Figure 3-3.
cate the rapid nicking of the transcribed gene regions up until 4-7 minutes of digestion since chromatin bound, nascent RNA is concentrated within the transcribed areas of chromatin. As seen from the digestion profile, 80% of the chromatin-bound RNA is released by 16 minutes after digestion when phase 2, which represents a plateau of RNA release, is reached. For the isolation of AFB₁-adducted DNase II-sensitive chromatin DNA sequences, a 5 minute digestion period was chosen, as this represents the maximum of the phase 1 release of RNA.

A similar enzyme digestion profile was found upon DNase II digestion of liver chromatin fractions isolated from AFB₁-treated animals additionally administered [³H]-thymidine (Figure 3-4). Again, as in the case of RNA, [³H]-thymidine release was rapid during the initial 4 minutes of digestion (phase 1) which subsequently began to decrease, reaching a second phase by 18 minutes of digestion. Again given the predilection of DNase II to cut within transcribed gene sequences, this kinetic profile suggested a five minute digestion period for maximal separation of the transcribed component of liver chromatin from AFB₁-treated animals.

To determine the enrichment of the DNase II-solubilized chromatin fraction in transcribed gene sequences, samples of DNA from fraction S2 (DNase sensitive chromatin) and fraction P1 (DNase-insensitive chromatin) were bound to nitrocellulose filters and hybridized with
[32p]-end labelled ribosomal RNA or transfer RNA as detailed by Mertz and Gurdon (199, 200) (Figures 3-5 and 3-6). As shown in Figure 3-5, nitrocellulose filters containing 1.5 micrograms of DNA from each of the two DNA fractions were hybridized for increasing periods of time with 7.5 micrograms rRNA (0.025 mg/ml). Saturation hybridization for both types of DNA was reached by 24 hours of hybridization. The saturation values were 0.21% and 0.04% of total nuclear DNA for fraction S2 and P1, respectively, indicating a 5.1-fold enrichment of the ribosomal RNA genes in the Mg2+-solubilized nuclease-sensitive fraction.

Similarly, nitrocellulose filters containing 1.5 micrograms of each DNA fraction were hybridized to [32P]-end labeled transfer RNA isolated from rat liver. Filter-bound DNA was again incubated with 7.5 micrograms of RNA for increasing periods of time and the percent DNA bound as a DNA-RNA hybrid calculated from the bound [32P]-radioactivity, after extensive washing with buffer and incubation with Ribonuclease A to aid in the removal of nonspecifically attached probe tRNA. As shown in Figure 3-6, saturation of hybridization was reached for fraction P1 DNA by 20 hours post-incubation, while DNA from fraction S2 did not reach saturation of hybridization until 28 hours post incubation. The saturation values were 0.0084% for DNase-sensitive chromatin DNA and 0.002% for nuclease-insensitive chromatin DNA indicating a 4.2-fold enrichment of the tRNA genes in the Mg2+-solubilized fraction of nuclear chromatin.
Figure 3-5 Hybridization of DNase II-sensitive and DNase II-insensitive chromatin DNA fractions to $^{32}\text{P}$-end labelled ribosomal RNA.
Figure 3-6  Hybridization of DNase II-sensitive and DNase II-insensitive chromatin DNA fractions to $^{32}$P-end labelled rat liver transfer RNA.
Binding of Aflatoxin B\textsubscript{1} to DNase II-sensitive, DNase II-Insensitive, and Unfractionated Nuclear DNA.

Using the protocol outlined in Figure 3-1, DNA from unfractionated, nuclease-sensitive and nuclease-insensitive liver chromatin was isolated from animals administered a 1 mg/kg $[^3]$H-AFB\textsubscript{1} dose i.p. The number of AFB\textsubscript{1} residues bound to DNA in these chromatin fractions was measured as a function of time as illustrated in Figure 3-7 and Table 3-3. DNA, purified from the respective chromatin preparations by cesium salt density centrifugation, was assayed for bound AFB\textsubscript{1} radioactivity 2, 6, 12 and 24 hours post-dosing. Each experiment consisted of the pooling of the livers from four sacrificed animals. Values presented for each timepoint represent the average of three separate experiments ± the standard deviation; each data point is presented in units of picomoles of AFB\textsubscript{1}/mg DNA and AFB\textsubscript{1} residues per 10\textsuperscript{6} nucleotides.

AFB\textsubscript{1} residues were found to preferentially bind to DNA within DNase II-sensitive chromatin fractions from 2 hours to 24 hours post dosing. As shown in table 3-3, 3-fold more AFB\textsubscript{1} residues were bound, on a picomole per milligram basis, to nuclease-sensitive chromatin DNA at the two hour time-point. While the preferential binding of AFB\textsubscript{1} to the DNA in this fraction was observed at all timepoints, the
Figure 3-7  Adduction of DNase II-sensitive, -insensitive, and unfractionated chromatin DNA by $AFB_1$.

Animals were administered a 1 mg/kg $[^3]HAFB_1$ dose and sacrificed at the indicated timepoints after toxin administration. Chromatin DNA fractionation and isolation procedures are given in the text.
Table 3-3  Comparison of the Distribution of Aflatoxin B₁ Residues within Total Nuclear DNA versus DNase II-sensitive and -insensitive Chromatin DNA

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Ratio A/B</th>
<th>A DNase II-sensitive</th>
<th>B DNase II-insensitive</th>
<th>Whole Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>722 ± 29**</td>
<td>241 ± 19</td>
<td>308 ± 21</td>
</tr>
<tr>
<td>2 Hours</td>
<td>3.0</td>
<td>(247)***</td>
<td>(83)</td>
<td>(105)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>566 ± 44</td>
<td>188 ± 14</td>
<td>247 ± 16</td>
</tr>
<tr>
<td>6 Hours</td>
<td>3.0</td>
<td>(194)</td>
<td>(64)</td>
<td>(85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>358 ± 26</td>
<td>128 ± 13</td>
<td>138 ± 19</td>
</tr>
<tr>
<td>12 Hours</td>
<td>2.8</td>
<td>(123)</td>
<td>(44)</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164 ± 19</td>
<td>72 ± 9</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>24 Hours</td>
<td>2.3</td>
<td>(56)</td>
<td>(25)</td>
<td>(29)</td>
</tr>
</tbody>
</table>

** In Units of picomoles of AFB₁/mg DNA. Values represent the average ± S.D. of 3 separate experiments. Each experiment consists of the pooling of the livers of 3 rats killed at the indicated times post-administration of a 1 mg/kg [³H]AFB₁ dose.

***In units of AFB₁ residues per 10⁶ nucleotides.
ratio of binding between the nuclease-sensitive and
-insensitive fractions decreased to 2.3 at 24 hours post-
dosing. Binding of AFB\textsubscript{1} within the nuclease-insensitive
fraction approximated binding levels within unfractionated,
nuclear chromatin. At two hours post-dosing, small but
statistically-significant differences were apparent between
the levels of AFB\textsubscript{1} binding within these two fractions; this
difference in binding decreased with time, AFB\textsubscript{1} binding
becoming essentially equal within nuclear and P1 DNA at the
24 hour time point. This finding parallels similar studies
of N-OH-acetylaminoflurene binding to putative euchromatic
and heterochromatic rat liver chromatin fractions; in this
case, levels of N-OH-AAF residues in euchromatic DNA frac-
tions were found to be similar to binding levels within
total nuclear DNA (192).

The decrease in the ratio of AFB\textsubscript{1} binding noted between
nuclease-sensitive and -insensitive chromatin DNA fractions
was evidence for differences in the rate of removal of AFB\textsubscript{1}
residues. Figure 3-8 illustrates the half-life of AFB\textsubscript{1} adduction
within DNase-sensitive, -insensitive, and whole chromatin DNA.
Within the DNase-sensitive fraction of rat liver chromatin DNA,
ABF\textsubscript{1} not only binds to greater levels, but also is removed
at a slightly faster rate; while AFB\textsubscript{1} residues have a
half-life of 10.7 hours in unfractionated nuclear chroma-
tin DNA, AFB\textsubscript{1} residues in the DNase-sensitive regions of
chromatin are removed with a half life of 9.1 hours, 16\%
faster than nuclear chromatin as a whole.
Figure 3-8  Half-life of AFBl adduction within DNase II-insensitive, and whole chromatin DNA.
The increased rate of AFB₁ adduct removal from nuclease-sensitive regions of chromatin becomes more apparent when the rate of AFB₁ removal is expressed per unit time. Table 3-4 compares the rate of AFB₁ residue removal, as expressed in units of picomoles AFB₁ removed per milligram DNA per hour, for whole nuclear, nuclease-sensitive and nuclease-insensitive chromatin DNA; these numbers were calculated from the AFB₁ binding data presented in table 3-3. During the period between two hours and six hours post-administration, AFB₁ adducts are removed from DNase-sensitive chromatin DNA at a three-fold greater rate as compared to DNA from DNase-insensitive chromatin DNA. While the rate of adduct removal from both of these DNA fractions decreases with time over the 24 hours observed after toxin administration, aflatoxin residues continue to be preferentially removed from nuclease-sensitive chromatin DNA over this time period.
Table 3-4  Rate of Removal of $\text{AFB}_1$ Residues from Nuclear DNase II-sensitive, and DNase II-insensitive Chromatin DNA

<table>
<thead>
<tr>
<th>Nuclear DNA Fraction</th>
<th>Rate of $\text{AFB}_1$ Residue Removal*** Between Each Timepoint of Binding Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-6</td>
</tr>
<tr>
<td>Unfractionated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td>(A)DNase II-sensitive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>39</td>
</tr>
<tr>
<td>(B)DNase II-insensitive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Ratio A/B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.0</td>
</tr>
</tbody>
</table>

*** Expressed in units of picomoles $\text{AFB}_1$ removed per hour post-dosing per mg DNA.
Chapter 3 - Section B

Introduction and Perspective

Investigations summarized in the previous section indicated that all regions of nuclear DNA are not equally susceptible to modification by the carcinogen AFB$_1$. Deoxyribonuclease II - sensitive regions of rat liver chromatin, enriched in transcribed chromatin DNA as shown by hybridization to rRNA and tRNA probes, are preferentially susceptible to AFB$_1$ addition as compared to deoxyribonuclease II - insensitive chromatin DNA or total nuclear DNA.

Evidence from the literature, however, strongly suggests that all gene sequences are not equally susceptible to carcinogen addition. One particular set of genes, the genes coding for ribosomal RNA, are sequestered from all other nuclear genes (221). They are contained in the nucleoli, the RNA-rich, intranuclear organelles whose function, in part, is to transcribe the rRNA genes and process the resulting transcripts into 18S and 28S rRNA species.

Several factors indicate that rDNA (the genes coding for the 45S ribosomal RNA precursor) are a preferential target for AFB$_1$ addition. First, ribosomal DNA is enriched in guanine as compared to nuclear or nucleolar DNA. As summarized in Table 3-5, nuclear and nucleolar DNA are simi-
Table 3-5  **Nucleotide Base Composition of Nuclear, Nucleolar, and Ribosomal DNA***

<table>
<thead>
<tr>
<th>Base (% Total Residues)</th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear DNA</td>
<td>28.3</td>
<td>29.2</td>
<td>21.0</td>
<td>21.5</td>
</tr>
<tr>
<td>Nucleolar DNA</td>
<td>28.6</td>
<td>28.9</td>
<td>21.4</td>
<td>21.1</td>
</tr>
<tr>
<td>Ribosomal DNA</td>
<td>17.7</td>
<td>17.7</td>
<td>32.3</td>
<td>32.3</td>
</tr>
</tbody>
</table>

***Taken from reference # 203.
lar in nucleotide base composition. Ribosomal DNA, however, is enriched in quanine as well as cytosine; this characteristic gives rDNA a greater density as compared to whole nuclear DNA. Since guanine is the nucleotide base to which greater than 90% of aflatoxin residues bind \textit{in vivo}, it would be expected that these guanine-rich genes would be preferentially susceptible to adduction by AFB$_1$ as compared to other transcribed genes which are not quanine enriched.

Second, biological evidence also strongly implicates the ribosomal RNA gene regions within the nucleolus as preferential targets for AFB$_1$ adduction. As discussed in chapter 2, numerous investigators have shown that AFB$_1$ treatment inhibits nucleolar RNA synthesis to a greater extent than nuclear RNA synthesis (111-115). Further mechanistic studies found that the inhibition of nucleolar RNA synthesis results from impaired nucleolar template function while extranucleolar RNA synthesis inhibition results primarily from impaired RNA polymerase activity. These findings support the hypothesis that the preferential inhibition of nucleolar RNA synthesis is caused by the preferential adduction of the transcribed regions of nucleolar as compared to nuclear DNA.

As discussed in chapter two, changes in DNA sequences, as products of carcinogen-DNA interactions, provide a mole-
cular mechanism for the observed alterations in gene expression which accompany the neoplastic transformation of cells by chemicals (3,4). Because of the emphasis on DNA as the critical macromolecular target for chemical carcinogens, the enhanced susceptibility of transcribed sequences becomes an important issue with respect to constructing models, both theoretical and mathematical, relating the levels of carcinogen adducts to perturbations in gene expression and gene sequence integrity.

The experiments outlined in section B of chapter 3 were designed to determine whether the ribosomal gene sequences in nucleolar DNA, as a subset of the transcribed genes in a liver cell, are preferentially susceptible to carcino- gen adduction in comparison to other transcribed genes. We have designed protocols which permit the measurement of $\text{AFB}_1$ adduction to nucleolar, extranucleolar, and nuclear DNA as a function of time after $\text{AFB}_1$ administration. In addition, we have further adapted the DNase II-Mg$^{2+}$ protocol outlined in section A to isolate the DNase II-sensitive and -insensitive fractions of nucleolar chromatin which are enriched in ribosomal DNA sequences. Using this protocol, we have examined the distribution of $\text{AFB}_1$ residues within these DNase II-sensitive and -insensitive regions of nucleolar chromatin DNA. The results, summarized below, clearly suggest that DNase-sensitive regions of nuclear chromatin differ in their susceptibility to carcinogen adduction.
Materials and Methods

Preparation of Nuclei

Purified nuclei were prepared by a modification of the Hymer and Kuff method (222). All procedures were performed at 0-4°C. Groups of 4 animals were stunned and decapitated, and their livers were perfused in situ via the portal vein with 10 ml of TSC buffer [0.025 M Tris-HCl, pH 7.0 : 0.25 M Sucrose : 3.3 mM CaCl₂]. The excised livers were blotted, weighed, and minced into TSC buffer + 1.0% Triton X-100 (v/v) (7.5 ml buffer per gram liver) with a tissue press. The suspension was homogenized by hand with 12 strokes of glass dounce homogenizer with a tight pestle, filtered through 2 layers of cheesecloth, and finally filtered through a layer of 260 gauge nylon mesh. The filtrate was centrifuged 10 minutes at 650 x g at 4°C, and the resulting pellet was washed twice with TSC buffer only. Between each wash, the nuclear pellets were thoroughly resuspended via 10 strokes of a glass homogenizer with a tight pestle. The yield of nuclei, measured by the quantitative recovery of DNA from the original liver homogenate, was 88-92%.
Isolation of Nucleoli

Nucleoli were isolated from liver nuclei by the method of Muramatsu and coworkers (223 – 225). Three-fourths of the nuclear pellet was resuspended in 0.34 M Sucrose (1 ml/gram of liver) with a pasteur pipette and homogenized with 6 strokes of a glass dounce homogenizer using a tight pestle. Light microscopic observations of toluidine blue preparations from the nuclear homogenate were made to ensure the nuclei were well resuspended and not clumped together; large amounts of nuclear clumps dictated further homogenization.

The nucleoli suspension (10 ml aliquots) was transferred to a 50 ml beaker on ice and sonicated with a brannsonic 1510 sonicator, equipped with a large 1.5 cm diameter probe. The sonication protocol employed was as follows:

- sonicate 25 seconds, 400 watts, half-power wait 5 seconds
- sonicate 10 seconds
wait 5 seconds
- sonicate 10 seconds
The five second waiting periods between sonication blasts help to prevent heat build-up at the end of the probe. Frothing of the sonicate was also avoided; since chromatin protein denaturation can occur. A toluidine blue-stained sample of the sonicate was examined via oil-immersion light microscopy to ensure complete nuclear disruption; if intact nuclei were observed (greater than 1 or 2 nuclei out of 10 observed fields), the mixture was sonicated for additional 10 second bursts. The sonicate (10 ml) was layered over 10 ml of 0.88 M sucrose in a pre-cooled, 30 ml Corex centrifuge tube (Corning, Inc., Corning, N.Y.) and centrifuged 20 minutes at 4°C, 650 x g. The resulting pellet is the liver nucleolar fracion while the supernatant is the liver extranucleolar fraction.

**Isolation of DNA from Nuclear Fractions**

DNA was purified from the nuclear, nucleolar, and extranucleolar fractions by a modification of the method of Marmur (226). The nuclear fraction (one-fourth of the nuclear pellet) was adjusted to 1% SDS and 0.4M NaCl by adding appropriate volumes of 10% SDS and 5 M NaCl . 1.2 volumes of phenol : chloroform : isoamyl alcohol (50:50:1,
v/v/v) was added, and the resulting emulsion was vigorously agitated for 20 minutes at ambient temperature. The phases subsequently were separated by centrifugation at 20,000 rpm in a 40 Beckman rotor at 4°C for 15 minutes. The DNA fraction was recovered from the aqueous epiphase by precipitation with three volumes of ethanol cooled to -70°C.

Nucleoli were resuspended in TSC buffer at a concentration of 1 ml/5 grams of liver, homogenized in a tight-fitting teflon -glass homogenizer, and extracted as described above. The extranucleolar supernatant was also made 1% in SDS and 0.4 M NaCl and extracted as described.

The DNA from each of the nuclear, nucleolar, and extranucleolar fractions was resuspended in sarcosyl buffer at approximately 0.4-0.7 mg/ml and isolated free of contaminant RNA and protein by CsCl/Cs2SO4 gradient centrifugation as Aliquots of the DNA fractions isolated on cesium salt gradients were taken for determination of bound radioactivity and DNA content. DNA was again measured by absorbance at 260 nanometers and by the diphenylamine colorimetric reaction (198).
Isolation of Nucleolar Chromatin Fractions Enriched in Ribosomal RNA Gene Sequences

DNase II-sensitive and -insensitive nucleolar chromatin fractions were isolated from liver nucleoli as schematically summarized in Figure 3-9. Rat Liver nucleoli, isolated from the liver nuclei of 6 AFB₁-treated animals, were resuspended in 20 mM Tris-HCl, pH 6.9 at a concentration of 1 ml/0.8-1.0 A₂₆₀ units of DNA and RNA and digested with ribonuclease by a modification of the method developed by Bombik et al. to yield a crude nucleolar chromatin fraction accessible to DNase digestion (227,228). RNase A (Sigma Chemical Co.) was heat treated at 90°C for 15 minutes to eliminate contaminant DNase activity. The nucleolar suspension was incubated with 1 mg/ml RNase A (added from a 10 mg/ml stock in 20 mM Tris-HCl pH 6.9) at 24°C for 10 minutes with intermittent shaking. After the RNase A digestion, the nucleolar suspension was cooled to 2°C and pelleted at 5000 x g for 20 minutes.

