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FACTORS INVOLVED IN TARGET SPECIFICITY OF THE HEPATOCARCINOGEN N-2-ACETYLAMINOFLUORENE WITH REGARD TO CELL TYPE AND LOCATION WITHIN THE GENOME

By

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ABSTRACT

Factors Involved in Target Specificity of the Hepatocarcinogen N-2-Acetylaminofluorene with Regard to Cell Type and Location within the Genome

by

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Carcinogenesis as a result of chemical exposure often exhibits organ-specific characteristics. The objective of this investigation has been to discern initial molecular events critical to target-specific carcinogenesis induced by the hepatocarcinogen, N-2-acetylaminofluorene (AAF). The following parameters were investigated: 1) the binding of AAF and its N-hydroxy metabolite (N-OH-AAF) to DNA of target cells, hepatic parenchymal (PC), and nontarget cells, hepatic nonparenchymal cells (NPC); 2) the location of carcinogen binding within the genome with regard to transcriptionally active as compared to inactive regions of chromatin in target and nontarget cells; 3) the effect of progressive carcinogen treatment on digestion of DNA from rat hepatic PC and NPC with base sequence-specific restriction endonucleases.

AAF selectively binds to DNA of target PC due to a relative decreased ability of the nontarget NPC to N-hydroxylate the procarcinogen. Once N-hydroxylation has occurred, both cell types are able to carry out remaining steps in the metabolic activation and repair of adducts to a similar extent.

Analysis of the binding of AAF to transcriptionally active vs. inactive regions of chromatin (as delineated by the nuclease DNase I) of target cells (PC) compared to nontarget cells (NPC) indicated that, at the time of peak binding, AAF selectively binds to DNA of transcriptionally inactive regions of target cell (PC) DNA and to transcriptionally active regions of nontarget cells (NPC). Pretreatment of rats daily with AAF for up to 5 days had no influence on the subsequent binding of tracer doses of [ring-^{3}H]-AAF to specific regions of DNA from PC and NPC.

Treatment of rats with AAF (15 mg/100 g) for 1, 3, 5 or 7 days did not alter the ability of the restriction enzyme, Eco Rl, to act at sites specific to this endonuclease, but resulted in inhibition of the restriction enzyme, Kpn I, to recognize sites for which this enzyme is specific immediately following treatment or 7 days following treatment.

These results indicate that factors such as metabolic capabilities of target cells and structural characteristics of chromatin allowing for binding of carcinogen to discrete regions might be more important in determining carcinogenic susceptibility of a particular tissue than total binding and persistence of carcinogen.

to Joseph, Mary

and Jim

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ABBREVIATIONS

AAF	N-2-acetylaminofluorene
N-OH-AAF	N-hydroxy-N-2-acetylaminofluorene
C8-gua-AAF	N-(deoxyguanosin-8-yl)-2-acetylaminofluorene
C8-gua-AF	N-(deoxyguanosin-8-yl)-2-aminofluorene
N^2-gua-AAF	N-(deoxyguanosin-N^2-yl)-N-2-acetylaminofluorene
DMN	dimethylnitrosamine
PC	parenchymal cells
NPC	nonparenchymal cells
NI	nuclei of parenchymal cells
NII	nuclei of nonparenchymal cells

INTRODUCTION

1. Background

Modern epidemiological studies indicate that 80-90% of all human cancers are caused by environmental factors (11,50). Chemicals seem to be the most probable causative factors, in view of the facts that oncogenic viruses are not highly contagious (50) and radiation is fairly uniformly distributed (50,86). Epidemiologists have found that the total percent of population mortality due to cancer varies little from country to country (86). However, mortality ascribed to cancers of specific tissues types varies a great deal between countries (86). As an example, cancers considered to be rare in the United States such as primary carcinoma of the liver, Burkitt's lymphoma or Kaposi's sarcoma are common in Africa (61). U.S. nonwhites resemble U.S. whites more than they do African groups of similar heredity in cancer susceptibility (61). With westernization of lifestyle in Africa, cancer patterns have become more like those of the Western world.

Many cancers have been found to be higher among recent immigrants to the U.S. as opposed to native U.S. whites (61). Migrants from Poland, Czechoslovakia, Norway and the U.S.S.R. exhibit higher incidences of stomach cancer than U.S. whites and these incidences correlate with those of their homeland (61). The lack of changes in cancer incidence following emigration may be due to a continuance of dietary

customs and habits once in the United States (61). Incidence of breast cancer was ranked highest among U.S. white females, yet quite reduced among women of Italy and Poland (61). However, data on Polish migrants indicate a rise in breast cancer incidence when women remain in the U.S. compared to the incidence observed in urban and rural Poland. An extensive review by Haenszel and Kurihara on mortality from cancer among Japanese migrants to the U.S. revealed that U.S. Japanese experienced higher cancer mortality than native Japanese for cancer within the intestines, gall bladder, pancreas, lung, ovary, prostate, nervous system and breast (47).

It must be kept in mind that although geographic variations in cancer incidence may be attributable to environment, environment can include lifestyle influences such as dietary, social and cultural habits (51). As noted by Higginson, "Clinical cancer is the end result of numerous influences at the cellular level, many of which cannot be investigated easily in epidemiologic studies..." (51).

Carcinogenesis due to chemical exposure was first noted in humans over two hundred years ago when Percival Pott, a general surgeon in England, noted a high incidence of cancer of the scrotum in chimney sweeps who began their careers in early childhood (101). In questioning the etiology of such cancers, he recognized that the problem was related to many years exposure to coal soot and tars, along with poor hygiene habits (101). Several years before Pott's observation, Dr. John Hill, a physician in London, published an article entitled, "Cautions Against the Immoderate Use of Snuff" (108), in which he reported cases of nasal polyps and lesions resembling cancer in

persons using tobacco snuff frequently and for prolonged periods (108). This report in 1761 was probably the first implication of tobacco as a cause of cancers.

In the late part of the 19th century, Rehn observed the development of urinary bladder cancer among several workers following prolonged exposure to aromatic amines in a German aniline dye factory (see reviews in Ref. 86 and 85). More recently, many associations have been made with cancer and occupational, medical and societal exposures to chemicals (29,50,84). The International Agency for Research on Cancer (IARC) has reviewed evidence on nearly 500 chemicals, and their association with cancer (52). Their objective was to elucidate carcinogenic risk of chemicals to humans. Due to limited human evidence and uncertainties concerning extrapolations of animal data to man, only 18 chemicals, groups of chemicals and industrial processes fell into Group 1, i.e., substances that are carcinogenic for humans (52).

Attempts to set up experimental animal models to study in depth factors associated with development of cancer following exposure to various chemicals dates back to the early 20th century. Yamagiwa and Ichikawa are credited with the first successful production of cancer in experimental animals by producing malignant epithelial tumors in ears of rabbits following coal tar applications (46). These types of studies were carried a step further by Rous and Kidd in 1941 (109). These investigators set up a series of experiments for the purpose of studying the relationship between benign growths which arise following applications of tar to ears of rabbits and cancerous lesions. They

noted that the definition of cancer at that time did not allow for neoplasms which depended upon permissive conditions for existence and growth, nor the fact that some stage or stages of neoplastic growth may be reversible (109). A first set of experiments was designed to determine if tar-induced tumors which regress upon cessation of tar treatment reappear from the same cells when tar treatment is resumed. Rous and Kidd found that not only do tumors reappear in the same areas, but following a shorter latency period, along with appearance of numerous new tumors (109). These studies suggested the condition of preneoplastic cells produced by the first tar treatment. In a second set of experiments, Rous and Kidd found that following cessation of a round of treatment with tar, application of a noncarcinogenic agent (turpentine) which induced a superficial inflammation and cell proliferation resulted in reappearance of original tumors and appearance of new ones (109). In addition, the process of wound healing itself caused neoplastic developments at the boundaries of scars following previous tar treatment (109). Lastly, when no further treatments were given following one period of tar application, the epithelium of rabbit ears healed, and any "carcinomatoids" regressed (109).

Mottram (89) proposed several factors which appeared to be required for production of abnormal cells following carcinogen exposure: 1) a "sensitizing factor" to produce an initial change from normality in a cell, 2) a specific cellular reaction to "fix" the consequent changes following exposure to a "sensitizing factor", and 3) a developing factor to produce an abnormal population of cells through growth and multiplication. This investigator was primarily concerned

with the role of hyperplastic agents as developing factors. Experiments were designed in which one flank of a mouse was treated with benzpyrene followed by croton oil treatment (20 weeks) and the other flank was painted with benzpyrene followed by vehicle treatment. A predominance of malignant tumors developed on the flanks of mice treated with benzpyrene and croton oil (89). These results led Mottram to conclude that, "The part played by hyperplasia and chronic irritation in the genesis of cancer is readily explained if prolonged stimulation of cell division is a necessary pre-requisite before cells will become neoplastic" (89). The existence of latency periods for carcinogenesis and the dormancy of altered cell populations following treatment with carcinogens were raised by these investigations.

Berenblum and Shubik (12) reported a series of experiments designed to determine the importance of time sequence for application of carcinogen and hyperplastic agent, and to determine whether a single application of carcinogen was adequate to result in neoplastic changes in cells following treatment with croton oil. There was no difference in the total numbers of tumors which developed or the latent period for tumor induction when rats were pretreated with croton oil, then given benzpyrene followed by 20 weeks of croton oil treatment or when rats were treated similarly, but without croton oil pretreatment (12). These studies confirmed earlier results by Mottram (89) that croton oil treatment following a single application of carcinogen can induce tumors (12).

From these early investigations, the notion of stages within the carcinogenic process evolved. Berenblum (13) explained his results in

terms first proposed by Friedewald and Rous (13) as an "initiation process" in which normal cells were converted into latent tumor cells, and a promoting process in which latent tumor cells were caused to develop into frank tumors. Fundamental characteristics of the two stages of carcinogenesis were first coalesced into a unified theory by Berenblum (13). The initiation phase was considered to be a process specific to the chemical applied, as well as being irreversible and rapid. The promotion phase required multiple, repeated applications (12,13), was not specific to a chemical (12,13,109) and was reversible (109). These conclusions remain as the basis for the understanding of chemical carcinogenesis today. As knowledge has increased, further divisions within the two stages has occurred (122), as well as an attempt to understand the process of progression (97).

Present day definitions of initiation, promotion and progression have taken into account the advances in field of molecular biology over the last 30 years. An initiating agent is defined as an agent (chemical, physical, or biological) which is capable of directly and irreversibly altering the native molecular structure of DNA (97). Alterations may or may not involve covalent binding of the agent to DNA, or may involve distortion of DNA structure, scission of the DNA chain, elimination of a base or sugar, or errors in DNA repair (97). A promoting agent is described as an agent which alters expression of genetic information of a cell (97). This event is generally considered to be epigenetic, brought about by such agents as hormones, hyperplastic agents, drugs, etc. (97). Finally, progression is defined as that stage of neoplastic development characterized by visible

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karyotypic alterations within tumor cells (97). These karyotypic changes correlate with increased tumor growth rate, invasiveness, metastases and neoplastic biochemical and morphologic alterations (97). Additional investigations (123) have revealed that in some cases, a single exposure to carcinogen is adequate for initiation, and that the effects of initiators can be additive (97). Furthermore, a round of cell proliferation seems to be required to confer irreversibility on the initiation lesions (21,34,59). Proliferation may "fix" some change in that it is now permanent. Thus, initiation may occur in at least 2 steps: 1) interaction of the carcinogen as an activated derivative with DNA, 2) fixation of the lesion by one round of cell replication (21). It has been shown that DNA containing MNU-, DMN- and N-OH-AAF-produced lesions can replicate in vivo (see ref. 21).