The nucleolar chromatin pellet was washed once with 20 mM Tris-HCl, pH 6.9 and once with 25 mM Sodium Acetate, pH 6.6. After the second wash, the chromatin suspension was adjusted to give an A₂₆₀ nm of approximately 10 (measured in 0.9 N NaOH). The suspension was brought to 24°C, and DNase II was added from a stock solution of 1000 units/ml to yield
Figure 3-9 Isolation of DNA from DNase II-sensitive
and DNase II-insensitive Nucleolar chromatin.
Isolation of DNA from DNase II-Sensitive and Insensitive Nucleolar Chromatin Fractions

\[ ^{3}H \] AFB\textsubscript{1} \rightarrow \text{Rat} \rightarrow \text{Liver} \rightarrow \text{Nuclei} \rightarrow \text{Sonicate (Refs. 32, 36)}

\text{Nucleoli} \rightarrow \text{Ribonuclease (1mg/ml)} \rightarrow \text{Incubate 10 minutes at 24°C. (heat treated)} \rightarrow \text{Nucleolar Chromatin Pellet}

\text{DNase II (40 units/ml)} \rightarrow \text{25 mM NaAcetate, ph 6.6 Incubate 2.0 minutes at 24°C} \rightarrow \text{Centrifuge (25,000 g, 20 min.)}

\text{Pellet (DNase II-Insensitive)} \rightarrow \text{Chromatin} \rightarrow \text{Supernatant (DNase II-Sensitive)} \rightarrow \text{Chromatin}

\text{Solubilize Macromolecules with Sarkosyl Detergent Buffer} \rightarrow \text{Add 6M CsCl, 3M Cs\textsubscript{2}SO\textsubscript{4}} \rightarrow \text{Centrifuge} \rightarrow \text{Isolate DNA} \rightarrow \text{Quantitate AFB\textsubscript{1} Binding}
a final concentration of 40 units/ml. The reaction was allowed to proceed for 2 minutes after which the reaction mixture was cooled in an ice slush and adjusted to pH 7.2 by the addition of 500 mM Tris-HCl, pH 11.

Nuclease-resistant chromatin (labelled P1 fraction) was removed by centrifugation in a SW 56 Beckman rotor for 20 minutes at 16,000 rpm, 4°C. The nuclease-sensitive supernatant (labelled S1 fraction) was made 0.5% in Sarkosyl and subjected to cesium chloride/cesium sulfate centrifugation as described previously to isolate DNA free of RNA and protein contamination. The P1 fraction was resuspended in Sarkosyl buffer (2.5 ml) and similarly subjected to cesium salt centrifugation for DNA isolation. AFB1 binding levels were quantitated as described.
Results and Discussion

Isolation of Nucleolar, Extranucleolar, and Nuclear Rat Liver DNA from AFBl-Treated Animals

To examine the distribution of AFBl residues within the nucleolar and extranucleolar fractions of nuclear DNA, a sonication-fractionation protocol, originally developed by Muramatsu and coworkers, was adapted for studies using liver nuclei from AFBl-treated animals. A clean liver nuclear preparation was isolated and subjected to repeated bursts of sonication in an isotonic sucrose solution. Intact nucleoli which survived sonication were liberated from nuclear chromatin by careful homogenization; a clean nucleolar pellet, free of nuclei and chromatin as judged by light microscopy of stained preparations, was subsequently isolated from the sonicate by centrifugation through a 0.88M sucrose underlayer. The nucleolar DNA fraction yielded an average of 0.92% of nuclear DNA, a figure in agreement with DNA yields from nucleoli in other published reports (105-207).

DNA from each of the three fractions (nuclear, nucleolar, and extranucleolar) was isolated from animals administered a 1 mg/kg $[^3]$HAFBl dose i.p., and the level of AFBl adduction was determined as a function of time as illustrated in Figure 3-10. DNA, purified from the respective nuclear fractions by cesium salt density centrifugation, was assayed for bound AFBl
Figure 3-10  Time course of AFB$_1$ adduction within nuclear, nucleolar, and extranucleolar rat liver DNA isolated from animals administered a 1 mg/kg $[^3]$H]AFB$_1$ dose. Experimental details are given in the text.
radioactivity 0.5 to 48 hours post dosing. Each experiment consisted of the pooling of livers of three sacrificed animals. Values presented for each timepoint represent the average of five separate experiments ± S.D. for the 0.5 and 2 hour time points, and three separate experiments ± S.D. for the remaining time points.

Similar levels of AFB₁ adduction were observed in nucleolar and extranucleolar DNA 0.5 to 48 hours post-dosing. The levels of adduction within these two DNA fractions do differ beyond one S.D. at the 0.5 hour time point; from 2 to 48 hours post-dosing, AFB₁ adduct levels within nucleolar and extranucleolar DNA differ by amounts within standard deviation error ranges. Two explanations can be formulated for the failure of nucleolar DNA to be preferentially adducted by AFB₁ over the time course observed in the face of chemical and biological evidence that nucleolar template function is preferentially impaired after AFB₁ administration. First, the sonication of a hyper-adducted nucleolar DNA fraction might preferentially dislodge the bound AFB₁-residues. This seems unlikely since the primary sonication-labile bonds (carbon-oxygen) are not involved in binding the AFB₁ moiety to nucleotide bases.

The second explanation comes from an examination of the base compositions of nucleolar, nuclear, and ribosomal DNA (Table 3-5). Nucleolar DNA itself is not guanine enriched.
Aflatoxin B₁ residues, however, could be preferentially bound within the transcribed regions of nucleolar DNA which are guanine-enriched.

Since the ribosomal RNA genes are the only genes in nucleolar DNA, these regions of chromatin could be expected to be preferentially susceptible to digestion by DNase II. Investigators using other biological systems have shown ribosomal DNA to be sensitive to digestion by deoxyribonucleases (219,220). Therefore, an adaptation of the DNase II/Mg⁺⁺ procedure outlined in section A was developed to preferentially liberate the transcribed ribosomal DNA regions of nucleolar chromatin.

As outlined in Figure 3-10, nucleoli from AFB₁-treated animals were briefly digested with ribonuclease A to breakdown the DNA - protein - RNA network which is the basis for nucleolar structural integrity (203). This procedure for nucleolar chromatin preparation has been shown to maintain the structural as well as the functional integrity of the ribosomal RNA genes; a similar protocol employed by Bombik and coworkers produced a nucleolar chromatin fraction which retained transcriptional integrity as assayed in an in vitro assay with E. coli RNA polymerase (208).

The RNase-treated nucleolar chromatin pellet was subsequently sheared by treatment with DNase II for 2 minutes at 40% of the enzyme concentration used to digest whole
nuclear chromatin. Previous studies by Yu have shown ribosomal DNA to be extensively sensitive to DNase digestion when isolated as nucleoli (43). Decreases in both the time of incubation as well as the DNase II concentration were thus made to compensate for the possible increased sensitivity of the ribosomal DNA regions in nucleolar preparations. The DNase-sensitive nucleolar supernatant and DNase-insensitive nucleolar pellet accounted for 14% and 83.6%, respectively, of the total nucleolar chromatin DNA.

To determine the enrichment of the DNase II-solubilized nucleolar chromatin DNA fraction in ribosomal RNA genes, samples of DNA from both nuclease fractions were bound to nitrocellulose filters and hybridized with $^{32}$P-rRNA as detailed in section A. As shown in Figure 3-11, saturation hybridization for both types of DNA was reached by 16 hours of hybridization incubation. The saturation values were 3.1% and 0.55% for nuclease-sensitive and -insensitive fractions, respectively, indicating a 5.6-fold enrichment of the ribosomal RNA genes in the DNase II-sensitive fraction.

Using this DNase II protocol, DNA from nuclease-sensitive and -insensitive liver nucleolar chromatin was isolated from animals administered a 1 mg/kg $^{3}$HAFB$_1$ dose i.p. and sacrificed 2 hours post-dosing; the specific activity of
Figure 3-11  Hybridization of DNase II-sensitive and DNase II-insensitive nucleolar chromatin DNA fractions to $^{32}$P-end labelled rat liver ribosomal RNA.
DNase II-Sensitive Chromatin DNA

NUCLEOLAR CHROMATIN:
RIBOSOMAL RNA PROBES

DNase II-Insensitive Chromatin DNA

% DNA Hybridized

Time - Hours
DNA-bound AFB₁ residues in these nucleolar chromatin fractions is given in Table 3-6. Consistent with the higher levels of adduction observed in DNase-sensitive nuclear chromatin, preferential binding of AFB₁ to nucleolar DNA enriched in rRNA genes was observed. Two hours post-dosing, DNA from the DNase II-sensitive fraction of nucleolar chromatin bound 5.3-fold more AFB₁ residues, on a picomole per milligram basis, than DNase II insensitive nucleolar chromatin DNA and 4.3-fold more AFB₁ residues than unfractionated nucleolar chromatin.
Table 3-6  Comparison of the Distribution of AFB₇ Residues Within Total Nuclear DNA with DNA Isolated from DNase II-sensitive and Insensitive Chromatin Fractions from Nuclei and Nucleoli

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Picomoles AFB₇ per Milligram DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase II-sensitive Nucleolar DNA</td>
<td>1040 ± 70</td>
</tr>
<tr>
<td>DNase II-insensitive Nucleolar DNA</td>
<td>196 ± 22</td>
</tr>
<tr>
<td>Total Nucleolar DNA</td>
<td>241 ± 24</td>
</tr>
<tr>
<td>DNase II-sensitive Nuclear DNA</td>
<td>722 ± 29</td>
</tr>
<tr>
<td>DNase II-insensitive Nuclear DNA</td>
<td>241 ± 19</td>
</tr>
<tr>
<td>Total Nuclear DNA</td>
<td>321 ± 22</td>
</tr>
</tbody>
</table>

*Values represent average ± S.D. of 3 experiments

Each experiment consists of pooling the livers of 6 rats killed two hours post-administration of a 1 mg/ kg [³H]AFB₇ dose.
Part II
Summary and Discussion

We have determined the time course of \( \text{AFB}_1 \) adduction within regions of liver nuclear and nucleolar chromatin DNA sensitive and insensitive to attack by deoxyribonuclease II. To investigate the distribution of \( \text{AFB}_1 \) residues within rat liver DNA fractions enriched in transcribed sequences, we have adapted the DNase II-MgCl\(_2\) chromatin fractionation procedure of Gottesfeld and coworkers (19) for our studies using liver chromatin from \( \text{AFB}_1 \)-treated animals. Liver chromatin was digested with DNase II, and DNA from the DNase-sensitive and -insensitive chromatin fractions was isolated, free of RNA and protein contamination, by cesium salt density centrifugation. The DNase-sensitive chromatin DNA fraction, which accounted for 11.9\% of liver nuclear DNA, was shown to be enriched in transcribed gene sequences, as compared to DNase II-insensitive chromatin DNA, by hybridization to nuclear RNA probes. When hybridized with ribosomal RNA, DNase-sensitive chromatin DNA was found to be 5.1-fold enriched in ribosomal RNA genes versus DNase-insensitive chromatin DNA; similarly, nuclease-sensitive chromatin DNA was shown to be 4.2-fold enriched in transfer RNA genes versus nuclease-insensitive chromatin DNA when both were hybridized with transfer RNA probes.
Using this protocol, AFB₁ adduction to DNA from unfractionated, DNase-sensitive, and DNase II-insensitive liver chromatin was determined as a function of time in animals administered a 1 mg/kg [³H] AFB₁ dose. As illustrated in Figure 3-7 and Table 3-3, AFB₁ preferentially bound to DNA from DNase-sensitive chromatin fractions from 2 to 24 hours post-dosing; compared to nuclease-insensitive chromatin DNA, DNA from nuclease-sensitive chromatin bound 2.3-to 3.0-fold more AFB₁ residues over the time period studied.

DNA from DNase II-sensitive chromatin was also found to differ from both unfractionated and DNase II-insensitive chromatin DNA in the rate of AFB₁ adduct removal in treated animals. Over the 24 hour period post-dosing that was observed, AFB₁ residues were removed from nuclease-sensitive chromatin DNA at a 3-fold faster rate than from DNase II-insensitive chromatin DNA.

We have extended these studies in nuclear chromatin to focus on one particular set of genes, the genes coding for ribosomal RNA. Two pieces of evidence suggested that rDNA would be preferential targets for AFB₁ adduction. First, ribosomal DNA is enriched in guanine compared to nucleolar or nuclear DNA; since guanine is the base to which greater than 90% of aflatoxin residues bind in vivo, these guanine-rich genes could be expected to be preferentially susceptible to AFB₁ adduction. Second, biological evidence indicates the ribosomal RNA genes as preferential targets in nuclear DNA.
for AFB₁ adduction. Subsequent to AFB₁ administration, ribosomal RNA synthesis is inhibited approximately 2-fold more than nuclear RNA synthesis as a whole. However, while extranucleolar RNA synthesis inhibition is primarily due to RNA polymerase inactivation, ribosomal RNA synthesis inhibition is due to DNA template inactivation. These findings supported the hypothesis that preferential inhibition by AFB₁ of nucleolar as compared to nuclear RNA synthesis is caused by the preferential adduction of transcribed regions of nucleolar as compared to nuclear DNA.

We therefore determined the time course of AFB₁ adduction within regions of liver nucleolar chromatin DNA sensitive and insensitive to attack by DNase II. The DNase II-MgCl₂ chromatin fractionation procedure of Gottesfeld and coworkers (19), developed for studies with nuclear chromatin, was adapted to isolate DNase II-sensitive and -insensitive fractions of nucleolar chromatin from liver nucleoli of AFB₁-treated animals. The DNase-sensitive nucleolar chromatin DNA fraction, which accounted for 14% of nucleolar chromatin DNA, was shown to be enriched in transcribed gene sequences, as compared to DNase II-insensitive chromatin DNA, by hybridization to ribosomal RNA probes: DNase II-sensitive chromatin DNA was 5.6-fold enriched in ribosomal RNA genes versus DNase II-insensitive chromatin DNA.
Using this protocol, AFB<sub>1</sub> adduction to RNA from unfractionated, DNase-sensitive, and DNase-insensitive liver nucleolar chromatin was determined two hours post dosing in animals administered a 1 mg/kg [³H] AFB<sub>1</sub> dose. DNA from DNase II-sensitive nucleolar chromatin was found to bind 4.2-fold more AFB<sub>1</sub> residues than total nucleolar DNA and 30% more AFB<sub>1</sub> residues than DNase II-sensitive nuclear chromatin DNA. These findings contrasted strikingly with studies on the adduction of AFB<sub>1</sub> within nucleolar and extranucleolar DNA fractions: no major differences in AFB<sub>1</sub> adduction were observed. Coupled with the DNase II-nucleolar studies, these results would indicate that nucleolar DNA is not preferentially susceptible to AFB<sub>1</sub> adduction: only the ribosomal RNA gene sequences are.

The results from our studies of both nuclear and nucleolar chromatin DNA are in agreement with other investigations of carcinogen distribution within deoxyribonuclease-sensitive and -insensitive regions of nuclear chromatin. In these studies, the organization and structure of chromatin have been shown to influence the accessibility of regions of DNA to carcinogen modification in vivo. As discussed in the introduction, the eucaryotic genome is organized into basic subunits consisting of DNA periodically folded around a histone core creating a string of DNA-protein particles called nucleosomes. These core particles are connected by lengths
of DNA, termed linker DNA, which also provide binding sites for histones. Digestion of isolated nuclei with micrococcal nuclease (MNase), or mild autodigestion of nuclei by endogenous Mg\(^{++}\),Ca\(^{++}\)-activated nuclease, preferentially cleaves the linker regions liberating nucleoprotein particles whose molecular weights are multiples of the monomeric DNA-protein subunit.

The relative accessibilities of core and linker DNA to MNase digestion correlate with the susceptibility of these DNA regions to carcinogen modification: when nuclei from carcinogen-treated animals are MNase digested, the levels of adduction within linker DNA are found to exceed the levels of binding to core DNA. Metzger and coworkers have investigated the distribution of the hepatocarcinogen N-hydroxy-acetylaminofluorene (N-OH-AAF) in liver nuclei (193). In liver DNA isolated from animals administered this compound, N-OH-AAF adduct residues were found to bind at a 2.2-fold greater level to linker DNA relative to core DNA.

Bailey and coworkers have similarly determined the distribution of AF\(_B\)\(_1\) in MNase-sensitive and -insensitive liver chromatin DNA (195). AF\(_B\)\(_1\), which like N-OH-AAF binds predominately at guanine residues, was found to bind at a 5-fold greater level to linker versus core DNA. Small alkylating agents also preferentially bind to linker as compared to core DNA. Dimethylnitrosamine is a hepatocarcinogen which methylates DNA at many sites.
In animals administered this compound, dimethylnitrosamine was found by Ramanathan and coworkers to methylate linker regions of liver chromatin at a 2.6-fold greater level than core DNA (196).

Our studies of carcinogen adduction within nuclease-sensitive regions of target organ chromatin were prompted by observations of the levels of chromatin organization which are responsible for the accessibility of specific gene sequences for transcription. As summarized in the introduction, morphological and biochemical evidence suggest that transcribed regions of nuclear chromatin are maintained in a discrete, active conformation distinguishable from the majority of nontranscribed DNA. We postulated that the active conformation of transcribed DNA, containing sites of RNA synthesis which are in a more diffuse and extended conformation, would make these regions of DNA more susceptible to carcinogen adduction. Our results with deoxyribonuclease II-sensitive regions of nuclear and nucleolar chromatin, enriched in transcribed DNA as shown by hybridization to rRNA and tRNA probes, supports the hypothesis that, in fact, transcribed genes are preferentially susceptible to carcinogen modification and damage. However, to properly approach and investigate the question of localized adduction of transcribed DNA sequences by chemical carcinogens, we needed to develop
a model system permitting the study of carcinogen adduction within specific gene sequences. We therefore focussed our attention from the study of DNase-sensitive chromatin fractions to the study of one specific group of gene sequences--the sequences coding for ribosomal RNA. These studies are summarized in Part II.
Part III

Chapter 4: Aflatoxin B₁ Adduction of Ribosomal DNA

Chapter 5: Mechanisms of AFB₁-Induced Inhibition of Ribosomal RNA Synthesis
Chapter 4

Aflatoxin B₁ Adduction of Ribosomal DNA Sequences

Introduction and Perspective

Our experimental results presented in chapter 3 supported the conclusion that not all regions of the nuclear genome are equally susceptible to covalent modification and damage by the hepatocarcinogen aflatoxin B₁. In section A, deoxyribonuclease II-sensitive regions of rat liver chromatin, enriched 3 to 5 fold in transcribed gene sequences as demonstrated by filter hybridization to ribosomal and transfer RNA probes, were found to be preferentially susceptible to adduction in AFB₁-treated animals: DNA from DNase II-sensitive chromatin bound 3-fold more AFB₁ residues than DNA from DNase II-insensitive chromatin.