Promotion may occur as a result of an agent which provides a selective environment in which initiated cells can be expressed either by inhibited growth of surrounding cells but not of initiated cells, or by preferentially stimulating growth of initiated cells (34). In addition, there is evidence that promoters exhibit a threshold and maximum effect (97). These characteristics may allow for opportunities to control human exposure, and thereby reduce levels of and exposure to these substances rather than completely eliminating them from the environment.

It is widely accepted that tumors evolve as clones of a single altered cell (110). In many cases the malignant cells in a primary tumor mass exhibit the same abnormal karyotype (110). Tumors might arise from one of many neoplastic cells that had a selective growth advantage over normal cells. Some neoplastic cells may be eliminated

as a result of metabolic disadvantage or immunologic destruction (110), and the mutant which has more selective advantage may ultimately give rise to a new subpopulation of tumor cells. The biology of neoplastic cells remains uncertain. Initiation may involve altered gene expression rather than structural mutation as indicated by the absence of unique gene products in tumor cells and the reversibility of transformation in some cell culture systems (110). Neoplastic traits generally reflect alterations in pre-existing genes which may result from mutations in regulatory genes or effects on gene dosage following chromosomal rearrangement. At present, evidence points to transcriptional control as the most frequent method for eukaryotic gene control (26). However, recent studies in mouse cells have indicated that treatment of cells with methotrexate induces a 350-fold increase in the gene which codes for dihydrofolate reductase (DHFR), the target enzyme for methotrexate (7). In addition, treatment of methotrexate-exposed cells with the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate increases the gene copy number for DHFR 16-fold (134). Thus, changes in gene dosage following drug or chemical exposure may be an important factor in neoplastic development.

The concept that cancer is the result of some change in the genetic material of cells, the somatic mutation theory, has been a central hypothesis in fundamental cancer research (92). Early research suggested that chromosomal mutation may be related to carcinogenesis. More recently, studies have been performed dealing with alterations of DNA, RNA and proteins by carcinogens, and how these events may be justified within the theory of somatic mutation. Fahmy

and Fahmy (30) found the mutations arising from direct intramolecular DNA damage, such as point mutations and chromosomal breaks may only be a small contribution to the types of DNA damage that occurs due to chemical carcinogens. In a series of investigations on a variety of chemical carcinogens, it was found that alkylating agents and nitroso agents often directly damage DNA resulting in point mutations and chromosomal breaks (30). However, hydrocarbons and aromatic amines induced small deletions of a chromosome in Drosophila, but not by point mutations or chromosomal breaks (30). The investigators suggest that these carcinogens may have induced the chromosomal deletions via interference with enzymes or regulators involved in DNA synthesis, replication or repair (30). They suggest that these results are in line with the somatic mutation theory, which can be expanded to include events which involved indirect mechanisms for induction of mutations, rather than exclusively direct DNA attack (30).

Cairns presented evidence that somatic mutation may not be an ideally inclusive explanation for cancer (20). He explained that subjects exhibiting deficiency in DNA repair capabilities should exhibit high incidences of tumors. Xeroderma pigmentosum (XP) patients are deficient in DNA repair in all of their tissues, only malignant melanomas are seen to be induced in these patients following exposure to UV light. It is conceivable that these patients may smoke cigarettes, and may be exposed to other carcinogens in the diet or workplace, yet the incidence of cancers other than of the skin is not strikingly high (20). It may be reasonable to conclude from this evidence that cancers commonly found in the general population may not

be caused in the same manner as are malignant melanomas in XP patients (20).

Other recent reports (5,110) have indicated that factors other than mutagenic potency must be considered in evaluating carcinogenicity of a chemical. A number of noncarcinogenic substances, including physiologic concentrations of oxygen, are mutagenic in the Salmonella reverse mutation assay of Ames (3) suggesting that mutagenic potency as measured in bacterial systems and carcinogenicity do not always correspond (110). In addition, many other factors are involved in determining the carcinogenicity of a chemical such as absorption, distribution, metabolic activation and inactivation and species-related factors (5). The somatic mutation theory may be adequate to explain some aspects of the development of cancer. However, in view of rapid advances being made in the field of molecular biology in terms of the nature of genetic transposition (20) and alteration of gene expression (32), alternative explanations must be considered to explain the diverse nature of mechanisms for chemically-induced carcinogenesis. In view of the multiple explanations for carcinogenesis, it is probable that there are multiple causes. It is possible to view the development of cancer as the result of an interaction of factors such as faulty differentiation as a consequence of altered DNA structure due to chemical damage or by viral modifications of the genome equivalent to mutations (102). Thus, 3 major theories of carcinogenesis (somatic mutations, viral modifications, faulty differentiation) may be integrated to explain the end result of cancer, rather than each theory being mutually exclusive.

2. Organ Specificity of Chemical Carcinogens

It has become evident from the extensive research that has been done in the field of chemical carcinogenesis that many carcinogens exhibit specificity for one or several organs. Epidemiologic evidence, discussed above, for environmental factors being induced at least in part for cancers in various regions of the world indicates that cancers of particular tissues are predominant in certain parts of the world (47,51,61). Thus, regional predominance of certain types of cancers may be the result of human exposure to different industrial chemicals or to various substances in the diet or environment of people in these regions.