In further experiments outlined in section B, we determined the distribution of AFB₁ adducts within DNase II-sensitive and -insensitive regions of nucleolar chromatin DNA. The reasons for focusing on these chromatin fractions from the nucleolus were two-fold. First, ribosomal DNA (the nucleolar DNA sequences coding for 45S precursor ribosomal RNA) is enriched in guanine, the nucleotide base to which greater than 95% of AFB₁ residues bind both in vivo and in vitro (39). These gene sequences thus could be expected to preferentially bind AFB₁ residues as compared to gene sequences not enriched in guanine.
Second, as summarized in chapter 2, experiments reported in the literature have shown that \( \text{AFB}_1 \) treatment inhibits nucleolar RNA synthesis to a greater extent than it inhibits nuclear RNA synthesis (43,44). Further studies in the literature have shown that \( \text{AFB}_1 \) inhibits RNA synthesis by different mechanisms in nucleolar versus extranucleolar fractions: the inhibition of nucleolar RNA synthesis results from impaired nucleolar template function, while extranucleolar RNA synthesis inhibition results principally from impaired RNA polymerase activity - not impaired template function. These findings therefore supported the hypothesis that preferential inhibition by \( \text{AFB}_1 \) of nucleolar as compared to nuclear RNA synthesis is caused by the preferential adduction of transcribed regions of nucleolar DNA as compared to transcribed regions of extranucleolar DNA.

To investigate the distribution of \( \text{AFB}_1 \) residues in transcribed regions of nucleolar DNA, Deoxyribonuclease II-sensitive regions of liver nucleolar chromatin, enriched 5-fold in ribosomal RNA gene sequences (as demonstrated again by filter hybridization to rRNA probes) were isolated from \( \text{AFB}_1 \)-treated animals. DNA from the nuclease-sensitive nucleolar chromatin was found to bind 3.3-fold more \( \text{AFB}_1 \) residues than total nuclear DNA and 4.3-fold more \( \text{AFB}_1 \) residues than total nucleolar DNA. These results from studies of both nuclear and nucleolar chromatin DNA indicate that nuclease-sensitive
regions of the liver cell genome, enriched in transcribed DNA sequences, are more susceptible to structural modification by AFB₁.

To adequately investigate the structural and functional impairment of transcribed DNA regions by chemical carcinogens, an experimental system was designed in which:

1. carcinogen-DNA adducts can be measured within specific transcribed gene sequences.
2. the qualitative and quantitative relationships between carcinogen-DNA adduction and functional endpoints can be investigated in specific gene sequences.

This model was schematically summarized in Figure 1-1. In this chapter, the development of methodologies for completing point (1) above are described. As shown in Figure 4-1, ribosomal RNA gene sequences are selectively isolated from total liver DNA by hybridization to ribosomal RNA. Total AFB₁ modification is then determined as a function of time after AFB₁ administration and as a function of AFB₁ dose. Hydrolysis of the AFB₁-adducted ribosomal DNA sequences and analysis of the hydrolysates by high pressure liquid chromatography further permits identification of the bound aflatoxin adduct species.
Figure 4-1  Schematic summary of the experiments designed to describe, in quantitative and qualitative terms, the adduction of ribosomal DNA sequences in AFB₁-treated animals.
Treat Male Fischer Rat with $^3$H-AFB₁ (i.p.)

Isolate Liver Nuclear DNA

Isolate Ribosomal DNA Sequences

Determine Levels of Bound AFB₁ Residues

Determine Identity of Major AFB₁ Adduct Species
Methods, Materials and Animals

The chemicals used in this experimental section were the same as those described in chapter 3.

Male CDF Fischer Rats (Charles River Breeding Labs, Wilmington, Mass.) were housed and fed as described previously. Carcinogen dosing of rats (125-160 grams) was by intraperitoneal injections of $[^3\text{H}]$-AFB$_1$ dissolved in 50$\mu$l of glass-distilled dimethylsulfoxide (Burdock and Jackson Labs., Muskegon Michigan).

Preparation of Ribosomal DNA Isolates From Total Rat Liver

Nuclear DNA of AFB$_1$-Treated Animals.

The protocol designed for the isolation of a nuclear DNA fraction enriched in ribosomal DNA sequences is schematically summarized in Figure 4-2. Each phase of this protocol is described in detail below.
Figure 4-2 Schematic outline of the protocol designed for the isolation of a nuclear DNA fraction enriched in ribosomal DNA sequences.
Phase 1: Nuclear DNA Isolation and AFB<sub>1</sub> Adduct Stabilization

\[ \text{[H]} - \text{AFB}_1 \rightarrow \text{Rat} \]

↓

Sacrifice and Perfuse Liver in situ

↓

Isolate Clean Nuclei Fraction

↓

Isolate Liver Nuclear DNA via Cesium Salt Density Centrifugation

↓

Selectively Retain Lower (More-Dense) 10% of DNA Peak in Cesium Salt Gradients:

Ribosomal DNA Enriched Fraction

↓

Sample

Determine Tritium Loss

↓

Alkali Treat DNA: Adduct Stabilization

0.1 M Glycine NaOH, pH 10.5

30 Minutes, 37°C

↓

Reprecipitate DNA

↓

Phase 2: Ribosomal DNA Isolation

Hybridize rDNA-Enriched DNA with IBS and 28S Ribosomal RNA

↓

Preparatively Isolate rDNA: rRNA Hybrids by CsCl Gradient Centrifugation

↓

Reisolate Purified rDNA: rRNA Hybrids by Second Round of CsCl Gradient Centrifugation

↓

Digest RNA with Ribonuclease (DNase Inactivated)

↓

Quantitate AFB Binding
Phase 1: Isolation of Rat Liver Nuclear DNA from AFB$_1$-Treated Animals and Stabilization of Bound AFB$_1$ Adducts.

(1) Preparation of Rat Liver Nuclei

Purified nuclei were prepared by a modification of the Blobel-Potter Method (197). All procedures were performed at 0-4°C. Animals were injected with a [$^3$H]-AFB$_1$ dose and killed at appropriate periods after dosing by stunning with a blow to the head followed by decapitation with a guillotine. The livers were perfused in situ via the portal vein with 10 ml of ice-cold TSC buffer [0.025M Tris-HCl, pH 7.0:0.25M Sucrose: 3.3mM CaCl$_2$], blotted dry, weighed, and minced into TSC buffer + 1.0% Triton X-100 (v/v) (7.5 ml buffer per gram of liver) with a tissue press. The suspension was homogenized by hand with 12 strokes of a glass dounce homogenizer (tight pestle), filtered through two layers of cheesecloth, and finally filtered through a layer of 260 guage nylon mesh. The filtrate was centrifuged for 10 minutes at 650 x g at 4°C, and the resulting pellet was washed twice with TSC buffer alone.
(2) Crude Nuclear DNA Isolation

The crude nuclear pellet was subsequently extracted with phenol as described by Marmur (202). The nuclei were brought up in 25mM Tris-HCl, pH 7.0, 3.3mM CaCl\textsubscript{2} at a concentration of 2.5 milliliters per gram of liver tissue. The nuclear suspension was adjusted to 1% SDS and 0.3M NaCl by adding appropriate volumes of 10% SDS and 5M NaCl. 1.2 volumes of phenol:chloroform:isoamyl alcohol (50:50:1, v/v/v) was added, and the resulting emulsion was vigorously agitated for 20 minutes at ambient temperature. The phases subsequently were separated by centrifugation at 20,000 rpm in a 40 Beckman rotor at 4℃ for 15 minutes. The DNA fraction was recovered from the aqueous epiphase by precipitation with 3 volumes of -70℃ ethanol.
(3) Isolation of Ribosomal DNA-Enriched Fraction of Nuclear DNA

The crude nuclear DNA pellet was washed once with -70°C ethanol and resuspended in HEPES buffer (50 mM HEPES, pH 7.0: 5 mM Na₂EDTA) at a concentration of 0.5 mg DNA/ml. The DNA suspension was transferred to a glass homogenizer with a tight fitting teflon pestle and homogenized with 15 up and down strokes. Sodium Sarkosyl was added to 1% and the homogenate was further homogenized with 6 up and down strokes. The following mixture was then composed:

- 2.0 ml DNA homogenate
  (equivalent to 1.0 mg of DNA)
- 0.5 ml Sarkosyl-HEPES buffer
- 13.96 ml 6 M CsCl
- 1.54 ml 3 M Cs₂SO₄
Total volume: 18.0 ml

The DNA-cesium salt mixture was transferred to Quickseal 38 ml rotor tubes (Beckman Instruments), and the tube was filled with heavy mineral oil and sealed. Note: Care must be taken to wash all the mineral oil from the outside of the centrifuge tube. Any oil residue remaining will cause the tubes to collapse during centrifugation.

The DNA-cesium salt mixtures were centrifuged for 12 hours, 45,000 rpm, 20°C in a Beckman VTi 50 rotor. Both slowed acceleration and deacceleration were employed as described in chapter 3. Each tube was fractionated using an ISCO, Inc. density gradient fractionator monitoring flow absorbance at 254 nanometers; fractions were manually read for their 254 nm absorbance, and the most dense 10% of each DNA peak was kept.
(4) Chemical Stabilization of AFB₁ Adducts.

The retained DNA fractions were briefly dialyzed against TE buffer and the DNA recovered by precipitation with ethanol as previously described. The DNA pellet was washed twice with an 80% ethanol/20% 0.3 M NaCl mixture and transferred to a glass Dounce homogenizer. Glycine buffer (0.11M glycine-NaOH, pH 10.5) was added to a final concentration of 0.7 to 0.9 mg DNA/ml. The solution was homogenized until the DNA was solubilized (2-5 minutes was initially allowed for the DNA to become wetted with the buffer) and subsequently incubated for 30 minutes at 37°C. After incubation, the DNA solution was made 0.3M in NaCl by adding an appropriate volume of 5M NaCl, and the DNA recovered by the addition of -70°C ethanol. Samples of the DNA pellet 0.05 to 0.1 milligrams) are taken before and after glycine treatment to accurately determine the loss of bound AFB₁ tritium radioactivity during base treatment; bound AFB₁ was determined by liquid scintillation and DNA is quantified by the diphenylamine colorimetric reaction (198).
Phase 2: Preparation of a Ribosomal DNA Isolate by Hybridization to Ribosomal RNA

The procedures outlined in phase 2 of the ribosomal DNA isolation procedure were adapted from the procedures of Wellauer and Dawid (211,212).

(1) Hybridization of Ribosomal DNA-Enriched DNA with 18S and 28S Ribosomal RNA

The base-treated nuclear DNA pellet was washed twice with -70°C ethanol/0.3M NaCl (9:1, v/v) and brought up in formamide hybridization buffer:

70% formamide (BRL, Inc.)
0.3M NaCl
10mM Tris-HCl, pH 7.2
1mM EDTA
at a DNA concentration of 15 micrograms/ml. Ribosomal RNA, isolated as described in the appendices, was added to a final concentration of 15 micrograms/ml each of 18S and 28S rRNA. The DNA-RNA mixture was transferred to 1 ml ampoules previously silanized and treated with diethylpyrocarbonate to inactivate residual ribonuclease activity; each ampoule contained 0.8 ml. The ampoules were incubated at 50°C for 3 hours, after which the contents were cooled to 4°C in an ice slush and dialyzed against 10mM Tris-HCl, pH 7.2, 2.5mM Na₂EDTA.
(2) Isolation of RNA-DNA Hybrids by Cesium Chloride Density Centrifugation

To the dialyzed RNA-DNA mixture, cesium chloride was added to bring the density of the solution to 1.71. The mixture was centrifuged for 12 hours in a VTi50 Beckman rotor (18 ml per gradient tube), and the gradients were fractionated as previously described. The more dense 20-25% of each DNA peak was retained, readjusted to a density of 1.72, and recentrifuged as above. To locate the DNA-RNA hybrids after the second round of centrifugation, 1/25 th of each fraction was bound to nitrocellulose filters as described in chapter 3 and hybridized to $^{32}$P-end-labelled 18S and 28S ribosomal RNA. The ribosomal DNA peaks were pooled and dialyzed against 10mM Tris-HCl, pH 7.0, 2.5mM Na$_2$EDTA.

Ribonuclease A, dissolved to a concentration of 0.6 mg/ml in 75mM Tris-HCl, pH 7.0, was heated for 10 minutes at 95°C to destroy DNase activity. To the dialyzed ribosomal DNA isolate, RNase was added to a final concentration of 20 micrograms/ml and the mixture was heated at 44°C for 30 minutes. An equal volume of distilled phenol was added and the mixture was shaken at room temperature for 15 minutes. The aqueous phase was cooled for 10 minutes, made 0.2M in NaCl, and the DNA recovered by precipitation with -70°C ethanol. The ribosomal DNA isolate was washed twice with -70°C ethanol/0.3M NaCl (9:1, v/v) and brought up in 1 ml of 20mM Tris-HCl, pH 7.0, 2mM EDTA. Samples were taken for radioactivity determination by liquid scintillation, and DNA was quantified either by absorbance at 260 nanometers or by the diphenylamine colorimetric reaction.
Determination of the Purity of the Ribosomal DNA Isolate

Two methods were employed to test the purity of the rDNA isolate:

(a) Nucleotide Base Composition
Samples of the rDNA isolate were hydrolyzed in 70% perchloric acid for 1 hour at 95°C as described by Hall (216) and Essigmann and coworkers (217). The hydrolysates were subsequently neutralized with 4N NaOH and injected onto a μC_{18} reverse phase column (Waters, Assoc.) eluted isocratically with 0.8% ETOH/H_2O. The area under each peak was quantified by means of an electronic integrator in the peak + height mode. Integrator units were converted to %DNA bases by determining the integrator units/μg of nucleic acid base standard.

(b) Eco RI Digestion Pattern and Southern Hybridization to rRNA
Samples of the rDNA isolate were digested with Eco RI restriction endonuclease (BRL, Inc.) under conditions specified by the manufacturer. The hydrolysate was layered onto a 1% agarose gel and electrophoresed employing a 0.09M borate running buffer. The gel was stained with ethidium bromide and photographed under a UV transilluminator. The gel contents were transferred to nitrocellulose filters and hybridized with \[^{32}P\]rRNA according to a modification of the Southern protocol as described in the appendices.
\textbf{AFB}_1 \textit{dose for Time-Course and Dose-Response Studies of AFB}_1 - \textit{DNA Adduction}. \\

For studies of the level of AFB\textsubscript{1} adduction to DNA as a function of time after administration, a 1 mg/kg \textsuperscript{3}H- \textit{AFB}_1 dose was used, with the following amounts of \textsuperscript{3}H- \textit{AFB}_1 per rat:

\begin{center}
\begin{tabular}{|l|c|}
\hline
\textbf{Timepoint} & \textbf{\textsuperscript{3}H}AFB\textsubscript{1} injected with 1 mg/kg \textit{AFB}_1 dose \\
\hline
2 Hours & 300 \\
6 Hours & 450 \\
9 Hours & 600 \\
12 Hours & 750 \\
\hline
\end{tabular}
\end{center}

For studies of the level of AFB\textsubscript{1} adduction to DNA as a function of dose 2 hours after AFB\textsubscript{1} administration, 600 microcuries of \textsuperscript{3}H- \textit{AFB}_1 was administered per rat.
Time Course of AFB₁ Adduct Species Within Ribosomal DNA Isolates

After sampling the rDNA isolates for the total quantity of bound AFB₁ residues, the DNA samples were made in 0.1N HCl (by adding 1 N HCl) and heated for 15 minutes at 90°C to liberate AFB₁-purine adducts. The samples were neutralized by the addition of 1 N NaOH, made 50 mM in KOAC and 0.1 mM in ZnCl₂, and incubated with nuclease P₁ (Sigma Chemical Co.) for 3 hours at 37°C at an enzyme concentration of 1 mg nuclease P₁ per 50 mg of DNA.

The hydrolysate was subsequently made 5% in ethanol and injected onto a C₁₈ column (Waters, Inc.) eluted with a 13% to 18% ethanol gradient over 45 minutes (39,40); the aqueous phase of this ethanol gradient was buffered to approximately pH 5 by the addition of 2 drops of 88% formic acid per liter of water. Thirty drop fractions of the gradient were collected, and the amount of radioactivity per fraction was determined by liquid scintillation.

A second set of ribosomal DNA isolates was obtained from total nuclear DNA as summarized in Figure 4-3. Nuclear DNA, enriched in rDNA sequences by one round of cesium salt density centrifugation, was incubated in 20 mM sodium phosphate,
1 mM Na₂EDTA, pH 6.8 at 85 - 88°C for 20 minutes at a DNA concentration of 0.7 mg/ml. As originally described by Lawley (213), heat treatment of N⁷-substituted guanines causes depurination under near neutral conditions. Therefore, after heat treatment of AFB₁-adducted DNA, only pyrimidine AFB₁ adducts (formed by scission of the 7,8,9 ring of AFB₁-substituted guanines) should remain. The hot buffer-treated DNA was subsequently hybridized to ribosomal RNA according to phase two of the rDNA isolation procedure outlined in Figure 5-2. The hot-buffer treated rDNA isolate was subsequently hydrolyzed and analyzed by HPLC as described previously.
Results

Purification of a Ribosomal DNA Isolate from Total Rat Liver Nuclear DNA of Aflatoxin B₁-Treated Animals

(1) Liver DNA Isolation

To examine the distribution of AFB₁ residues within DNA sequences coding for ribosomal RNA, we purified a ribosomal DNA isolate of rat liver nuclear DNA by modifications of the methods developed by Wellauer and Dawid (211,212). Liver nuclei were isolated by the method of Blobel and Potter (181) and total nuclear DNA was preparatively extracted by the method of Marmur (189). Approximately 26 - 32 milligrams of nuclear DNA was obtained from the livers of three 125 - 160 gram rats.

Total nuclear DNA was sheared by 15 strokes in a teflon-glass homogenizer and banded in cesium chloride/cesium sulfate density gradients as summarized in chapter 3. The cesium salt density centrifugation served two purposes. First, cesium salt centrifugation of DNA yields a clean DNA isolate free of contaminate RNA and protein. Second, mammalian ribosomal RNA genes have a high guanine + cytosine content and thus band to the lower (more-dense) side of the total nuclear DNA peak. Therefore, to preparatively isolate a ribosomal DNA-enriched fraction from nuclear DNA as a prelude to RNA:DNA hybridization, the crude nuclear DNA fraction was sheared by homogenization and banded in cesium salt gradients. By collecting only
the lower region of the nuclear DNA band, ribosomal DNA can be preparatively separated from the bulk of nuclear DNA.