From the time carcinogen exposure occurs (either via the environment or through administration to an experimental animal) to the development of malignant tumors, a number of events may occur within various tissues. Organ specificity of chemical carcinogens may be influenced by the distribution of the carcinogen within the body, the relative activities of toxifying and detoxifying enzymes in various organs, the presence of cellular components to which an ultimate carcinogen may bind before it reaches the critical intracellular target (presumed to be DNA), and the repair capacity of various tissues (79). In some cases, the route of administration may influence the site of tumor development due to factors sited above. When given in the diet, N-OH-AAF produced tumors of the forestomach (88). When injected intraperitoneally, N-OH-AAF produced peritoneal sarcomas (88). This is probably due to N-OH-AAF being one metabolic step closer than the parent compound, AAF, to the ultimate carcinogenic form.

Dimethylnitrosamine (DMN) causes primarily liver, kidney and some lung tumors (79). Since the nitrosamines appear to distribute uniformly throughout the body water following oral administration to rats (79), it is unlikely that distribution of the carcinogen per se is involved to any great extent in organotropism of these carcinogens. Similarly, N-methylnitrosourea (MNU) can induce tumors in a variety of tissues depending upon conditions of administration, yet this carcinogen rapidly distributes throughout total body water following administration (79). However, streptozotocin, a glucose derivative of MNU, has been shown to selectively accumulate in pancreatic islet cells of mice, an area in which tumors develop following administration of single, large doses (79).

Metabolic activation and detoxification appear to contribute extensively to organotropism of carcinogens. The observation that many carcinogens of diverse chemical structure caused similar types of tumors led investigators to investigate binding of carcinogens to cellular components, and metabolic activation of carcinogens (85). DMN is more readily activated by hamster lung slices than by rat lung preparations, and these results correlated with the greater susceptibility of hamster respiratory tract to carcinogenesis from DMN (79). AAF, the parent compound from which N-OH-AAF is derived, does not cause tumors of the forestomach upon oral administration, nor does this precarcinogen cause peritoneal sarcomas upon i.p. injection (88). Guinea pigs are resistant to AAF-induced carcinogenesis, and there is evidence that this may be due to a low rate of N-oxidation of AAF in the guinea pig relative to other species (69,87). When N-OH-AAF is

administered to guinea pigs, tumors develop at the site of administration (87). Mouse and hamster are susceptible to AAF-induced hepatocarcinogenesis, and both species were found to N-hydroxylate AAF *in vivo* (87).

Metabolic detoxification may be important in reducing the susceptibility of a tissue to carcinogenicity by a chemical. It is well known that the liver is capable of metabolically activating carcinogens to reactive forms (69,85-87,103). These reactive metabolites may then undergo conjugation reactions, such as N-glucuronidation, to render them more stable and water-soluble, and thus, excretable (58,69,103). However, these water-soluble metabolites may be transported through the kidney to the bladder, wherein the acidic environment of the urine (pH of 4-6 in dog and human urine) may cause acid hydrolysis of the glucuronide conjugate (58,103). Such is the case with some glucuronide conjugates of arylamine N-hydroxylation products (58,103). These hydrolysis products can be protonated at the N-hydroxy position, forming a highly reactive arylnitrenium ion capable of binding to nucleophilic macromolecules of the urinary bladder epithelium (58,103). Thus, a precarcinogen which undergoes extensive metabolism at one site is carcinogenic predominantly at a distant site.

Induction of different metabolic pathways in the liver may reduce or intensify the carcinogenicity of some chemical carcinogens. When rats were fed 3-methylcholanthrene in the diet (0.003% w/w), the incidence of tumors following AAF administration decreased by two-thirds (88). A predominance of 1-, 3-, 5- and 7-ring-hydroxymetabolites occurred, while very little of the N-hydroxy metabolite was

produced (88). The ring hydroxy metabolites of AAF have little to no carcinogenic potential (88). When phenobarbital was fed during 10 weeks of diethylnitrosamine (DEN) administration to rats via drinking water, a decrease in hepatic tumor yield was observed compared to 10 weeks of DEN treatment without concomitant phenobarbital administration (143). Induction of detoxifying metabolic pathways may have been responsible for decreased tumor incidence. However, when phenobarbital treatment was begun one week following cessation of DEN treatment, an increased tumor incidence was observed (143). In this case, phenobarbital was most probably acting as a promoter, resulting in expression of damage caused by DEN pretreatment. More recently, it has been shown that pretreatment of rats with polybrominated biphenyls (PBBs) followed by AAF administration decreased the incidence of mammary tumors in female rats (117).

Subsets of cells within a target organ may be differentially susceptible to carcinogensis by various compounds. Several factors may influence the susceptibility of subsets of liver cells to carcinogens. Cells closest to the portal triad (zone 3 of the hepatic acinus model) are higher in microsomal enzyme activity (this may, in part, be due to differences in oxygen tension across the acinus) (129). Substrates for detoxifying metabolic reactions such as glutathione conjugation may not be evenly distributed across the acinus (129). Susceptibility of protein synthesis to toxic damage may show regional inequalities. There can be a graded clearance of toxic substances from hepatic cells within an acinus to the blood (129). Treatment of rats with the hepatocarcinogen N-nitrosomorpholine results in

development of large hepatocyte subpopulation with enlarged nuclei (139). The process of centrifugal elutriation, i.e., isolation of populations of cells based on cell size and density, allows for isolation of subpopulations of hepatocytes of varying size and ploidy. By employing this procedure on suspensions of liver cells from rats treated with N-nitrosomorpholine, higher proportions of hypertrophied and polyploid cells were found in fractions of elutriated hepatocytes consisting of the large cell subpopulations following N-nitrosomorpholine treatment (139). Additionally, when 5 simple aliphatic nitrosamines were fed to Fischer F344 rats, varying patterns of carcinogenesis were observed (76). Using centrifugal elutriation to separate populations of hepatic parenchymal and nonparenchymal cells, Lewis and Swenberg found damage to nonparenchymal target cells as a result of treatments of rats with the carcinogen 1,2-dimethylhydrazine to persist, while damage was repaired in parenchymal nontarget cells (74). Nitrosodimethylamine gave rise to hemangiosarcomas of the liver, while nitrosodiethylamine gave rise to hepatocellular carcinomas at an approximately equivalent dose (76). Nitrosomethylethylamine induced both hemangiosarcomas and hepatocellular carcinomas; all 5 nitroso compounds caused esophageal tumors (76). Nitroso-di-n-propylamine induced tumors of the esophagus and forestomach, but not of the liver (76).