Figure 4-4 illustrates this principle. Liver nuclear DNA from a rat administered a 1 mg/kg AFB₁ dose was isolated 2 hours post dosing by phenol extraction of a nuclear homogenate. The crude DNA fraction was sheared by 15 strokes in a teflon-glass homogenizer and subsequently banded by CsCl/Cs₂SO₄ density centrifugation (1.0 mg/18 ml of gradient). Aliquots of the DNA peak were collected (0.5 ml); one microgram samples of each aliquot were bound to nitrocellulose filters and hybridized with [³²P]-end labelled 18S and 28S ribosomal RNA as described in the appendices. The solid line represents flow monitoring of the gradient at 254 nanometers, while the dotted line represents the [³²P] radioactivity profile. As shown, the ribosomal DNA sequences band in the lower (more dense) side of the nuclear DNA peak. Repeated trials indicated that, in sheared liver DNA preparations, greater than 90% of the ribosomal DNA fraction could be recovered in the lower 10% of the nuclear DNA peak. Thus, nonribosomal DNA can be selectively removed from nuclear DNA, leaving a DNA fraction enriched in ribosomal RNA gene sequences which is suitable for subsequent hybridization to ribosomal RNA.
Figure 4-3  Protocol to selectively isolate a liver nuclear DNA fraction enriched in rDNA sequences.
$[^3\text{H}] - \text{AFB}_1 \rightarrow \text{Rat}$

- Sacrifice and Perfuse Liver *in situ*
- Isolate Clean Nuclei Fraction
- Isolate Liver Nuclear DNA via Cesium Salt Density Centrifugation
- Selectively Retain Lower (More Dense) 10% of DNA Peak in Cesium Salt Gradients: Ribosomal DNA Enriched Fraction
Figure 4-4

Preparative separation of ribosomal DNA sequences from total nuclear DNA by centrifugation in CsCl/Cs₂SO₄ density gradients. The experiment is described in the text. The solid line represents absorbance at 254 nanometers (left scale). The dotted line represents bound \(^{32}\)P radioactivity from end labelled 18S and 28S ribosomal RNA hybridized to gradient DNA fractions on nitrocellulose filters as described in the appendices (right scale).
AFB₁ Adduct Stabilization

As discussed in chapter two, the predominant initial adduct in AFB₁-modified DNA, AFB₁-N⁷-guanine, is chemically unstable and undergoes spontaneous depurination, as shown in Figure 4-5. A positive charge created on the 7,8,9 ring of guanine destabilizes the N⁹-C₁' glycosidic bond, and thus, the AFB₁ adduct itself. During the long periods of time and the range of chemical conditions required for ribosomal DNA isolation, the instability of the bound aflatoxin radioactivity would interfere with the accurate determination of AFB₁ binding levels.

To stabilize this adduct for further DNA manipulations, AFB₁-adducted nuclear DNA, enriched in ribosomal DNA sequences by one round of cesium salt density centrifugation, was dialyzed against TE buffer (20 mM Tris-HCl, pH 7.0, 2 mM Na₂EDTA), precipitated with -70°C ethanol, and treated with 0.11 M glycine-NaOH, pH 10.5 at 37°C for 30 minutes at a DNA concentration of 0.7 to 0.9 mg DNA/ml. As shown in Figure 4-5, basic treatment of N⁷-substituted DNA-bound guanines leads to hydroxide attack at the C-8 guanine carbon breaking the C₈-N⁹ bond and neutralizing the destabilizing positive charge generated across the N⁷, C₈, N⁹ bonds. The new adduct forms are a 4.5:1 mixture of 2 ring-opened AFB₁ adduct species:
Figure 4-5  Fate of the AFB₁-N⁷-guanine adduct under neutral and alkaline pH conditions.
AFB₁-FAPyr - 2,3-dihydro-2-(N⁵-formyl-2',5',6'-triamino-4'-oxo-N⁵-pyrimidyl)-3-hydroxyaflatoxin B₁

AFB₁ Peak F - tentatively identified as

2,3-dihydro-2-(8,9-dihydro-8-hydroxy-N⁷-guanyl)-3-hydroxyaflatoxin B₁

Figure 4-6 illustrates the conversion of AFB₁-N⁷-guanine to these stable, ring-opened forms. Chromatographic analysis was performed on glycine-treated and untreated DNA adducted in vitro to a level of 1550 picomoles of AFB₁/mg DNA (equivalent to 530 AFB₁ residues per 10⁶ nucleotides). The adducted DNA samples were hydrolyzed in 0.1 N HCl, digested with Nuclease P₁, and the AFB₁ adduct species were preparatively isolated on a C₁₈ sep-pak column (Waters Assoc.). The preparative adduct isolate was analytically chromatographed on a C₁₈ column eluted isocratically with 18% ethanol as described (92).

As shown in Figure 4-6A, in vitro AFB₁-adducted DNA contains predominately AFB₁-N⁷-guanine. This adduct also predominates in the adduct profile of DNA adducted in vivo by AFB₁ (39,40).

As shown in Figure 4-6B, after a 30 minute treatment with 0.11 M glycine-NaOH, pH 10.5 at 37°C, AFB₁-N⁷-guanine is converted almost completely to the two ring-opened derivatives AFB₁-FAPyr and AFB₁-PeakF. Approximately 1.3% of the radioactivity as AFB₁-N⁷-guanine is not converted and thus lost as bound AFB₁ radioactivity.
Figure 4-6 Chromatographic analysis of AFB₁-adducted DNA treated with 0.11 M glycine-NaOH, pH 10.5. Sample preparation and chromatographic conditions are given in the text.

DNA adduction level: 1550 picomoles AFB₁/mg DNA

= 530 AFB₁ residues per 10⁶ nucleotides

A: untreated DNA sample
B: glycine-treated DNA sample
In addition, the exposure of the AFB₁ molecule to alkaline conditions causes further labilization of AFB₁ tritium radioactivity. The AFB₁ used in these studies has tritium incorporated in the carbon alpha to the carbonyl of the terminal cyclopentanone ring, as shown in Figure 4-7. During glycine treatment, tritium is lost from the bound AFB₁ adduct species. An average of 18% (range 14% to 24%) of the bound tritium radioactivity was found to be lost from nuclear DNA samples treated with glycine-NaOH under the stated condition of adduct stabilization.

Parallel studies in which DNA adducted with $[^{14}\text{C}]-\text{AFB}_1$ was similarly treated for adduct stabilization showed that of the average 18% tritium loss, 6% was due to hydrolysis of the bound AFB₁ moieties (either loss of bound AFB₁-guanine adducts or the aflatoxin moiety itself.) Therefore the remaining 12% of tritium radioactivity loss was determined to be due to tritium exchange. As shown in the summary protocol diagram of Figure 4-2, samples of nuclear DNA, enriched in ribosomal DNA by one round of cesium salt density centrifugation, were sampled before and after 0.11 M glycine-NaOH treatment to determine the exact amount of tritium radioactivity retained. This sampling would permit accurate eventual quantitation of AFB₁ residue bound to ribosomal DNA isolates.
Figure 4-7 Alkalinelabilization of aflatoxin B$_1$-bound tritium.
Alkaline Conditions

Neutral Conditions

$^3\text{H}_2\text{O}$

$^3\text{H}_2\text{O}$
Hybridization of Nuclear DNA, Enriched in Ribosomal DNA by Cesium Salt Density Centrifugation, to Ribosomal RNA

The glycine-treated DNA isolates were incubated with ribosomal RNA to form rRNA:rDNA R-Loop hybrids. R-Loops form upon incubation of DNA and RNA in high formamide at temperatures approaching the $t_m$ of DNA (214). The R-Loop DNA hybrid is more dense than double stranded DNA and can be separated from single and double stranded DNA in cesium chloride gradients; the extent of the shift in density depends on the RNA:DNA ratio within a particular RNA:DNA hybrid molecule. Wellauer and Dawid have shown the formation of rRNA:rDNA hybrids at a temperature of 45°C and a pH of 8.0. Because of the need to lower the pH of the incubation mixture near neutrality (to stabilize bound $AFB_1$ tritium), the incubation temperature was raised to 50°C.

Figure 4-8 illustrates the gradient profile of nuclear DNA, enriched in rDNA by one round of cesium salt centrifugation, hybridized with 18S and 28S ribosomal RNA under these high formamide-hybridization conditions. The hybridization mixture was chilled, dialyzed against TE buffer, adjusted to a density of 1.71 by the addition of solid cesium chloride, and centrifuged for 12 hours at 45,000 rpm in a VTi Beckman rotor. The dotted curve represents the absorbance at 260 nm of 0.5 ml fractions of the gradient. Samples of each fraction were bound to nitrocellulose filters and hybridized to $[^{32}\text{P}]$ rRNA to visualize
Figure 4-8  Gradient profile of nuclear DNA, enriched in rDNA by one round of cesium salt centrifugation, hybridized with 18S and 28S ribosomal RNA in 70% formamide buffer.

Figure 4-9  Profile of 254 nm absorbance and $^{32}$P radioactivity of the second round of cesium salt centrifugation to separate rRNA:rDNA hybrids from nonribosomal DNA.
the ribosomal DNA fraction; the cross-hatched curve represents these values. The rRNA:rdNA hybrid bands to a higher density that the main band of nonribosomal DNA. As shown however, nonribosomal DNA, which is largely denatured under these high temperature-formamide conditions, forms a broad band which overlaps with the ribosomal DNA hybrid band.

The lower (more dense) 20-25% of each DNA peak fraction was collected, readjusted to a density of 1.72, and centrifuged again. To precisely locate the rRNA:rdNA hybrid, 1/25 th of each fraction from this second gradient was bound to nitrocellulose filters and hybridized with $^{32}$P ribosomal RNA. An illustrative profile of both absorbance at 254 nm and $^{32}$P radioactivity is given in Figure 4-9. As shown, the ribosomal RNA:ribosomal DNA hybrid is separated from the broad peak of single-stranded DNA in the denser portion of the gradient. Over the course of the two cesium salt density centrifugation steps, 15% of the AFB$_1$-bound tritium was lost from the rDNA fraction; the levels of bound AFB$_1$ radioactivity in rDNA were thus adjusted to reflect this tritium loss.
The hybrid fractions were pooled, dialyzed against TE buffer, and treated with ribonuclease A under low salt conditions. This ribonuclease treatment effectively removes the hybridized rRNA leaving a double stranded DNA fragment. Yields of the resulting ribosomal DNA fraction ranged from 1.7 to 3.2 micrograms from an original DNA isolate of 22 to 32 milligrams of rat liver DNA.
Analysis of the Ribosomal DNA Isolate

Samples of the ribosomal DNA isolate obtained from the gradient peak fractions of Figure 4-9 were analyzed for purity by two methods. First, analysis of the nucleotide base content was performed. As previously discussed, the genes coding for the 45S precursor ribosomal RNA are distinctive in having a 64.6% content of guanine and cytosine as compared to nuclear DNA as a whole which has a guanine and cytosine content of 42.5%; this enrichment of G and C was used to selectively separate ribosomal DNA sequences from sheared total nuclear DNA. Therefore the G and C content of the ribosomal DNA isolate can serve as an indirect index of the purity of the isolate.

Table 4-1 compares the base composition of the ribosomal DNA isolate to the base composition of ribosomal DNA, as calculated from the base composition of 45S rRNA, and total nuclear DNA. The rDNA isolate was 46% enriched in guanine and cytosine bases, indicating indirectly a near 50% enrichment of ribosomal RNA genes in this nuclear DNA isolate. Other investigators who have isolated and studied ribosomal RNA genes from Drosophila (211) and human DNA (212) have reported purified rDNA fractions of 50% or greater; therefore these nucleotide base studies of the rat ribosomal DNA isolated from AFB$_1$-treated animals are in line with these values.
Table 4-1  Nucleotide Base Composition of Ribosomal DNA Isolate of Rat Liver DNA from Aflatoxin B₁-Treated Animals

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal DNA Isolate</td>
<td>23.5</td>
<td>23.6</td>
<td>25.8</td>
<td>27.0</td>
</tr>
<tr>
<td>Nuclear DNA</td>
<td>28.3</td>
<td>29.2</td>
<td>21.0</td>
<td>21.5</td>
</tr>
<tr>
<td>Pure Ribosomal DNA**</td>
<td>17.7</td>
<td>17.7</td>
<td>32.3</td>
<td>32.3</td>
</tr>
</tbody>
</table>

**From Reference 203
The purity of the rat ribosomal DNA isolate was also determined by digesting the DNA with Eco Rl restriction endonuclease and separating the hydrolysate on a 1% agarose gel. Figure 4-10A illustrates the Eco Rl digestion pattern of the rat ribosomal RNA gene as determined by Tantravahi and coworkers (215). Eco Rl restriction endonuclease cleaves the rat rRNA gene into 3 fragments: 11.2 kilobases (fragment A), 6.7 kilobases (fragment B), and 4.6 kilobases.

As shown in Figure 4-10B, Eco Rl digestion of the ribosomal DNA isolate produced a digestion pattern comparable to the published rDNA restriction map. Three major bands are seen with sizes of 11.4 kb, 6.7 kb, and 4.9 kb; each band is clear and distinct with little ethidium bromide-staining DNA observable in the background of the DNA sample. Curiously, no additional band(s) are visible representing the nontranscribed spacer region separating adjacent 45S ribosomal DNA genes. This could be for two reasons. First, the nontranscribed spacer regions between 45S genes could be variable in length, as in the case of Drosophila (211). This would lead to variable length Eco Rl fragments which would not appear as distinctive bands.

Second, the hybridization conditions employed to isolate ribosomal DNA sequences may select for only those fragments which do not contain intact nontranscribed spacer regions. The basis for the separation of rRNA: rDNA hybrids from total nuclear DNA was the greater density of the hybrid compared to single- and
Figure 4-10A  Eco R1 restriction endonuclease map of the ribosomal RNA gene locus in the rat.

(Ref. # 215)
Ribosomal DNA Restriction Map

11.2 KB (Fragment A)  6.6 KB (Fragment B) (Fragment C)

NTS - Nontranscribed Spacer DNA
ETS - External Transcribed Spacer DNA
ITS - Internal Transcribed Spacer DNA
18S - DNA Region Coding for 18S Ribosomal RNA
28S - DNA Region Coding for 28S Ribosomal RNA
KB - Kilobases
Figure 4-10B  Purity of the ribosomal DNA isolate as determined by gel electrophoresis of Eco Rl-rDNA digests and subsequent hybridization to [32P]rRNA via southern hybridization

A: Separation of Eco Rl-rDNA digest on a 1% agarose gel

B: Southern hybridization of the restriction fragments separated in the gel shown in A to [32P]rRNA

C: Sizes of the Eco Rl restriction fragments in kilobases. The sizes of each fragment were determined from Eco Rl and Hind III cut lambda DNA standards.
double-stranded DNA. Ribosomal DNA fragments, containing intact or nearly-complete spacer regions might be expected to form an rRNA:rDNA hybrid of lower density than fragments containing a higher proportion of 18S and 28S sequences, and thus, a higher proportion of 18S and 28S rRNA to DNA. Examining Figure 4-9 shows that some ribosomal DNA, not within the major peak of ribosomal RNA-hybridizing material, migrates in the broad single-stranded DNA peak. It is plausible to assume this ribosomal DNA fraction, not represented in the ribosomal DNA isolate, may represent spacer-enriched ribosomal DNA fragments.

To obtain higher sensitivity, the electrophoretically-separated Eco R1 digest of the ribosomal DNA isolate was transferred to a nitrocellulose filter. The filters were then hybridized with purified 18S and 28S ribosomal RNA that had been highly labelled by incubation with $^{32}$P-gamma-ATP and polynucleotide kinase. This transfer and hybridization was performed by modifications of the Southern technique as summarized in the appendices. As shown next to the gel photograph in Figure 4-10B, the $^{32}$P-rRNA probes bound only to DNA in the principal EthBr-staining bands. No other rRNA-hybridizing bands were evident within the background DNA smear.
Time Course of AFB₁ Adduction to Ribosomal DNA

Using the protocol described in Figure 4-2, we determined the time course of AFB₁ adduction to ribosomal DNA isolates from the total liver DNA of AFB₁-treated animals. Figure 4-11 compares the level of AFB₁ modification within ribosomal DNA isolates and total nuclear DNA from two to twelve hours after the administration of a 1 mg/kg [³H]-AFB₁ dose. The results presented confirm earlier studies summarized in Chapter 3 suggesting that transcribed sequences within the nuclear genome are preferential targets for AFB₁ adduction. At two hours post-dosing, 4.8-fold more AFB₁ residues were bound to ribosomal DNA as compared to total nuclear DNA. This preferential binding is persistent over the 12 hour time course, decreasing to a 4.1-fold enrichment in binding at 12 hours post-dosing.

As evident from the slopes of the two AFB₁ adduction curves in Figure 4-11, the rates of AFB₁ adduct removal differ between the ribosomal DNA and nuclear DNA fractions. Figure 4-12A summarizes the removal of AFB₁ adducts within these two DNA fractions. To compare further the time course and half-life of AFB₁ adduction within ribosomal DNA to whole nuclear fractions enriched in transcribed sequences, the persistence of AFB₁ adduction within DNase II-sensitive and -insensitive nuclear DNA has been given in Figure 4-12B. During the first 12 hours post-dosing, the half-life of bound AFB₁-residues within the ribosomal DNA isolate is only 6.1 hours, while the half-life of adducts within DNase II-sensitive nuclear DNA is 9.1 hours, and the $t_{1/2}$ within whole nuclear DNA is 10.7 hours.
Figure 4-11  Time course of $\text{AFB}_1$ adduction within ribosomal DNA isolates and total nuclear DNA from the liver nuclei of animals administered a $1 \text{ mg/kg}$ $[^3\text{H}]\text{AFB}_1$ dose. Each data point represents the average ± standard deviation of three experiments. Each experiment consists of pooling the livers from three rats administered a $1 \text{ mg/kg}$ $[^3\text{H}]\text{AFB}_1$ dose.
Figure 4-12A  Half-life of AFB₁ adduction within ribosomal DNA isolates and total nuclear DNA from the liver nuclei of animals administered a 1 mg/kg [³H] AFB₁ dose. Experimental details are given in the introduction to Figure 5-11.