Following treatment of rats with AAF in the diet for long periods or for short periods followed by treatment with phenobarbital, carcinomas develop primarily from hepatocytes as opposed to other liver cell populations (1,33,38,119,105). When rats are maintained on a 0.02% (w/w) AAF-containing diet, a dosage which causes minimal oval

cell proliferation, altered foci become apparent at 3 weeks of treatment (145). Ultrastructural studies have revealed that cell organelles within cells of altered foci are characteristic of hepatocytes (145). These changes include abundant endoplasmic reticulum, glycogen and microbodies, prominent nucleoli, increased mitochondria and decreased parallel-arrayed rough endoplasmic reticulum (145). When rats are maintained on a 0.05% (w/w) AAF diet, different types of changes occurred in organelles of hepatocytes (38), including permanent changes. There is a permanent decrease in the amount of rough endoplasmic reticulum (38). Nucleoli and Golgi apparatus are hypertrophied, and mitochondria are decreased in size (38). There is evidence to suggest that hepatocellular carcinomas may arise from cells of altered foci (1,28,98,105). Convincing evidence reported by Rabes et al. (105) demonstrated that some cells within enzyme-altered foci are histologically and biochemically compatible with early carcinomas.

In view of the early, rapid proliferation of "oval" cells (33, 119,120) seen when rats begin an AAF-containing diet, malignant tumors in the liver are derived almost exclusively from hepatic parenchymal cells (146). Though hepatocytes constitute 90% of the liver weight, they represent only 65% of the total number of cells present. Blood-borne environmental carcinogens must traverse the Kupffer cell barrier of the sinusoids to reach the liver parenchyma, yet the target of many carcinogens is the hepatocyte (33). Therefore, there is a need for elucidation of events which result in carcinogenesis targeted to hepatocytes after AAF exposure.

Covalent interaction of carcinogens with DNA is thought to be a critical step in the initiation of chemically-induced carcinogenesis. Alkylation of the N-7 position of guanine by nitrosamines, while producing the major alkylation product, does not correlate with organ specificity of nitrosamines (79). The formation and persistance of the O^6-methylguanine adduct may play a more important role in carcinogenesis (10). Lewis and Swenberg found that following administration of 1,2-dimethylhydrazine which induces primarily malignant hemangioendotheliomas, initial alkylation of DNA bases was higher in hepatic parenchymal (nontarget) cells (74). However, removal of O^6-methylguanine was significantly slower from the nonparenchymal (target) cell DNA, resulting in an accumulation of this adduct in the target cell DNA (74). These results suggest that selective repair in various cell populations at a target organ may be an important factor in carcinogenesis. However, Beland et al. have recently reported that following biweekly treatment of rats with N-OH-AAF, certain DNA adducts (N-deoxyguanosin-8-yl-2-aminofluorene) persisted and accumulated in both target (liver) and nontarget (kidney) tissue DNA (10). Therefore, persistance of DNA adducts alone may not be sufficient for carcinogenesis.

3. Chromatin Structure and the Specificity of Carcinogens for Particular Genomic Locations

Carcinogen attack may be specific with regard to target site in ways other than specificity at the organ and cellular level. In view of the importance of carcinogen modification of DNA, it is worthwhile to consider organization of DNA in the mammalian cell and how

carcinogens may gain access to DNA. In eucaryotic cells, chromosomes at metaphase, which are visible under a light microscope, are complexes of DNA packaged tightly with histone and nonhistone proteins (71). At stages of the cell cycle other than metaphase, DNA and associated proteins are present as the more diffuse chromatin (36, 63,71). At present, it is recognized that DNA of eucaryotic chromosomes is arranged in at least 3 levels of organization (71). Firstly, the fundamental unit of a chromatin fiber is a nucleosome (36,63,71). Nucleosomes assemble in a chain to form the chromatin fiber (36,63, 71). Folding and/or coiling of the chromatin fiber into a compact chromosome is the third level of organization (71).

Recent studies of chromatin structure have revealed that the nucleosome consists of a specific length of DNA wrapped around an octamer of histone proteins termed the core particle (36,63,71). When chromatin fibers are unfolded in a low ionic strength environment, nucleosomes are arranged along DNA as "beads on a string" (36, 63,71). Each nucleosome particle is approximately 100 Å in diameter (36,71). The nucleosome consists of a core particle and spacer DNA (36,63,71). The core particle contains an octamer of histones composed of 2 each of the lysine-rich histones H2A and H2B, and two each of the arginine-rich histones H3 and H4 (36,71). Recent research has shown that the arginine-rich histones (H3 and H4) are essential for folding of DNA in a nucleosomal manner (36). The length of DNA associated with the core particle is 140 base pairs (bp), and is invariant among species (36,71). The linker (or spacer) region of DNA can vary between 10 to 70 bp in length, and is dependent on the species (36,71).

X-ray crystallographic data have revealed that the nucleosome core particle is probably a flat disk, approximately 100 Å in diameter and 50 Å in height, with DNA wrapped around the outside of the disk (36, 71). Dissociation of the nucleosome in 2 M NaCl has yielded 2 tetramers consisting of 1 molecule of each histone, suggesting that nucleosomal core particles possess a 2-fold axis of symmetry (71). There is evidence that a fifth histone protein, H1, is associated with DNA at the end of the nucleosome core, or between nucleosomal cores, and may form a link between 2 "beads" in a chromatin fiber (63). Removal of H1 has little effect on nucleosomal cores, but does affect the conformation of the chain of "beads" (63).

There is controversy over the arrangement of nucleosomal core particles within the chromatin fiber. Some evidence suggests a solenoid-type arrangement with a pitch of 100 Å and a diameter of 300 Å (71). Others suggest the nucleosomes arrange as clusters (71). Histone H1 appears to stabilize the arrangement possibly by cross-linking nonadjacent nucleosomes (36,71). The amino acid composition of the H1 histone subspecies has been found to vary a great deal more than that of the other histones (36). This variable histone is associated with the variable section of DNA within a nucleosome (36). It is conceivable that variability in these regions is related to species-specificity. Laemmli (71) has found that through treatment of metaphase chromosomes with competing polyanions (dextran sulfate), the chromosomal DNA held together by so-called scaffolding nonhistone proteins, can be isolated free of histone proteins. Their results have shown that a set of nonhistone proteins form a central scaffolding crosslinking DNA into a radial distribution of regular loops

(10-30 μm) (71). In this model, histones would serve to compact the DNA loops (71). The result is compaction of genetic information into the structure of a chromosome for distribution to daughter cells during mitosis.

With an increased understanding of the organization of genetic information in a eucaryotic cell, investigations on biological activity of chromatin could be undertaken. Generally, 10-20% of the entire genome is considered to be transcriptionally active chromatin (117,141). The exact mechanism for control of gene expression remains to be elucidated. It has been noted, however, that transcriptionally active regions of the genome are organized into nucleosomes, as are the transcriptionally inactive regions (141). Yet there is some alteration of this basic structure that renders some regions of chromatin transcriptionally active. Weisbrod and Weintraub have reported that there appear to be a group of proteins associated with the globin gene of erythrocytes which is preferentially sensitive to DNase I digestion (142). When these proteins of the high mobility group (HMG) proteins are removed, globin gene is no longer preferentially susceptible to DNase I. The investigators suggest that somewhere along the DNA near the globin gene, a recognition event occurs followed by a preparation event in which DNase I-sensitive structure is propagated along the chromatin region to be transcribed (142). These HMG proteins may be involved in propagating transcriptionally active chromatin structure (142).

Endonucleases have been used as tools to investigate the nature of chromatin structure in transcriptionally active and inactive

regions of the genome. Weintraub and Groudine (140) have found that pancreatic deoxyribonuclease 1 (DNase I) can digest DNA sequences corresponding to globin genes in chick erythrocyte nuclei and ovalbumin genes in hen oviduct nuclei, when only 10% of the total nuclear DNA is digested. However, treatment of erythrocyte nuclei with DNase I did not remove ovalbumin gene sequences (140). Furthermore, when staphylococcal nuclease (also known as micrococcal nuclease) was used to digest erythrocyte nuclei, there was no preferential digestion of active gene sequences (140). The erythrocyte genome was arranged in a nucleosomal structure, yet the DNase I sensitivity of globin gene sequences suggests that nucleosomes in active chromatin regions differ conformationally in some respect. These results were subsequently confirmed in analagous studies by Garel and Axel (42).

Gottesfeld and Bonner (44) have employed spleen deoxyribonuclease II (DNase II) to fractionate chromatin into regions of differing degrees of transcriptional activity followed by selective $MgCl_2$ precipitation. DNase II digests chromatin into segments 100-200 nucleotides in length. The nuclease-resistant chromatin (PI) is pelleted. Portions of the remaining chromatin can be selectively precipitated in the presence of a divalent cation (Mg^{2+}) resulting in the P2 pellet, and the Mg^{2+}-soluble portions (S2) which are thought to contain transcriptionally active regions of chromatin (44). The globin gene sequence has been localized in the S2, or putative transcriptionally active, region of Friend leukemia cell chromatin both prior to and after induction of hemoglobin synthesis (17,138). These results support those obtained with DNase I, i.e., there are unique

structural characteristics of transcriptionally active chromatin that confer upon them a susceptibility to endonucleases. Hyperacetylated histones have been found associated with DNase I-sensitive chromatin, suggesting that this mode of histone modification may be important in determining transcriptional activity (118). However, some evidence indicates this type of modification is not sufficient for gene activation (141). Additional evidence indicates that DNase-I sensitive chromatin in higher and lower eucaryotes contains genes transcribed by all 3 of the RNA polymerases (135). There are conflicting electron microscopic reports on whether or not transcribing chromatin remains in a nucleosomal conformation (81). Questions remain to be answered concerning the spacing of RNA polymerase along transcribing DNA, and whether the active structure is in a dynamic equilibrium with native chromatin structure (81).

Staphylococcal nuclease has been shown to digest preferentially linker vs. nucleosomal core DNA (36,75). Early in incubation of chromatin with staphylococcal nuclease, linker regions became acid soluble (75). However, core DNA can be digested by this enzyme at a much slower rate (36,75).

These endonucleases can be used as probes for localization of carcinogen binding within the genome of target cells.

The binding of representatives of various classes of chemical carcinogens to DNase I-sensitive regions of chromatin DNA has been extensively investigated. When bronchial epithelial cells and fibroblasts (target and nontarget cells, respectively) in culture were treated with the polycyclic aromatic hydrocarbon, benzpyrene,

carcinogen adducts were formed in DNase I-sensitive (transcriptionally active) regions of chromatin of both cell populations (4). Binding of benzpyrene to DNase I resistat areas of chromatin in target cells occurs rapidly, and adducts persist (4). However, in chromatin of fibroblasts, adduct formation occurs more slowly and adduct removal occurs more rapidly from DNase I resistant regions (4). These observations may be important in determining the eventual transformation of lung epithelial cells into carcinoma cells (4).