Figure 4-12B  Half-life of AFB₁ adduction within DNase II-sensitive, DNase II-insensitive, and total nuclear DNA from the liver nuclear chromatin of animals administered a 1 mg/kg [³H] AFB₁ dose.
The principal AFβ₁ adduct present in nuclear DNA, AFβ₁-N⁷-guanine, is relatively stable under neutral or slightly acidic conditions: its half-life has been calculated to be approximately 100 hours (92). The 17-fold decrease in the half-life of ribosomal DNA adduction therefore strongly implicates a role for enzyme activity in the removal of AFβ₁ moieties from these gene sequences. Analysis of the rate of adduct removal further supports this assertion. Table 4-2 compares the rate, measured in picomoles of AFβ₁ per milligram of DNA removed per hour, of AFβ₁ removal between each of the time points measured for both ribosomal DNA and whole nuclear DNA over the 12 hour period post-dosing. Thus ribosomal DNA sequences are not only preferentially susceptible to structural modification and damage by AFβ₁; these sequences may also be preferentially susceptible to the enzymatic removal of adduct moieties as compared to the nuclear genome as a whole.
<table>
<thead>
<tr>
<th>Table 4-2</th>
<th>Rates of Removal of AFB₁ Adducts from Ribosomal and Nuclear DNA</th>
</tr>
</thead>
</table>

Rate of Removal
In Picomoles AFB₁/Milligram DNA/Hour

<table>
<thead>
<tr>
<th>Hours Post-Dosing</th>
<th>2 - 6</th>
<th>6 - 9</th>
<th>9 - 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal DNA</td>
<td>118</td>
<td>98</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rDNA ave.: 86)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear DNA</td>
<td>14</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Nuclear DNA ave.: 16)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Dose Response Relationships of Aflatoxin B<sub>1</sub> Adduction to Ribosomal DNA

Because ribosomal DNA sequences appear to be preferentially susceptible to AFB<sub>1</sub> adduction, the question arises as to whether the binding levels observed are dose-related to the 1 mg/kg AFB<sub>1</sub> dose administered. The binding levels observed may represent a point at which AFB<sub>1</sub> binding to rDNA sequences has reached a plateau; adduct levels at doses below and above 1 mg/kg may not be related to the toxin dose administered. Thus, we determined the level of adduction to ribosomal DNA isolates from animals administered a 0.25 and 0.50 mg/kg [<sup>3</sup>H]-AFB<sub>1</sub> dose and sacrificed 2 hours post dosing. Figure 4-13 illustrates the dose-response relationship of AFB<sub>1</sub> binding to ribosomal DNA isolates under these time and dose conditions. Dose-dependent binding of AFB<sub>1</sub> to ribosomal DNA is observed over the four-fold range of toxin administration. No saturation is observed in the capacity of ribosomal DNA sequences to be adducted by AFB<sub>1</sub> metabolites.
Figure 4-13  Dose-dependent binding of AFB₁ to ribosomal DNA isolates from liver nuclei of animals administered a 0.25, 0.5, or 1.0 mg/kg \(^{3}\mathrm{H}\)AFB₁ dose. As in figure 4-11, each data point is the average \(\pm\) S.D. of three experiments. Each experiment consists of the pooling of the livers from three treated animals.
Time Course of AFB$_1$ Adduct Species within Ribosomal DNA

As summarized in chapter 2, the major in vivo and in vitro AFB$_1$-DNA adduct species is AFB$_1$-N$^7$-guanine (86,87). The instability of this adduct leads to (1) depurination of the AFB$_1$-N$^7$-guanine moiety creating an apurinic site, and (2) hydroxide attack at the C-8 position of guanine creating ring-opened, stable derivatives of AFB$_1$-N$^7$-guanine: AFB$_1$-FAPyr and AFB$_1$ peak F (Figure 4-5). The time course of these three major AFB$_1$-DNA adducts, taken from the data of Croy and Wogan (39,40), are summarized in Figure 4-14 for in vivo studies of whole nuclear DNA. As shown during the first 12 hours post-dosing, AFB$_1$-N$^7$-guanine is the major AFB$_1$-DNA adduct in nuclear DNA, accounting for 82% of the bound AFB$_1$ radioactivity. The proportion of this adduct decreases with time, and the two ring-opened derivatives of AFB$_1$-N$^7$-guanine become the predominant adduct species.

The need to convert AFB$_1$-N$^7$-guanine moieties to ring-opened derivatives as required for the isolation of ribosomal DNA sequences, however, prevents a direct determination of the rDNA adduct profile. An alternative procedure has therefore been devised to measure, as a function of time, the quantities of these three major AFB$_1$ adduct species in rDNA. Figure 4-15 summarizes this protocol. Nuclear DNA, enriched in rDNA sequences by one round of cesium salt density centrifugation, was incubated in 20 mM sodium phosphate, 1 mM Na$_2$EDTA, pH 6.8 at 85-88°C for 20 minutes. The glycosidic bond of N$^7$-substituted guanines is
Figure 4-14  Time course *in vivo* of the three predominant AFB$_1$ adduct species in total nuclear DNA. Values taken from the work of Croy and Wogan (39,40).

The quantity of each adduct species is expressed as a percentage of the total DNA hydrolysis products.
Figure 4-15 Protocol to quantify the amount of AFB₁-N⁷-guanine in ribosomal DNA isolates of liver nuclear DNA from aflatoxin B₁ treated animals.
$^{[3}H] - AFB_i \rightarrow \text{Rat}
\downarrow
\text{Isolate Liver Nuclear DNA}
\text{Enriched in Ribosomal DNA by}
\text{Cesium Salt Density}
\downarrow
\text{Heat DNA Isolate in}
20 \text{ mM Sodium Phosphate,}
1 \text{ mM EDTA, pH 6.8}
\text{For 20 Minutes at 85-88°C.}
\downarrow
\text{Isolate Ribosomal DNA}
\downarrow
\text{Determine Amount of Bound}
AFB_i - FAPyr and AFB_i - Peak F
\downarrow
\begin{bmatrix}
\text{Quantity of} \\
\text{AFB}_i - \text{FAPyr} \\
\text{AFB} - \text{Peak F}
\end{bmatrix}
\begin{bmatrix}
\text{in Alkali-Treated} \\
\text{Ribosomal DNA}
\end{bmatrix}
- \begin{bmatrix}
\text{Quantity of} \\
\text{AFB}_i - \text{FAPyr} \\
\text{AFB}_i - \text{Peak F}
\end{bmatrix}
\begin{bmatrix}
\text{in Hot Buffer-Treated} \\
\text{Ribosomal DNA}
\end{bmatrix}
= \text{Quantity of} AFB_i - N^7\text{-Guanine}
unstable as previously described and breaks upon heating at neutral pH (213). Treating AFB$_1$-adducted DNA under these conditions therefore should liberate the AFB$_1$-N$^7$-guanine adduct leaving the more stable ring-opened species bound to DNA. High pressure liquid chromatographic analysis of ribosomal DNA isolated from hot buffer-treated nuclear DNA would permit determination of the true quantities of AFB$_1$-FAPyr and AFB$_1$-peak F present. The difference in the quantities of these two adduct species between glycine-treated and hot buffer-treated rDNA isolates would thus represent the quantity of AFB$_1$-N$^7$-guanine present in rDNA. Therefore, by this methodology, the time-course of removal of the three principal AFB$_1$ adducts could be determined within ribosomal DNA.

Figure 4-16 illustrates the selective liberation of AFB$_1$-N$^7$-guanine under these hot buffer-treatment conditions. Chromatographic analysis was performed on hot buffer-treated and untreated DNA adducted in vitro to a level of 1550 picomoles AFB$_1$/mg DNA (equivalent to 530 AFB$_1$ adducts per $10^6$ nucleotides). To monitor more accurately the fate of AFB$_1$-FAPyr and AFB$_1$-Peak F under these conditions, the DNA samples used were a 50:50 mixture of alkali-treated and untreated AFB$_1$ adducted DNA; a chromatogram of this DNA mixture is shown in Figure 4-16A. After a 20 minute treatment with 20 mM sodium phosphate, 1 mM Na$_2$EDTA, pH 6.8 at 85-88°C, greater than 99% of AFB$_1$-N$^7$-guanine is liberated while the ring-opened adduct species are stable (Figure 4-16B). Investigations were also conducted with tritium and [$^{14}$C]-labelled
Chromatographic analysis of DNA adducted in vitro to a level of 1550 picomoles AFB\textsubscript{1}/mg DNA (equivalent to 530 AFB\textsubscript{1} adducts per $10^6$ nucleotides). The DNA samples were a 50:50 mixture of glycine-NaOH - treated and untreated AFB\textsubscript{1} adducted DNA.

A: Chromatogram of the DNA mixture

B: Chromatogram of the same mixture after treatment in 20 mM sodium phosphate, 1 mM Na\textsubscript{2}EDTA, pH 6.8 at 85°-88°C for 20 minutes. Chromatographic conditions are given in Materials and Methods.
AFB₁-adducted DNA to determine if this treatment regimen caused any loss of the AFB₁-bound radioactivity. It was found that 12% of the AFB₁ tritium liberated from AFB₁-FAPyr and AFB₁-Peak F under these conditions, and thus the levels of bound AFB₁ radioactivity in rDNA were adjusted to reflect this tritium loss.

Table 4-3 summarizes the quantities of AFB₁-FAPyr and AFB₁-Peak F in both glycine-treated and hot buffer-treated ribosomal DNA isolates. The difference in the total amounts of these two adducts is given as the quantity of AFB₁-N⁷-guanine; the time course of all three adducts species, as a percentage of the total bound hydrolysis products, is given in Figure 4-17A. In comparing the time course of each of the three adduct species in nuclear DNA versus ribosomal DNA (Figure 4-17B versus 4-17A), no substantial differences are observed. In ribosomal DNA as in nuclear DNA, AFB₁-N⁷-guanine is the predominant adduct species over the time period up to 12 hours post-dosing. As a percentage of the bound adduct species, the proportion of the two ring-closed species increases with time. Further as the quantity of AFB₁-N⁷-guanine decreases, the quantity of AFB₁-FAPyr increases; this indicates that AFB₁-N⁷-guanine is not only being removed, but it is also being converted to the more stable ring-opened adduct species.
Figure 4-17  Time course *in vivo* of the three predominant
AFB₁ adduct species in:
(A) ribosomal DNA isolates
(B) total nuclear DNA (repeat of Figure 4-14)
Table 4-3 Quantification of the Acid Hydrolysis Products of Glycine-Treated and Hot-Buffer Treated Ribosomal DNA Isolates

(Expressed in Units of Picomoles of Aflatoxin B₁/Microgram Ribosomal DNA)

<table>
<thead>
<tr>
<th>Time</th>
<th>Glycine-Treated</th>
<th>Hot Buffer-Treated</th>
<th>Quantity of AFB₁-N⁷-Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB₁ FAPyr (A)</td>
<td>AFB₁ FAPyr (C)</td>
<td>From FAPyr (A-C)</td>
</tr>
<tr>
<td></td>
<td>AFB₁ Peak F (B)</td>
<td>AFB₁ Peak F (D)</td>
<td>From Peak F (B-D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (A-C)+(B-D)</td>
</tr>
<tr>
<td>Post-Dosing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Hours</td>
<td>1.0</td>
<td>0.11</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>0.072</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>6 Hours</td>
<td>0.68</td>
<td>0.19</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td>12 Hours</td>
<td>0.49</td>
<td>0.20</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>0.33</td>
</tr>
</tbody>
</table>
Chapter 5
Mechanisms of AFB₁-Induced Inhibition of Ribosomal RNA Synthesis

Introduction

The results presented in chapter 4 indicate that ribosomal DNA sequences are preferential targets for AFB₁ adduction in the liver DNA of AFB₁-treated animals. However, one of the principal pieces of evidence which indicated these sequences to be preferential targets for AFB₁ adduction was the reported preferential inhibition of ribosomal RNA synthesis as compared to nuclear RNA synthesis (113-115). As summarized in chapter two, further mechanistic studies have shown that the inhibition of ribosomal RNA synthesis is due to the impairment of the ribosomal DNA template while inhibition of extranucleolar RNA synthesis is due primarily to inhibiton of RNA polymerase activity. The findings summarized in chapter 4 would suggest that this preferential impairment of the ribosomal DNA template is due to the preferential adduction by AFB₁ metabolites of ribosomal DNA sequences.

Experiments have been designed to further investigate the quantitative and qualitative relationships between AFB₁ adduction, and the functional impairment which results, within ribosomal RNA gene sequences. Nucleolar RNA synthesis inhibition has been determined; a mathematical representation of the transcriptional processes perturbed in
ribosomal RNA genes by $\text{AFB}_1$-DNA modification has been constructed to relate the inhibition of RNA synthesis by $\text{AFB}_1$ to the adduction of ribosomal DNA sequences.

Further, the mechanisms underlying the aflatoxin $\text{B}_1$-induced inhibition of nucleolar transcription have been investigated by two approaches. First, the nascent rRNA transcripts produced in vivo after $\text{AFB}_1$ administration have been characterized with respect to size. Second, an in vitro nucleolar transcription assay has been employed to estimate the percentage of in vitro RNA synthesis represented by RNA polymerase elongation versus polymerase initiation of transcription. Employing these two approaches, the mechanisms underlying the transcriptional effects of $\text{AFB}_1$ in the nucleolus, i.e. inhibition of polymerase initiation of transcription or the blockage of polymerase movement along the ribosomal DNA template, can be assessed, and the accuracy of the supporting assumptions of the derived mathematical representation can be tested.

**Materials, Methods, Animals**

The same chemical sources and animal procedures were employed in these experiments as in previous chapters.
Quantification of the In Vivo Inhibition of Nuclear and Nucleolar RNA synthesis

Male Fischer rats (125-150 grams) received a 1 mg/kg AFB₁ dose and were sacrificed 0.5 to 12 hours-post dosing. Thirty minutes prior to sacrifice, 200 microcuries of [³H]-orotic acid was injected intraperitoneally. After sacrifice, clean nuclei and nucleoli preparations were obtained as described in chapter three, and the RNA from these cell fractions was isolated by the hot phenol:SDS procedure of Penman described earlier (229). Counts per A₂₆₀ unit RNA were compared at each timepoint with the same value in untreated controls.

Characterization of the In Vivo Nascent Ribosomal RNA Transcripts after AFB₁ Administration

As in the previous section, animals were administered a 1 mg/kg AFB₁ dose and sacrificed 2, 6, 9, and 12 hours post dosing. Thirty minutes prior to sacrifice, 400 microcuries of [³H]-orotic acid was administered i.p. Clean nucleolar preparations were isolated from liver nuclei and total nucleolar RNA was extracted (229).

The phenol extracted nucleolar RNA isolate was subsequently glyoxylated, to eliminate secondary structure, as described by McMasters and Carmichael (246). The RNA isolates were brought up in 1.0 M glyoxal, 50% (v/v) dimethyl sulfoxide, and 10 mM sodium
phosphate, pH 7.0 at a concentration of 0.5-0.75 mg/ml. The samples were transferred to capped plastic Eppendorf tubes and heated for 1 hour at 50°C. After incubation, the samples were cooled to room temperature and made 10% in sucrose spin solution. The treated rRNA was layered onto 10-40% sucrose-sarkosyl gradients (composed as described in the appendices) and centrifuged in an SW40 Becman rotor for 13 hours at 40,000 rpm. The gradients were fractionated with a density gradient fractionator (ISCO, Inc.), and the fractions were assayed for radioactivity by liquid scintillation.

In Vitro RNA Polymerase Assay—
Isolation of Rat Hepatic Nuclear and Nucleolar Fractions

Rat liver fractions were isolated by the hypertonic sucrose method as described by Yu (42). All procedures were performed at 0-4°C. Animals were stunned and decapitated, and their livers were perfused in situ via the portal vein with 10 ml of ice-cold TSC buffer (0.02 M Tris-HCl-HCl, pH 6.9:0.25 M sucrose:3.3 mM CaCl₂). The livers were removed, weighed, and homogenized in STC buffer (2.0 M sucrose: 0.02 M Tris-Cl, pH 6.9: 3.3 mM CaCl₂), 2.5 ml/gram liver, in a loose glass dounce homogenizer. The homogenate was filtered through four layers of cheesecloth and diluted with 2 volumes of STC buffer. The homogenate was then centrifuged at 21,000 rpm (40,000 xg) in a Beckman 30 rotor for 1 hour. The
resulting nuclear pellet was resuspended in 0.34 M sucrose at 1 ml/gram original liver. The yield of nuclei, measured by quantitative recovery of DNA from the original liver homogenate, was 45-55%.

For the isolation of nucleoli, 10 ml aliquots of the nuclear suspension were subjected to the sonication protocol described in chapter three, yielding a clean nucleolar pellet. The resulting pellet was resuspended in 0.34 M sucrose at a concentration of 1 ml/10 grams of original liver. After resuspension, the nucleolar mixture was transferred to a chilled teflon-glass homogenizer and gently homogenized with 6-8 up and down strokes.

**Assay of RNA Polymerase Activity**

Total nucleolar polymerase activities were assayed *in vitro* with a standard polymerase reaction mixture as outlined in table 5-1. To a standard RNA polymerase reaction mixture, 0.20 ml of the nucleolar homogenate was added and the mixture incubated at 37°C for 20 minutes with constant shaking. The reaction was stopped by transfer of the reaction mixture to an ice slurry, followed by the addition of 6 ml of a cold 10% trichloroacetic acid: 1% sodium pyrophosphate
Table 5-1  Standard RNA Polymerase - Template Reaction Mixture

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final Reaction Mixture Concentration (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.2</td>
<td>50</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>1</td>
</tr>
<tr>
<td>MeSH</td>
<td>14</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>40</td>
</tr>
<tr>
<td>ATP</td>
<td>0.1</td>
</tr>
<tr>
<td>UTP</td>
<td>0.1</td>
</tr>
<tr>
<td>CTP</td>
<td>0.1</td>
</tr>
<tr>
<td>GTP</td>
<td>0.1</td>
</tr>
<tr>
<td>$[^{14}C]$GTP</td>
<td>0.5 μCuries</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 0.55 ml</td>
</tr>
</tbody>
</table>
(TCA:PP) mixture. After allowing 5 minutes for the precipitation of acid-insoluble material, the precipitate was collected on Whatman GF-C filters (the filters were first soaked in 5% TCA:PP mixture for 3 minutes and then in 10% TCA:PP for 3 minutes). The filters were washed five times with 5% TCA:PP and twice with 5 ml of 80% ethanol/H$_2$O (the latter cooled to $-20^\circ$C).

The filters were air dried in 20 ml glass (not plastic) scintillation vials for 3 hours. The radioactivity incorporated into the precipitated RNA was counted in 15 ml of Bray's solution. Samples of the nucleolar homogenate were assayed for DNA by the Burton colorimetric reaction as modified by Giles and Myers (200), described in chapter 3 and the appendices. The specific activity of the nucleolar RNA polymerase was expressed as picomoles of $[^{14}$C]-GMP (incorporated into TCA-precipitable RNA) per milligram of DNA in the polymerase reaction mixture.
Results

Mathematical Representation of the Ribosomal DNA Transcriptional Processes Perturbed by Aflatoxin-DNA Modifications.

To relate the observed inhibition of ribosomal RNA synthesis by AFB₁ to the adduction of rDNA sequences, a mathematical representation of the transcriptional processes perturbed by aflatoxin-DNA modifications has been constructed, and is described below.

1. Subdivide the population of rRNA genes into fractions which contain 0, 1, 2, 3, ..., N adducts/rRNA genes.

Specifically,

\[ P(0) = \text{fraction of rRNA genes containing 0 AFB}_1 \text{ adducts} \]
\[ P(1) = \text{fraction of rRNA genes containing 1 AFB}_1 \text{ adduct} \]
\[ P(N) = \text{fraction of rRNA genes containing N AFB}_1 \text{ adducts} \]

with \[ P(0) + P(1) + P(2) + P(3) + \ldots \ldots \ldots + P(N) = 1 \]

2. Let \( L \) = the average number of AFB₁ adducts per ribosomal RNA gene. Using the Poisson distribution, calculate \( P(N) \) for each value of \( N \) -- i.e. the fraction of rRNA genes which contain 0, 1, 2, 3, ..., N AFB₁ adducts:

the fraction of rRNA genes which contain \( N \) adducts given an average of \( L \) adducts in a rRNA gene

\[
= \frac{L^N e^{-L}}{N!}
\]
3. Assume that an RNA polymerase I molecule transcribing a ribosomal RNA gene is blocked from further elongation when it comes upon an AFB₁ adduct. The presence of an adduct in an rRNA gene will thus lead to premature termination of transcription and production of truncated nascent 45S rRNA molecules. This premise is supported by in vitro studies by Yu (45) which have suggested that the primary mechanism of AFB₁ transcriptional inhibition is to block the elongation stage of 45S ribosomal RNA synthesis.

4. Consider \( P(1) = \text{fraction of rRNA genes which contain 1 adduct}. \) While adducts will be uniformly distributed throughout the gene, the average distance from the promoter region to the first adduct will be \( \frac{1}{2} \) or \( \frac{1}{N+1} \) the length of the gene. Thus, \( P(1) \) rRNA genes will produce, on average, transcripts 50% of the length of 45S rRNA. The contribution of this subset of genes to the total rRNA synthesis is \( P(1) \times 50\% \), while the contribution of the subset containing no adducts is \( P(0) \times 100\% \).

5. Calculate the amount of rRNA transcription in a population of AFB₁-treated liver cells:

\[
\sum_{N=0}^{\infty} P(N) \left( 100\% \times \frac{1}{N+1} \right) = \frac{1}{L} (1 - e^{-L})
\]

compared to the transcription in untreated cells, which is 100%.
The model equation (#5) presented on the previous page thus establishes a direct relationship between the average level of modification within ribosomal DNA, L, and the amount of \textit{in vivo} transcription measured after the administration of an AFB\textsubscript{1} dose. Figure 5-1 summarizes the time course of nuclear and nucleolar RNA synthesis inhibition observed after a 1 mg/kg AFB\textsubscript{1} dose. Each data point represents the average of three experiments; each experiment consists of the pooling of livers from 4 animals. Consistent with published investigations reviewed in chapter two, nucleolar RNA synthesis was found to be preferentially inhibited, compared to nuclear RNA synthesis, after AFB\textsubscript{1} administration. Maximum inhibition in both fractions was observed at 2 hours post-dosing, when nucleolar RNA synthesis was 12.7\% of control levels.

To test the ability of the mathematical representation to relate the levels of ribosomal DNA adduction given in chapter 4 to the levels of RNA synthesis inhibition given in Figure 5-1, the model equation was employed to calculate the expected fraction of normal transcription as a function of the average number of AFB\textsubscript{1} adducts per ribosomal RNA gene (given on the next page).
Figure 5-1  Time course of nuclear and nucleolar RNA synthesis following AFB1 administration. Experimental details given in the text. Each data point represents the average of three experiments; error bars representing the standard deviation of each experiment are within the data symbols.
Time Course of Nuclear and Nucleolar RNA Synthesis following AFB$_1$ Administration.

![Graph showing the time course of nuclear and nucleolar RNA synthesis following AFB$_1$ administration. The graph plots percent control transcription against hours post dosing. There are two lines: one for nuclear and one for nucleolar RNA synthesis. The nuclear RNA synthesis shows a sharp decrease followed by a gradual increase, while the nucleolar RNA synthesis decreases more gradually.](image-url)
<table>
<thead>
<tr>
<th>Average # Adducts/rRNA Gene</th>
<th>Expected Fraction of Normal Transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>0.631</td>
</tr>
<tr>
<td>2</td>
<td>0.431</td>
</tr>
<tr>
<td>3</td>
<td>0.354</td>
</tr>
<tr>
<td>4</td>
<td>0.244</td>
</tr>
<tr>
<td>5</td>
<td>0.199</td>
</tr>
<tr>
<td>6</td>
<td>0.165</td>
</tr>
<tr>
<td>7</td>
<td>0.140</td>
</tr>
<tr>
<td>8</td>
<td>0.126</td>
</tr>
</tbody>
</table>

At two hours after a 1 mg/kg AFB₁ dose, 12.7% of normal (control) RNA synthesis is observed in liver nucleoli, as shown in Figure 5-1. Using the numbers above relating the number of AFB₁ adducts to expected amounts of transcription, a fraction of 0.127 normal transcription at two hours indicates 7.9 AFB₁ adducts per rDNA gene (equivalent to 1587 picomoles AFB₁/mg DNA). Experimentally, the value for AFB₁ adduction of rDNA at 2 hours post-dosing is 1494 pmoles/mg DNA (figure 4-11). Comparison of these two values, differing by 6%, would indicate that the mathematical representation can accurately relate ribosomal DNA transcriptional inhibition to ribosomal DNA adduction.
Table 5-2 further compares the time course of AFB$_1$ adduction presented in Figure 4-11 to the time course of adduction predicted by the mathematical representation of the model equation. The values for both the predicted and observed binding are given in number of adducts per ribosomal RNA gene; this includes only the strand of DNA which is transcribed during synthesis of a 45S rRNA molecule. As illustrated by the percent difference in the actual versus predicted ribosomal DNA adduct levels, the mathematical representation accurately relates AFB$_1$ binding within rDNA to the transcriptional inhibition which results: The level of modification within rRNA gene sequences at a specific time can thus be quantitatively related to the inhibition of ribosomal RNA synthesis at the same time.

The mathematical representation presented above was designed to specifically relate transcriptional inhibition and AFB$_1$ adduction within ribosomal RNA genes. To expand the usefulness of this mathematical approach to the investigation of transcriptional inhibition in other genes, a more general model approach can be taken as described below.

(a) as developed above, the fraction of control transcription in a population of AFB$_1$-treated cells

\[ \frac{1}{L} \left( 1 - e^{-L} \right) \]
Table 5-2  Comparison of the Time Course of AFB₁-Ribosomal DNA Adduction with the Time Course Predicted by the Mathematical Representation of AFB₁-Induced Nucleolar RNA Synthesis Inhibition

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>(A) Actual Level of rDNA Adduction (# adducts/gene)</th>
<th>(B) Predicted Level of rDNA Adduction (# adducts/gene)</th>
<th>%Difference (\frac{(B-A)}{A})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hours</td>
<td>7.4</td>
<td>7.9</td>
<td>6.8%</td>
</tr>
<tr>
<td>6 hours</td>
<td>5.1</td>
<td>5.7</td>
<td>12%</td>
</tr>
<tr>
<td>9 hours</td>
<td>3.7</td>
<td>4.1</td>
<td>11%</td>
</tr>
<tr>
<td>12 hours</td>
<td>2.9</td>
<td>3.2</td>
<td>10%</td>
</tr>
</tbody>
</table>
(b) given \[ L = \# \text{AFB}_1 \text{ adducts/gene} \]
\[ L' = \# \text{AFB}_1 \text{ adducts/microgram DNA} \]
\[ K = \# \text{basepairs per gene} \]
\[ Z = \# \text{genes per microgram DNA} \]
\[ X = \# \text{basepairs per microgram of DNA} \]

then \[ L = L'/Z \]
and \[ K = X/Z \]

(c) The size of a particular gene, \( S_0 \) (which is equal to \( K \), the number of base pairs per gene), will be the size of the primary RNA transcript. In \( \text{AFB}_1 \)-treated cell populations, the size of the primary gene transcript will be reduced, since a transcribing RNA polymerase will be inhibited when the enzyme comes upon a DNA-bound adduct. The length of the average truncated transcript will be given by \( S = \frac{K}{L+1} \)

so that the fraction of control transcription length will be given by

\[
\frac{S}{S_0} = \frac{K/L+1}{K} = \frac{1}{L+1} = \frac{1}{(L'/Z) + 1}
\]

equivalent to point (4) described in the previous rDNA model derivation.
(d) Substituting into the model equation (5), the fraction of normal RNA synthesis observed in AFB₁-treated cells is:

\[
\frac{1}{L} \left( 1 - e^{-L} \right) = z \frac{Z}{L'} \left( 1 - e^{-L'/Z} \right)
\]

\[
\Downarrow
\]

\[
\frac{S_o}{S} = \frac{(L'/Z)}{+1}
\]

\[\Rightarrow \quad \frac{S_o}{S} - 1 = \frac{L'}{Z}
\]

and \[
\frac{S_o - S}{S} = \frac{L'}{Z}
\]

(e) Substituting into the equation in point (a), the fraction of normal RNA synthesis observed in AFB₁-treated cells is

\[
= \left( \frac{S}{S_o - S} \right) \left( 1 - e^{-\left( \frac{S_o - S}{S} \right)} \right)
\]

and \[
\frac{S}{S_o} = \frac{\text{average size of truncated transcript}}{\text{size of primary transcript}} = \frac{1}{(L'/Z) + 1}
\]
Evaluation of the AFB₁-Induced Inhibition of Transcriptional Initiation and Transcriptional Elongation in Ribosomal RNA Genes

Analysis of the inhibition of nucleolar transcription (summarized in Figure 5-1) by the mathematical representation would indicate that premature termination is in fact the predominant mechanism by which AFB₁ adduction of ribosomal DNA templates leads to the inhibition of ribosomal RNA synthesis in vivo. We have approached the question of the role of inhibition of transcription initiation and elongation by employing an in vitro transcription assay which can differentiate between these two possibilities. Nucleoli, isolated from liver cell nuclei, contain RNA polymerase I molecules which are bound to the rDNA template and not bound and thus capable of transcriptional initiation. As just discussed, evidence from the literature (45) suggests that AFB₁ can interfere with the elongation of nascent transcripts. However, the role of inhibition of polymerase initiation may also contribute to the template-dependent inhibition of nucleolar RNA synthesis by AFB₁.

We have determined the capacity of nucleolar homogenates from AFB₁-treated animals to synthesize ribosomal RNA in vitro in the presence and absence of the detergent sarkosyl (sodium sarkosinate). Electron micrographic studies of
transcribing ribosomal RNA genes have shown that sarkosyl effectively removes all the bound proteins from the DNA strands—except transcribing RNA polymerase molecules (162). In sarkosyl-treated nucleolar homogenates, RNA polymerase molecules can continue to synthesize RNA until the enzyme reaches termination codons, after which it dissociates from the DNA strand. Reinitiation of transcription, however, cannot occur. Thus, in vitro transcription by nucleolar homogenates from AFB<sub>1</sub>-treated animals would represent synthesis by both initiating and elongating RNA polymerase molecules. The same in vitro transcription assay conducted in the presence of sarkosyl would represent transcriptional elongation only; the difference in results between these two systems (with and without sarkosyl) would indicate the amount of transcriptional initiation that is occurring in vitro. A comparison of the percentage of in vitro transcription due to initiation and premature termination using nucleoli from AFB<sub>1</sub>-treated versus untreated animals would permit an assessment of the effects of AFB<sub>1</sub> on each of these transcriptional processes.

Figure 5-2 summarizes the results of these in vitro experiments. Nucleolar homogenates from animals administered a 1 mg/kg AFB<sub>1</sub> dose were isolated 2,6,9, and 12 hours post-dosing as described in the Methods sections. The homogenates were incubated
Effect of AFB₁ administration *in vivo* on nucleolar RNA synthesis *in vitro*, measured in the presence and absence of sodium sarkosinate.
Effect of AFB Injected in vivo on Activity of Nucleolar RNA Polymerase Measured in vitro (Given as % Control Activity) → 7% 21% 32% 44%

RNA Polymerase Activity

Picomoles $[^{14}C]GTP$ Incorporated per Mg DNA

- Serkosal
- + Serkosal

Time Post-Dosing (Hours)

Untreated Control

2 6 9 12
for 20 minutes in the transcription assay mixture given in Table 5-1 along with $^{14}$C-GTP to label nascent ribosomal RNA synthesized in vitro. The rRNA was subsequently precipitated with trichloroacetic acid, collected on filters, and the incorporated $^{14}$C label quantified by liquid scintillation. These results are given by the shaded boxes of Figure 5-2. The percent decrease in transcription as compared to untreated controls is given by the percent figures across the top of the graph.

Each bar figure represents the average of three experiments; the error T on top of each bar represents the standard deviation of the experimental value. Each experiment consists of pooling the livers of 5 animals. As shown, AFB$_1$ treatment decreases the amount of in vitro transcription observed. The time course of RNA synthesis in vitro appears to mimic that observed in vivo in AFB$_1$-treated animals. Transcriptional inhibition is maximal at two hours post-dosing and decreases over the 12 hour period observed.

These in vitro transcription studies were repeated with the addition of sodium sarkosinate (ICN, Inc.) to 0.5% of the transcription mixture. The results of these experiments are given by the white boxes next to the previously described black boxes: black box (− sarkosyl), white box (+ sarkosyl). The percentage decrease between the + sarkosyl and − sarkosyl experiments is given inside the white bar.

Consider first the values for the untreated controls. Nucleoli from untreated animals incorporated 6,600 picomoles of
\(^{14}\)C-GTP (as GMP) in nascent RNA per milligram of nucleolar DNA. With the addition of sarkosyl, the level of \textit{in vitro} RNA synthesis dropped 36\% to 4200 pico moles \(^{14}\)C-GTP per mg DNA. This decrease is due primarily to the inhibition of RNA polymerase I initiation. Approximately 64\% of the observed \textit{in vitro} RNA synthesis is therefore due to bound polymerase elongation while approximately 36\% is due to \textit{de novo} initiation of transcription.

Next consider the values for the 12 hour timepoint. Nucleoli from animals administered a 1 mg/kg AFB\(_1\) dose and sacrificed 12 hours later incorporate only 2900 pmoles \(^{14}\)C GTP/mg DNA. This indicates a 56\% decrease in \textit{in vitro} transcription due to AFB\(_1\) adduction of the ribosomal DNA template. Upon the addition of sarkosyl to the assay mixture, the incorporation of labelled presursor dropped 32\% to 2000 pico moles/mg DNA. Therefore the percent decrease in \textit{in vitro} transcription observed with the 12 hour samples with and without sarkosyl addition is comparable to control preparations. This would indicate that the same percentage of \textit{in vitro} RNA synthesis is due to polymerase initiation in AFB\(_1\)-treated and untreated nucleoli preparations. Once polymerase initiation does occur, the polymerase comes upon an AFB\(_1\) adduct, and prematurely terminates transcription. Further examination of the values observed with the 2, 6, and 9 hour samples indicates that poly-
merase initiation is similarly not inhibited in these experiments as well.

Characterization of the In Vivo Nascent Ribosomal RNA Transcripts after AFB₁ Administration

To further evaluate the accuracy of the model equation to relate ribosomal DNA adduction and nascent rRNA transcript length, experiments were undertaken to characterize the nascent ribosomal RNA transcripts produced in vivo in the liver nucleoli of AFB₁-treated animals. Point #4 in the mathematical representation presented above states that polymerases transcribing an AFB₁-adducted rDNA template will stop when they encounter an AFB₁ residue. The presence of AFB₁ adducts would thus lead to premature termination of transcription and truncated nascent 45S rRNA molecules.

In vitro studies reported by Yu (45) have supported this premise. In these studies, Yu and coworkers isolated nucleoli from AFB₁-treated animals and incubated the nucleolar homogenates in an in vitro transcription assay similar to the system described in the methods section of this chapter. The nascent rRNA transcripts synthesized in vitro were hydrolyzed in KOH and the amount of GMP and guanosine incorporated into the RNA macromolecules was determined by anion exchange chromatography. The ratio of
3'-terminal guanosine to internal GMP was taken as an approximation of the length of the ribosomal RNA chains. Using this technique, the length of in vitro nascent rRNA chains was found to be almost directly proportional to the inhibition of in vitro ribosomal RNA synthesis: in vitro transcription inhibited to 10% of control levels was found to produce transcripts 12% of control length. These results were taken to indicate that RNA polymerase 1 molecules were inhibited from further elongation when they come upon an AFB₁ adduct.

We have designed experimental protocols to measure directly and more accurately the length of in vitro RNA transcripts produced after AFB₁ administration. When single stranded RNA and DNA molecules are treated with glyoxaldehyde, the glyoxylation (of principally purine moieties) removes all secondary structure; these molecules will migrate in agarose gels to distances directly proportional to the logarithm of the macromolecules molecular weight, as described by McMasters and Carmichael (246). We therefore adapted this technique to the characterization of ribosomal RNA molecules on sucrose gradients. Ribosomal RNA was treated with buffered glyoxaldehyde for one hour and separated on 10%-40% detergent sucrose gradients. As shown in Figure 5-3, single-stranded RNA species migrate to positions logarithmically related to their molecular weight in sucrose gradients as in agarose gels. Thus, in vivo nascent ribosomal RNA transcripts in AFB₁-treated animals can be isolated, glyoxylated, and similarly separated on detergent-sucrose gradients to investigate
Figure 5-3  Logarithmic relationship between the molecular weight of glyoxylated RNA standards and their equilibrium position in 10-40% sucrose-sarkosyl gradients.
the effects of ribosomal DNA template adduction on nascent ribosomal RNA size.

Figure 5-4 illustrates the nucleolar RNA profile from an untreated (laβellea control) animal. Total nucleolar RNA was extracted, glyoxylated, and separated on detergent-sucrose gradients; the gradients were subsequently fractionated and the RNA-bound tritium, representing only RNA species synthesized after aflatoxin B₁ administration, was quantified by liquid scintillation. As expected, the RNA profile contains some complete 45S rRNA molecules as well as a spectrum of small, incomplete molecules.

Also shown is the profile of nascent nucleolar RNA species produced two hours after a 1 mg/kg AFB₁ dose. Ribosomal RNA synthesis is significantly suppressed, with radioactivity appearing in RNA species smaller than 45S rRNA. The RNA profile at this two hour timepoint supports the previously discussed study by Yu (45) in which it was found that AFB₁ modification of template DNA induces premature termination of nascent RNA precursor molecules.