Similar studies have been undertaken employing the nitroso compound, dimethylnitrosamine (DMN) (102). Ramanathan et al. found that, following treatment of rats with DMN, DNase I-sensitive regions of liver chromatin contained a higher concentration of methylated products compared to DNase I accessible regions of chromatin (102). Studies of the removal of methylated products from fractions of liver chromatin revealed that methylated products were nearly completely lost from DNase I-sensitive chromatin 2 weeks following administration, while 14% of the methylated products remained in nuclease-inaccessible regions (102). These results suggest that conformational qualities that render regions of chromatin accessible to DNase I may also be responsible for increased susceptibility of these regions to carcinogen attack, and to repair enzymes.

The aromatic amine, N-OH-AAF, has been found to bind preferentially to DNase I-resistant regions of chromatin from liver cell nuclei following treatment of rats with this carcinogen in vivo (84). The concentration of carcinogen adducts remains in DNase I-resistant chromatin regions up to 72 hours following treatment (84). Ramanathan

et al. (106) have performed analogous experiments with N-OH-AAF, and found that there was a 4-fold concentration of adducts in DNase I-inaccessible regions of rat liver chromatin. This ratio persisted up to one week following carcinogen treatment. The biological significance of carcinogen localization in these regions of chromatin remains to be more fully elucidated. The fact remains, however, that representatives of various classes of known chemical carcinogens interact nonrandomly with DNA of target and nontarget cells, and this nonrandom interaction appears to be the result of the unique conformation of chromatin as determined by DNase I accessibility.

Drugs may interact with specific regions of chromatin delineated by DNase I susceptibility. Recently, it has been shown that the antitumor antibiotic, bleomycin, causes single strand breaks in DNase I sensitive regions of hen oviduct cell nuclei containing ovalbumin gene sequences, but not in DNase I-insensitive regions of chromatin in oviduct cell containing globin gene sequences (70). Thus, chromatin with a more open configuration (DNase I-sensitive) may be more susceptible to attack by a variety of damaging agents than inactive, condensed chromatin.

DNase II selectively attacks regions of transcriptional activity in chromatin. Following treatment of rats in vivo with the nitroso compounds methylnitrosourea (MNU) and dimethylnitrosamine (DMN), alkylation was found to be greater in DNase II digested, $MgCl_2$-soluble portions, the putative transcriptionally active regions of rat liver chromatin (35). These results are in agreement with similar studies using DNase I to fractionate rat liver chromatin following carcinogen treatment.

In contrast to the DNase I studies, a 16-fold concentration of carcinogen adducts has been found in putative transcriptionally active regions of rat liver chromatin digested by DNase II and solubilized in $MgCl_2$ following treatment of rats with N-OH-AAF in vivo (114). Similar results for preferential carcinogen binding to transcriptionally active regions of chromatin were confirmed following fractionation of N-OH-AAF-treated rat liver chromatin by the selective $MgCl_2$ chromatin precipitation procedure (115), a sucrose gradient chromatin fractionation procedure (90), and the glycerol gradient chromatin fractionation procedure (115). The latter study (115) revealed that carcinogen was preferentially lost from transcriptionally active regions of rat liver chromatin after 10 days. The discrepancy between DNase I and DNase II studies following treatment of rats with the aromatic amine N-OH-AAF may indicate that these endonucleases may act by different mechanisms.

Staphylococcal nuclease has been a useful tool for investigation of specificity of carcinogen binding and repair of adducts from specific regions of chromatin. When normal human fibroblasts are treated with the ultimate carcinogen N-acetoxy-AAF, carcinogen adducts occur preferentially in regions of nuclease sensitivity, i.e., linker regions (131). When ^{3}H-thymidine was present in the media to allow for radioactive labelling of repair-incorporated nucleotides, Tlsty and Lieberman (131) found that repair synthesis initially occurred in linker regions, but at later times, ^{3}H-thymidine-labelled repair patches were relocated within nucleosomal core regions. Similar rearrangement occurred following repair of UV-light-induced DNA

damage, indicating that chromatin structure, rather than type of carcinogen, may be the determining factor in carcinogen adduct repair. Kaneko and Cerutti (60) reported on an analogous study in which binding to DNA and persistance of adducts in Staphylococcal nuclease sensitive and resistant areas of chromatin from normal human fibroblasts was investigated following treatment of cells with a lower dose of N-acetoxy-AAF than that used in the former study (131). In accord with results of Tlsty and Lieberman (131), the initial concentration of carcinogen adducts was higher in linker vs. core (60), as was the initial loss (presumably due to repair) of adducts. With increasing time for repair, carcinogen adducts continued to be rapidly removed from linker regions, and were slowly removed from core regions (60). These results provided no support for nucleosomal rearrangement concomitant with repair of adducts, or as a result of the repair process. The 5- to 60-fold increase in carcinogen concentration to which fibroblasts were exposed in the studies of Tlsty and Lieberman (131) may have resulted in adduct concentration to an extent that nucleosomal rearrangement was induced. Nevertheless, results from these studies illustrate that arrangement of chromatin into nucleosomal core and linker regions can influence carcinogen binding and repair.

4. AAF-Induced Hepatocarcinogenesis

2-Acetylaminofluorene (AAF) was originally developed by the U.S. Department of Agriculture in 1940 to be used as an insecticide (146). AAF was found to have low acute toxicity in rats, mice and rabbits, but produced numerous tumors in various organs of rats upon long-term

feeding (146). This compound was never marketed, but has since been used as a model compound for study of carcinogenesis induced by aromatic amines. Extensive investigations have shown AAF to be carcinogenic for liver, urinary bladder, mammary gland, earduct, salivary gland, lungs, forestomach and small intestine (146, reviewed in ref. 37).