Using the peak fraction position (denoted by an *) of the treated RNA profile, the average length of the RNAs in this nucleolar isolate can be approximated. At two hours post-dosing, the mean length of nucleolar RNA from AFB₁-treated animals is 12% of the length of 45S rRNA. From the relationship
Figure 5-4  Profile of nascent RNA molecules separated on 10 - 40% sucrose-sarkosyl gradients after isolation from untreated animals and animals administered a 1 mg/kg AFB₁ dose and sacrificed tow hours post dosing.
between RNA chain length and AFB\textsubscript{1} adduction discussed under model statement (4) above, the average length of nascent ribosomal RNA molecules from genes containing N AFB\textsubscript{1} adducts is \( \frac{1}{N+1} \). Therefore with an average length of 0.12 at 2 hours post dosing:

\[
0.12 = \frac{1}{8.3} = \frac{1}{7.3 + 1}
\]

The estimated value of N = 7.3 compares favorably to the value of N predicted by the model equation, which is 7.9. Analysis of the truncated rRNA molecules produced two hours after AFB\textsubscript{1} administration therefore further supports the mathematical representation presented above which relates ribosomal DNA adduction and rRNA synthesis inhibition.

Figure 5-5 illustrates the radioactivity profile from nucleolar RNA isolates of animals sacrifices 6,9, and 12 hours after AFB\textsubscript{1} administration. As with the analysis of nucleolar transcripts isolated two hours post-dosing, the peak fraction (denoted by an *) of the treated RNA profiles was used to estimate the average length of RNA in the isolate. The levels of AFB\textsubscript{1} adduction within ribosomal DNA predicted from these average size estimations are given in Table 5-3 (column B). The values
Figure 5-5  Profile of nascent nucleolar RNA molecules on 10-40% sucrose-sarkosyl gradients after isolation from animals administered a 1 mg/kg AFB₁ dose and sacrificed 2, 6, 9, and 12 hours post-dosing.
Table 5-3

Levels of Ribosomal DNA Adduction Predicted From Analysis of Nascent Nucleolar RNA Molecules

<table>
<thead>
<tr>
<th>Time Point</th>
<th>(A) rDNA Adduction Predicted by the Model Equation (adducts/gene)</th>
<th>(B) rDNA Adduction Predicted by the Analysis of Nucleolar Transcript Sizes (adducts/gene)</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hours</td>
<td>7.9</td>
<td>7.3</td>
<td>-7.6%</td>
</tr>
<tr>
<td>6 Hours</td>
<td>5.7</td>
<td>4.9</td>
<td>-14%</td>
</tr>
<tr>
<td>9 Hours</td>
<td>4.1</td>
<td>3.0</td>
<td>-27%</td>
</tr>
<tr>
<td>12 Hours</td>
<td>3.2</td>
<td>2.0</td>
<td>-37%</td>
</tr>
</tbody>
</table>
calculated from the RNA profiles, the model equation, and the actual level of rDNA adduction are in good agreement.  

Some deviation appears, however, between the values predicted from the model equation and the RNA profiles at the later time-points. An examination of the ribosomal RNA profiles in Figure 5-5 shows that larger RNA species, particularly 45S rRNA, are not represented in the two hour profile from treated animals, but do appear at 9 and 12 hours post-dosing. One could perhaps conclude that during the periods of maximal AFB₁ adduction of rDNA, the poisson distribution accurately relates adduct levels to RNA synthesis inhibition (2-6 hours). At later timepoints, however, the processes which cause removal of AFB₁ residues, spontaneous depurination and enzymatic removal and/or repair, may cause the distribution of AFB₁ residues within ribosomal DNA genes to deviate from a strict poisson distribution. The mixture of small as well as large 45S truncated transcripts might thus reflect adduction of rDNA in a manner not perfectly predicted by the model equation.
This is the most complete text of the thesis available. The following page(s) were not included in the copy of the thesis deposited in the Institute Archives by the author: $z^3$, $z^3$
Part III
Summary and Discussion

The lines of evidence supporting genetic mechanisms of carcinogenesis have focussed attention on DNA as the critical macromolecular target for chemical carcinogens; this evidence includes the heritability of the tumor phenotype, the irreversibility of the tumor initiation, and the qualitative and quantitative correlations between compounds which exhibit both carcinogenic and mutagenic properties. This emphasis on DNA as a critical macromolecular target is based on the fact that changes in DNA sequences, as products of carcinogen-DNA interactions, provide molecular bases for the alterations in gene expression which accompany neoplastic transformation by chemicals.

The dynamics of chromatin structure during gene transcription led us to postulate that transcribed regions of the genome could be preferential targets for carcinogen modification and damage. Transcribed regions of nuclear chromatin are distinguishable, both biochemically and morphologically, from the majority of nontranscribed DNA by the maintenance of a discrete, active chromatin conformation. We hypothesized that the extended, more diffuse conformation of transcribed chromatin would make these regions of DNA preferentially susceptible to carcinogen adduction. We first approached this question by
determining the time course of AFB₁ adduction within deoxyribo-
nuclease II-sensitive nuclear and nucleolar chromatin DNA
fractions enriched in transcribed gene sequences. The results
of these initial studies, summarized in Part I of this thesis,
supported our hypothesis that, in fact, transcribed gene sequences
may be preferentially susceptible to carcinogen adduction: DNase II-
sensitive regions of nuclear and nucleolar chromatin DNA were
found to be adducted to higher levels in vivo by the hepatocarcinogen
aflatoxin B₁ as compared to unfractionated nuclear and nucleolar
chromatin DNA.

To more adequately investigate the structural, as well as the
functional, impairment of transcribed regions of chromatin DNA,
we have constructed an experimental model system in which:

1) carcinogen-DNA adducts can be measured within specific
transcribed gene sequences, and

2) the qualitative and quantitative relationships
between carcinogen-DNA adduction and functional
endpoints can be investigated in specific gene sequences.

Using this model system, schematically summarized in
Figure 1-1, we have selectively isolated ribosomal RNA gene
sequences from total liver DNA via hybridization to complementary
ribosomal RNA; the levels of AFB₁ modification as well as the
identity of AFB₁ adducts have been determined as a function of
time and dose within these rDNA isolates. Concurrently, nucleolar
RNA synthesis inhibition has been measured and the mechanisms
underlying the AFB₁-induced inhibition of rDNA transcription have been investigated. Using the information from these two lines of study, the time course of rDNA adduction as well as that of ribosomal RNA synthesis inhibition have been integrated into a mathematical representation which accurately relates AFB₁-rDNA adduction to rDNA template inactivation.

Chapter four summarizes our studies of the time course and dose-response of AFB₁ adduction to ribosomal DNA isolates and the identification of the predominant rDNA-bound AFB₁ adducts. A protocol taken from the methods developed by Wellauer and Dawid (211,212) has been designed to selectively separate a ribosomal DNA isolate from the liver DNA of AFB₁-treated animals. Total liver nuclear DNA was isolated from animals administered a [³H]AFB₁ dose and sheared by dounce homogenation. A rDNA enriched-nuclear DNA fraction was subsequently isolated by banding the crude liver DNA preparation in cesium salt density gradients and selectively retaining only the more dense 10% of the DNA peak.

The predominant AFB₁ adduct, AFB₁-N⁷-guanine, which is unstable to extremes of temperature and pH, was stabilized in these DNA isolates by treatment with an alkaline glycine-NaOH buffer. Under these alkaline conditions, AFB₁-N⁷-guanine was shown to be converted to stable, formamidopyrimidine derivatives permitting retention of the bound AFB₁ moieties and accurate quantitation of the DNA-bound radioactivity.
Ribosomal DNA sequences were subsequently isolated by hybridization of the alkali-treated nuclear DNA fractions in a 70% formamide buffer with 18S and 28S ribosomal RNA and separation of the rDNA:rRNA hybrids via two rounds of cesium chloride density centrifugation.

Using this protocol (summarized in Figure 4-2), we determined the time course of AFB₁ adduction to ribosomal DNA isolates from the total liver DNA of AFB₁-treated animals. As summarized in Figure 4-11, the results of these studies confirmed our earlier studies of Part I suggesting that transcribed sequences within the nuclear genome are preferential targets for AFB₁ adduction. Ribosomal DNA isolates were found to bind 4.1 - 4.8-fold more AFB₁ residues over the 12 hour period after administration of a 1 mg/kg [³H]AFB₁ dose. Further as shown in Figure 4-13, it was found that AFB₁ adduction to rDNA was dose responsive over the four-fold dose range tested.

In addition, it was found that AFB₁ residues are preferentially removed from rDNA sequences as compared to total nuclear DNA. Examination of the half-life of AFB₁ adduction within these two DNA populations found the t₁/₂ for AFB₁ binding to rDNA to be 6.1 hours, 43% less than that to nuclear DNA. Because AFB₁-N⁷-guanine, the predominant AFB₁ adduct in nuclear DNA, is relatively stable in vitro with a half-life of 100 hours, the 17-fold decrease in the in vivo versus in vitro half-life of ribosomal DNA adduction strongly implicates a role for enzymatic
removal of \( \text{AFB}_1 \) moieties from rDNA sequences. Analysis of the rate of adduct removal, in terms of picomoles of \( \text{AFB}_1 \) removed per milligram of rDNA over the 12 hour time period studied, further suggested the preferential removal of \( \text{AFB}_1 \) residued in rDNA sequences; as shown in Table 4-2, \( \text{AFB}_1 \) residues are removed at a 5.4-fold greater rate from rDNA versus nuclear DNA over the time observed.

We subsequently determined the time course of the three predominant \( \text{AFB}_1 \) adduct species in rDNA: \( \text{AFB}_1-\text{N}^7\)-guanine, \( \text{AFB}_1\)-FAPyr, and \( \text{AFB}_1\)-Peak F. Liver nuclear DNA from \( \text{AFB}_1\)-treated animals, enriched in rDNA sequences by one round of cesium salt density centrifugation, was heated in sodium phosphate - EDTA buffer to selectively liberate the unstable \( \text{AFB}_1-\text{N}^7\)-guanine adducts. Ribosomal DNA isolates were subsequently prepared by hybridization to rRNA as described in phase 2 of the isolation protocol. HPLC analysis of the bound \( \text{AFB}_1 \) adducts in these rDNA isolates therefore reflected the \textit{in vivo} levels of the two heat-stable adducts, \( \text{AFB}_1\)-FAPyr and \( \text{AFB}_1\)-Peak F. Subtraction of the amounts of these two adducts between glycine-treated and hot buffer-treated DNA was taken as a measure of the amount of \( \text{AFB}_1-\text{N}^7\)-guanine bound to rDNA \textit{in vivo}. As shown by comparison of the adduct profiles of rDNA and nuclear DNA (Figure 4-17), no differences in the predominant \( \text{AFB}_1 \) adduct species was evident. Coupled with the findings of preferential \( \text{AFB}_1 \) adduction within rDNA, the lack of observed differences in the adduct populations within rDNA versus nuclear DNA would suggest that the \( \text{AFB}_1 \)-induced impairment of ribosomal
DNA template function is due exclusively to the greater adduction of rDNA sequences --- not to differences in the profile of AFB₁ adduct species.

Having determined the time course and identity of AFB₁ adducts in rDNA, we next turned our attention to the investigation of the effects of AFB₁ adducts on the template integrity of ribosomal RNA genes. We first reestablished that liver nucleolar RNA synthesis is preferentially inhibited as compared to nuclear RNA synthesis in AFB₁-treated animals. As summarized in the review of studies by Sanders (113), Akrimiski (114), and Yu (42,43), the inhibition by AFB₁ of nucleolar transcription has been shown to result exclusively from inactivation of the ribosomal DNA template. We were therefore able to construct a mathematical model relating the adduction of the ribosomal DNA template to the inhibition of ribosomal DNA transcription which results and test this model using the data on in vivo AFB₁ adduction of rDNA and the in vivo inhibition of ribosomal RNA synthesis. The model we constructed assumes that AFB₁ adducts prevent the elongation of nascent rRNA transcripts leading to the production in vivo of truncated rRNA transcripts: based on this assumption, the levels of rDNA adduction predicted by the model equation were calculated from the time course of ribosomal RNA synthesis given in Figure 5-1. As shown in table 5-2, the close agreement of the actual versus the expected values suggests that the proposed mathematical representation does indeed describe with some accuracy the biochemical events
in the nucleoli of \( \text{AFB}_1 \)-treated animals which lead to the diminution of nascent ribosomal RNA synthesis.

Analysis of the inhibition of nucleolar transcription by the mathematical representation also suggested that the principal model assumption was in fact valid: premature termination is the predominant mechanism by which \( \text{AFB}_1 \) adduction of ribosomal DNA templates leads to the inhibition of ribosomal RNA synthesis \textit{in vivo}. We approached the question of the role of inhibition of transcription initiation and elongation in two ways. First, an \textit{in vitro} transcription assay was employed which could differentiate between these two possibilities. Nucleolar homogenates prepared from \( \text{AFB}_1 \)-treated animals were incubated in an \textit{in vitro} transcription assay as described by Yu (42,43) in the presence and absence of sarkosyl. The ability of this detergent to remove all nucleolar chromatin proteins except transcribing RNA polymerase molecules permitted the determination of the relative amounts of transcriptional initiation and premature termination in these nucleolar homogenates from \( \text{AFB}_1 \)-treated animals as a function of time. As summarized in Figure 5-2, \( \text{AFB}_1 \) administration \textit{in vivo} was not found to alter the amount of polymerase initiation as compared to untreated controls over the 12 hour period post-dosing that was studied. Indirectly, these results supported the model assumption of inhibition of polymerase elongation as the predominant mechanism underlying \( \text{AFB}_1 \)-induced inhibition of ribosomal RNA synthesis.
Second, experiments were undertaken to characterize the nascent rRNA transcripts produced in vivo in the liver nucleoli of AFB₁-treated animals to further evaluate the accuracy with which the model equation relates RNA transcript length to ribosomal DNA adduction. Animals previously administered AFB₁ were given [³H]orotic acid 30 minutes prior to sacrifice to label newly synthesized rRNA. Phenol extracted nucleolar RNA was subsequently glyoxylated to eliminate secondary structure and separated according to size by centrifugation through 10-40% sarkosyl sucrose gradients. Using the peak fraction position of the nucleolar RNA profile, we were able to back calculate the average number of AFB₁ adducts in rDNA from the average length of the rRNA population as given in the model point #4:

a group of rRNA genes which contain N AFB₁ adducts will produce, on average, rRNA transcripts \( \frac{1}{N+1} \) the length of the precursor 45S rRNA. As shown in Table 5-3, the values calculated from the rRNA profiles; the model equation calculations (Table 5-2), and the actual levels of rRNA adduction measured in experiments detailed in chapter four, are in good agreement. These findings, obtained from three separate experimental approaches, therefore strongly support the conclusion that AFB₁ adducts, when encountered by an RNA polymerase molecule, are efficiently able to block further elongation of nascent RNA synthesis and thus cause premature termination of nascent transcripts. The preferential inhibition of nucleolar RNA synthesis observed in AFB₁-treated animals can thus be attributed to the
preferential adduction of the ribosomal DNA template which prohibits elongation of engaged RNA polymerase molecules and causes premature termination of nascent transcripts.

The development of this model system presents the first opportunity to study in vivo both chemical damage within specific gene sequences as well as the template impairment which results in those same gene sequences. Our ability to implement this system and selectively examine the interaction of AFB$_1$ with ribosomal DNA has therefore presented us with a unique advantage in testing the principal question of this thesis:

are ribosomal RNA gene sequences, as a subset of transcribed gene sequences, preferentially susceptible to adduction and functional impairment in the liver nucleoli of AFB$_1$-treated animals.

Our results, summarized in chapter 4 and 5, strongly suggest that rRNA gene sequences, as a subset of transcribed gene sequences, are preferentially adducted by AFB$_1$; in addition, this preferential adduction is responsible for the preferential inhibition of nucleolar RNA synthesis observed in AFB$_1$-treated animals.
Suggestions For Future Research

Research in the following areas is recommended:

1. The studies summarized in chapters 4 and 5 dealt with the study of a specific transcribed gene sequence, the ribosomal RNA gene. In order to adequately compare the distribution within specific transcribed and nontranscribed sequences, the appropriate technology should be developed to measure AFB₁ adduction to one of the classes of repetitive, nontranscribed DNA sequences. The genomes of primates contain a highly repeated DNA sequence, termed alpha DNA, that would be especially suitable to carcinogen studies. In cultured cells of the African green monkey, alpha sequences comprise 15 - 20% of the total nuclear DNA (Maio, 1971). This DNA has a base composition similar to that of the bulk of nuclear DNA and is not transcribed (Kuff et al., 1978).

The basic repeating unit of alpha in these cells is a sequence of 172 bp arranged primarily in tandem arrays on several chromosomes. Digestion of purified monkey DNA with the restriction endonuclease Hind III and separation of the DNA fragments by preparative electrophoresis through agarose or acrylamide gels would allow the isolation of about 8% of the cellular DNA as these 172 bp alpha monomer fragments; these alpha monomers have been shown to be at least 90% homogeneous with respect to sequence and could therefore be used as specific cellular probes for DNA damage and repair in specific nontranscribed sequences.


2. The technology presented in chapter four for the isolation of ribosomal RNA gene sequences could be adapted for the isolation of other repetitive, transcribed gene sequences. Both histone and transfer RNA genes are present in multiple copies in nuclear DNA. The wide range of hybridization technology now available should make these gene sequences good candidates for successful isolation from the target organ DNA of carcinogen-treated animal cells. These other transcribed genes could provide comparisons for the relationships between transcriptional activity, guanine content, and carcinogen adduction within the subset of transcribed gene sequences.
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Appendix I

Determination of DNA by the Diphenylamine Colorometric Reaction

I. Introduction

This protocol outlines the diphenylamine procedure for DNA quantitation of Giles and Meyers (198).

II. Materials and Methods

1) DNA standards
   
   Calf Thymus DNA (Sigma Type 1) is weighed out and dissolved in 20 ml Lysis buffer:
   
   Lysis Buffer: 50 mM HEPES pH 7.0
   1% Sarkosyl
   1 mM EDTA

   This sample is centrifuged in 6M CsCl, 3M Cs₂SO₄ gradients as explained in chapter 3. The DNA band is located by flow monitoring of absorbance at 254 nm, dialyzed against 20 mM Tris-HCl pH 7.0, 2 mM Na₂EDTA and precipitated by the addition of 5 M NaCl to
0.1 M and 2.5 volumes of -70°C ethanol. A 1 mg/ml stock solution is made in 20 mM Tris-HCl pH 7.0, 1 mM Na₂EDTA and stored frozen in 0.1 ml aliquots in 1.5 ml ependorf tubes.

2) Perchloric Acid Solutions

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Volume of 70% PCA</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 N PCA</td>
<td>8.6 ml</td>
<td>500 ml</td>
</tr>
<tr>
<td>0.6 N PCA</td>
<td>25.6 ml</td>
<td>500 ml</td>
</tr>
<tr>
<td>1 N PCA</td>
<td>43.0 ml</td>
<td>500 ml</td>
</tr>
<tr>
<td>1.2 N PCA</td>
<td>51.6 ml</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

3) Diphenylamine, Reagent Grade, Matheson, Coleman, and Bell, Inc. Norwood, Ohio (DO NOT use Kodak brand).

4) 20 mM Tris-HCl, pH 7.0, 0.25 M Sucrose (TS buffer)

5) 0.3 N KOH

6) Glacial Acetic Acid, full strength

7) Acetaldehyde, 1.6 mg/ml (KEEP REFRIGERATED AT ALL TIMES)

Matheson, Coleman, and Bell, Inc.

III. Procedure

PART A

1) Add TS buffer to sample to a final volume of 5.0 ml

2) Add 2.5 ml ice cold 0.6 N PCA. Mix well.
3) Let stand in ice for 10 minutes.

4) Spin 5 minutes, 1400 rpm, International PR-6 centrifuge, 0°C.

5) Wash pellet once with 5 ml 0.2 N PCA spin down as in step 4.

**PART B**

1) To pellet from part A, add 4 ml 0.3 N KOH. Vortex.

2) Heat for 1 hour at 37°C, vortexing every 5-10 minutes.

3) Cool in ice 10 minutes.

4) Add 2.5 ml 1.2 N PCA. Vortex.

5) Cool in ice 10 minutes.

6) Centrifuge at 2200 rpm, 0°C, 10 minutes, PIC-6.

7) Wash pellet twice with 3 ml ice cold 0.2 N PCA.

**PART C**

1) Add 2 ml IN PCA. Dissolve pellet.

2) Transfer to tall, disposable test tube and cover top with a marble.

3) Heat 20 minutes, 70°C.

4) Cool in ice 10 minutes.

5) Pellet precipitant by centrifugation, 2000 rpm, PR-6, 10 minutes, 0°C.

6) To supernatant add 2 ml 4% diphenylamine in glacial ace-
tic acid, made fresh. Mix well.

7) Add 0.1 ml aqueous acetaldehyde (1.6 mg/ml).

8) Let stand at room temperature overnight (minimum 12 hours).

9) Read $A_{595} - A_{700}$ versus a 1 N PCA blank.

Linearity of this assay is 10 to 100 micrograms of DNA.
Appendix II

Isolation of 18S and 28S Ribosomal RNA Used for RNA-DNA Hybridizations

I. Preparation of Total Liver RNA

Cytoplasmic and nuclear RNA from the livers of male Fischer rats (125-175 grams) was prepared by an adaptation of the methods of Yang and Novelli (35).

1. Stun, decapitate, and bleed animals. Remove liver to a beaker of cold homogenizing media.

   Homogenizing media: 0.4 M sucrose
   (H media) 10 mM Tris-Cl (pH 7.5)
   0.1 M KCl
   0.3 M NaCl
   0.02% Diethylpyrocarbonate (DEP)
   (Sigma Chemical Company)

2. Mince liver tissues with a hand-held tissue press into H media (4 ml/gram tissue).

3. Homogenize with a glass Dounce Homogenizer using a tight pestle. Apply 12 strokes per 40 ml.

4. Add 6 ml/gram tissue Mixing media.

   Mixing media: 0.15 M NaCl
   (M media) 10 mM Tris-Cl (pH 7.5)
5. Add 10 ml/gram tissue 80% redistilled phenol/20% M media.
6. Add 1 drop of DEP per 200 ml.
Transfer to Erlenmeyer flasks. Shake briskly at 4°C for 30 minutes.

NOTE: From step 6 onward, contact of RNA with ribonuclease becomes a menacing problem in the recovery of intact 18S and 28S rRNA. The following precautions must be exercised.

1. All glassware must be
   a. rinsed with 0.1% DEP followed by sterile, distilled H₂O.
   b. covered with aluminum foil, washed with 0.1% DEP and sterile H₂O.
   c. autoclaved greater than 2 hours. After treatment, glassware must be handled with glove covered hands.

2. Sterile, disposable glass pipets should be used.

7. Separate phases by centrifuging 15 minutes, 30,000 x g, 4°C.
8. Remove supernatant to a beaker, prerinsed with 0.1% DEP, at room temperature. Add 0.3 grams KOAc/gram of liver tissue and dissolve by swirling.
9. Add 25 ml EtOH/gram liver tissue. (Ethanol should be precooled for at least 24 hours in a -70°C freezer). Stir, cover beaker with parafilm, store in a -20°C freezer overnight.

10. Pour off ethanol. Transfer precipitant to polyallomer tubes prewashed with 0.1% DEP and ethanol.

11. Spin 15 minutes, 20,000 x g, -5°C. Drain pellets.

II. Separation of 18S and 28S Ribosomal RNA

1. Resuspend DNA pellets in 10% RNA Spin Solution (10% RSS):

$$10\% \text{ RNA Spin Solution: } 10\% \text{ RNAase-free Sucrose (BRL, Inc.)}$$

0.1 M NaCl

1 mM Na$_2$EDTA

10 mM NaOAc

0.02% DEP

1% Sarkosyl NL-30 detergent

(ICN Pharmaceuticals, Inc.)

Standardize to pH 5.1.

2. Layer 2-5 mg (2 mg/ml) of RNA onto a 10-40% sucrose gradient made from 10% and 40% RNA Spin Solutions.

$$40\% \text{ RNA Spin Solution: same as } 10\% \text{ RNA Spin Solution, substituting } 40\% \text{ RNAase-free sucrose.}$$

36-38 ml gradients are made in polyallomer SW 27 tubes (Beckman,
Figure AII-1 Separation of 18S and 28S rRNA. Two mg samples of total liver RNA were separated on 10-40% sucrose gradients centrifuged in a SW27 rotor centrifuged for 16 hours at 27,000 rpm at 4°C.
3. Gradients are centrifuged 16 hours, 27,000 rpm, 4°C.

4. Gradients are then fractionated with an Instrumentation Specialties, Inc. density gradient fractionator permitting separation of the 18S and 28S peaks (figure AII-1).

III. Removal of Contaminant mRNA species from 18S and 28S RNA by Oligo(dT) Cellulose Chromatography

1. Add 1 M KOAc pH 5.1 to 3% volume of 18S and 28S fractions in 1.5 ml ependorf tubes.

2. Add 2 volumes -70°C ethanol and allow to stand at -20°C overnight. The precipitate is pelleted by centrifuging for 20 minutes, 4°C in an ependorf centrifuge.

3. Wash RNA pellet twice with ethanol -0.2 M NaCl (2:1).

4. Contaminant mRNA is removed by the method developed by Aviv and Leder (36). The RNA pellet is resuspended in oligo-dT application buffer at a concentration of 0.5 A260 units/ml.

   Oligo-dT Application Buffer: 0.5M KCl

   10 mM Tris-Cl, pH 7.5

   1.5 mM Na₂EDTA

   0.5% Sarkosyl NL-30

   0.01% DEP

5. 70 to 100 A260 units of RNA are applied to a 5 ml (1.0 gram, dry weight) oligo(dT)-cellulose column (BRL, Inc.) previously washed with application buffer.

6. The nonabsorbed 18S and 28S rRNA are eluted with con-
Figure AII-2 Oligo (dT)-cellulose chromatography of crude ribosomal RNA isolated from rat liver and preparatively purified by sucrose density gradient centrifugation. Peak A, material not retained by the column.
continued washing with application buffer. Material retained by the column is eluted with the oligo(dT) elution buffer:

Oligo(dT) Elution Buffer: 10 mM Tris-Cl, pH 7.5
1.5 mM Na₂ EDTA
0.5% Sarkosyl NL-30
0.01% DEP

7. Figure AII-2 shows a typical elution pattern for RNA applied to an oligo(dT) column. Greater than 95% of the applied RNA is recovered in the early eluting peak.

8. The 18S and 28S rRNA peak is made 3% in KOAc and precipitated in 1.5 ml ependorf tubes by the addition of -70°C ethanol.

9. The ethanol precipitate is washed as described in step 3 and stored in ethanol at -20°C.

IV. (32P)-end labeling of 18S and 28S Ribosomal RNA

18S and 28S rRNA were end labelled with 32PO₄ using the procedure of Baker and Ziff (37).

A. Alkali digested rRNA

1. 10-15 micrograms of rRNA is dissolved in 0.1 ml of ETS buffer.

ETS Buffer: 10 mM Tris-Cl, pH 7.9
10 mM Na₂ EDTA
0.2% SDS
2. Add 0.025 ml of NaOH. Let sit on ice for 30 minutes in an eppendorf tube.

3. Add 0.05 ml unbuffered HEPES and mix well. Add 0.4 ml -70°C EtOH.

4. Let RNA precipitate in dry-ice methanol. Pellet precipitate in eppendorf centrifuge, 15 minutes.

5. Wash RNA with 100% EtOH twice.

B. Phosphatase Treatment of Whole RNA

1. 10-15 micrograms of rRNA is dissolved in 0.045 ml H2O.

2. Add 5 micrograms 0.5 Tris-Cl, pH 8.0; 20 mM MgCl2.

3. Add 200 units of Bacterial Alkaline Phosphatase (BRL, Inc.) Let sit at 37°C for 30 minutes.


5. Remove aqueous phase and reextract as in step 4.

6. Remove aqueous phase and extract with an equal volume of chloroform.

7. Remove aqueous phase. Add 0.06 ml 1 M NaOAc.

8. Cool. Add 0.6 ml -70°C ethanol. Let sit in dry ice - methanol ice math to precipitate RNA. Pellet RNA in eppendorf centrifuge, 15-20 minutes. Discard supernatant.

9. Add 0.075 ml ET buffer, 0.02 l M NaOAc, pH 5.2. Resuspend.

10. Add 0.225 ml -70°C ethanol. Precipitate and pellet as above.
C. Kinase Treatment

1. ATP-gamma-32p, 5 mCi/ml, New England Nuclear.

Resuspend RNA in 0.06 to 0.08 ml gamma-ATP.

Add 7 microliters of 10x Kinase Buffer: 0.5 M Tris-Cl pH 8.8

\[
\begin{align*}
0.1 \text{ M MgCl}_2 \\
50 \text{ mM Dithiothreitol} \\
1 \text{ mM Spermidine} \\
1 \text{ mM EDTA}
\end{align*}
\]

Mix well.

Note: Protective measures must be taken in working with 32p. Principle of these precautions is to shield one's body from the kinase sample by a plexiglass box (i.e. electrophoresis box).
Appendix III

Southern Hybridization Protocol

This protocol summarizes the steps involved in transferring restriction enzyme-digested DNA samples to nitrocellulose filters for hybridization with 32p-labelled ribosomal RNA. Additional information for other adaptations of this procedure can be found in references by Southern (52).

I. Running Digested DNA Samples

1. 1-5 micrograms of restriction enzyme digested DNA is loaded per lane of a vertical BioRad gel apparatus. A 1% Agarose gel is employed (BRL, Inc.) with a Borate running buffer:

   10X Borate running buffer: 0.9 M Tris-Cl
   -
   0.9 M Boric Acid - final pH 8.2
   25 mM Na₂EDTA
   -

Hind III-digested lambda bacteriophage DNA can be run simultaneously as molecular weight markers.

2. Run gel 16-20 hours, 75 volts till dye front is at the bottom of the gel.

3. Stain gel with ethidium bromide in Borate buffer (0.5
micrograms EthBr/ml) for 30 minutes. (Approximately 500 ml of EthBr-buffer is sufficient per gel). After staining, the gel is photographed using a short wavelength ultraviolet transilluminator and polaroid type 57 (black and white) film. A transparent ruler is placed beside the gel to measure the Hind III-lambda bands for graphing the log molecular weight versus distance migrated.

II. Preparation of Gel for Transfer
1. Notch gel at one corner to permit correct orientation with photograph for future reference.

2. Denature the gel by placing it in an 8 inch x 12 inch glass cooking dish, supported by 2 sheets of saran wrap, containing 200 ml of D solution: 0.5 M NaOH

\[ 0.6 \text{ M NaCl} \]

The gel is denatured for 45 minutes with intermittent rocking every 5-7 minutes.

3. Gently pour off the denaturation solution and treat further for 1 hour with N solution: 1 M Tris-HCl, pH 7.4

\[ 1.5 \text{ M NaCl} \]

III. Transfer of DNA to Nitrocellulose Filter
1. Set-up the transfer apparatus as follows:
   a. Oblong plastic box, approximately 12 inches x 8
inches x 5 inches deep.

b. 2 absorbant plastic sponges in box (4 x 6 x 2 inches thick when wet).

2. Pour 10x SSC into the pan and saturate the sponges well. Then add more 10x SSC till the fluid is level with the sponges.

20xSSC: 0.3 M Sodium Citrate, pH 7.2

3M NaCl

3. Cut two sheets of Whatman 3 MM filter paper the same width as the sponge and 3 times the length and saturate with 10x SSC. Place both sheets onto the sponges and remove any air pockets with gloved fingers.

4. Place the gel on the middle of the sponges, again removing any air bubbles between the gel and the paper towels with gloved fingers.

5. Cut Bio-Rad 3 mM gel spacers to form a rectangle around the gel with the spacers 3-5 mm from the edge of the gel all the way around. **DO NOT** let the spacers touch the gel.

6. **Gently** cut a piece of Millipore nitrocellulose (# HAHY 304-FO) to the size of the gel (include the notch cut in the top corner) plus the gel spacers around the gel. Leave a little extra room around the edges; this will be removed later. Also cut 15 sheets of Whatman 3 mm paper to these same dimensions.

7. Wet the nitrocellulose in 2x SSC by layering it onto a glass pan containing 2x SSC; the filter is thus wet from one side only avoiding trapped air bubbles.

8. Cut strips of saran wrap and cover the sponge surfaces
from the gel spacers to the sponge edges.

9. Place the filter paper onto the gel removing any trapped air bubbles. With a sharp razor blade, cut any excess nitrocellulose hanging over the edges of the gel spacers.

10. Wet 1 sheet of cut Whatman 3 MM and place onto the nitrocellulose, again removing any trapped air bubbles.

11. Place 7 sheets of dry 3 MM on top and weigh down with a glass plate. Wait about 10 minutes until these are wet as visible through the glass. Then place the other 7 sheets on top and repeat.

12. When all sheets of nitrocellulose are wet, layer 2½ to 4 inches of paper towels, cut to the size of the filter, on top and weigh them down with additional glass weights. Capillary action will pull 10 X SSC thru the gel and solubilize the DNA fragments which are now single stranded. These DNA fragments will stick to the nitrocellulose filter.

13. Allow transfer to go 12 hours or until at least 500 ml of 10x SSC has been absorbed.

**Binding DNA to Nitrocellulose**

1. Remove the layers of paper filters and towels. Trim nitrocellulose filter to size of gel with the top of the filter lining up with the bottom of the well slots.

2. Rinse both sides of filter well with 2x SSC.

3. Dry the filter at room temperature on paper towels for 10-15 minutes. Then place the filter within a folding of alumi-
num foil and bake at 80°C in a vacuum oven for two hours.

4. Restain the gel to determine how efficiently the transfer proceeded.

**DNA-RNA Hybridization**

1. Place the filter in a 8 inch x 10 inch Dazy Seal-A-Meal bag. Wet it, using a long stem pasteur pipette, with presoak solution:

   **Presoak solution:** 1 mM Adenosine (Sigma)
   
   10 mM Na₂HPO₄ pH 7.0
   
   30 mM NaCitrate pH 7.0
   
   0.3 M NaCl
   
   0.5 mg/ml yeast RNA (Sigma)
   
   0.5% Sodium Dodecyl Sulfate

2. Add 20 ml additional presoak solution, remove as much air as possible, and seal 3 times. (Careful, one can easily burn one's fingers).

3. Saturate 4 paper towels with 6x SSC, 0.25% SDS and place on each side of the bag containing the filter. Place all of this in a larger 10 inch wide sealable bag and add 10 ml of 6x SSC, 0.25% SDS. Remove as much air as possible and seal 3 times.

4. Place in a 67°C H₂O bath for 2 hours.

5. Rinse filter with 2x SSC. Blot filter dry with paper
towels, then air dry for 2 hours at room temperature.

6. Make up fresh hybridization solution (15 ml):

0.75 M NaCl
7.5 mM Na Citrate
0.2% SDS
0.5 milligrams/ml yeast RNA
0.1 micrograms/ml $^{32}$P-ribosomal RNA probe

7. Place filter in a small sealable bag and apply hybridization solution containing the ribosomal RNA probe (20 μl/cm$^2$ of filter). Use a clay-adams micropipet for doing this, pasteur pipets will not do.

8. Remove all air and seal three times. Repeat procedure described in (3.) and place in larger sealable bag. Put in 67°C H$_2$O bath for 24 hours.

9. Rinse filter will with 2x SSC at room temperature. Place filter in the plastic box used for the transfer already containing 300 ml of wash solution equilibrated to 67°C.

Wash solution: 0.75 M NaCl
75 mM NaCitrate pH 5.5
10 mM EDTA
0.3% SDS

After one hour, remove filter, replace wash solution, and repeat.

10. Rinse the filter well with 2x SSC.

11. Rinse twice in 100 ml wash solution. Blot dry with Kimwipes, wrap in saran wrap, and place in film holder with one sheet of Kodak XOMAT X-ray film. The length of exposure will be
determined by the efficiency of background RNA removal. If background problems do occur, the filter can be treated between steps 10 and 11 with 100 ml of 0.5 micrograms/ml RNAase A in 2x SSC (pH 7.4) at 37°C for 1 hour.
Biographical Sketch

Rick Irvin was born in Gainesville, Georgia on October 16, 1955, the son of Thomas E. and Annie Ruth Irvin. He was educated in the public school system of Athens, Georgia and was graduated as a member of the first senior class of Cedar Shoals High School in 1973. He subsequently attended the University of Georgia and received the B.S. degree, summa cum laude, with a double major in Biochemistry and Microbiology, in June, 1977. While at UGA he was a member of the Honors Program and graduated with General University Honors. He also was elected to the Phi Beta Kappa National Honor Society, as well as the Phi Kappa Phi and Alpha Epsilon Delta National Honor Societies.

In September, 1977, he matriculated as a graduate student at the Massachusetts Institute of Technology. Presently, he is a candidate for the Ph.D. degree in Toxicology with Professor Gerald N. Wogan, Underwood-Prescott Professor of Food Science and Professor of Toxicology in the Department of Nutrition and Food Science serving as his thesis advisor. While at MIT, Mr. Irvin was elected to the MIT chapter of the Sigma Xi National Honor Society. Upon graduation, he will assume the position of Assistant Professor of Veterinary Public Health in the College of Veterinary Medicine at Texas A&M University with a joint appointment as Assistant Professor in the interdepartmental program of Food Science and Technology. At Texas A&M, he research interests will center within the areas of Food Toxicology, Chemical Carcinogenesis and Teratogenesis, and Environmental Law.