There are a number of changes in rat liver which consistently occur during and after long-term feeding of a 0.05% (w/w) AAF-containing diet to rats. At 3-4 weeks of AAF treatment, rat livers appear pale, and light microscopy reveals vacuolization of the hepatocyte cytoplasm, an increase in agranular endoplasmic reticulum, and a distortion and dilation of bile canaliculi (136). By 12 weeks of AAF feeding, nodular hyperplasia appears, distorting the normal pattern of liver structure, along with the continuation of changes that had occurred at 3-4 weeks of treatment, as well as glycogen accumulation and the appearance of large lysosomes (136). At 8-10 months of treatment, livers were visibly enlarged and finely nodular; the lobular structure was entirely lost, and there was evidence of widespread hyperplasia of hepatic parenchymal cells, along with loss and dilation of agranular and granular endoplasmic reticulum (136). By the end of 10 months on the AAF diet, there was 100% incidence of liver tumors in treated rats (38).

Studies involving maintenance of rats on an AAF-containing diet (0.04% w/w) revealed that the earliest change observed was proliferation of oval cells in portal areas (33). Autoradiographic studies of appearance and proliferation of oval cells as rats are maintained on a 0.05% AAF-containing choline-devoid diet revealed that oval cells do

not arise from hepatocytes, but rather, arise from a few portally-situated oval cells, and contain α-fetoprotein (119,120). By 5 weeks of maintenance of rats on an AAF-containing diet, hyperplasia of hepatic parenchymal cells was observed (148). This hyperplasia was only observed within hepatic nodules (148).

In summary, AAF-induced hepatocellular carcinomas develop in several stages as rats are maintained on an AAF-containing diet (145). Foci, small islands (8-10 cells in diameter) of altered hepatocytes, develop within 3 weeks of carcinogen exposure (145). These cells stain positively for gamma-glutamyl transpeptidase, and are deficient in adenosine triphosphatase, glucose-6-phosphatase, glycogen accumulation, hyperbasophilia following staining with toluidine blue, and are resistant to iron accumulation (145). Functionally, cells of foci characteristically became resistant to toxic effects of chemicals requiring metabolic activation (145). This properly may allow for the selective growth advantage of these altered cells. When carcinogen is removed at this stage, altered foci disappear (145). However, if carcinogen treatment is continued, islands of foci develop into nodules, which are round, elevated groups of cells approximately the size of several lobules that compress normal, surrounding liver tissue (145). The cells of nodules continue to exhibit the same functional abnormalities and enzyme alterations as those of foci (145). Nodules develop 2 to 4 weeks following foci development (145). They display few oncologic properties compared to carcinomas (145), however, it is clear that they are composed of abnormal hepatocytes which are resistant to the cytotoxic effects of carcinogens. Finally, after continued

exposure to AAF hepatocellular carcinomas arise, probably from hyperplastic nodules (145) which display enzyme and functional alterations. Additionally, these carcinomas display progressive growth upon cessation of carcinogen, tumor invasiveness and metastasis (145).

From early studies by the Millers and co-workers (87,88), it became clear that AAF was metabolized to an active form as part of its carcinogenic action. The N-hydroxy metabolite of AAF produced tumors at the site of injection (peritoneum), or in the forestomach following inclusion of AAF in the diet of rats, while similar treatment with AAF did not produce tumors at these sites (87,88). Furthermore, guinea pigs were resistant to AAF-induced carcinogenesis, yet developed tumors at the site of administration when N-OH-AAF was given (87). Ring-hydroxylated metabolites produced to a great extent following pretreatment of rats with 3-methylcholanthrene had little to no carcinogenic activity (88). Thus, the minor metabolite, N-OH-AAF, was considered to be an important metabolite of AAF in the carcinogenic process. It is now recognized that the hepatic microsomal P-450 monooxygenase system metabolizes AAF to its N-hydroxy derivative (67,69,85).

The sulfate ester of N-OH-AAF has been found to be important in AAF-induced carcinogenesis. From early studies, the level of liver cytosolic sulfotransferase activity paralleled the level of susceptibility to tumor induction by N-OH-AAF (28). These studies suggested that another metabolic step was necessary to convert N-OH-AAF to a carcinogenic form. Female rats are less susceptible to AAF-induced hepatocarcinogenesis than male rats, and female rats possess one-fifth

the cytosolic sulfotransferase activity of male rats (28). More recently, inhibition of sulfate conjugation of N-OH-AAF by pentachlorophenol or low availability of sulfate has been shown to result in an increase in the percent of the administered dose excreted as a glucuronide conjugate compared to the percent of dose normally excreted as such (83). In addition, inhibition of sulfate conjugation in vivo by injecting rats with pentachlorophenol prior to injection with N-OH-AAF resulted in a 26% reduction of total DNA binding, most notably reducing the total amount of N-acetylated adducts produced (82).

Other enzymic pathways have been recognized for conversion of AAF to a reactive electrophile (85). N-OH-AAF may undergo a peroxidase-catalyzed oxidation resulting in a free nitroxide radical, followed by disumutation of two of these radicals to yield N-acetoxy-AAF and 2-nitrosofluorene, both being reactive electrophiles (85). Secondly, cytosolic acetyltransferase may transfer the acetyl group from N-OH-AAF to the oxygen of the hydroxylamine, resulting in formation of the reactive N-acetoxy-aminofluorene (85). Lastly, AAF can be converted to the O-glucuronide and/or the N-glucuronide conjugates (58,85). These forms may enable AAF to be stably transported to another tissue, such as the urinary bladder, wherein the conjugate can be hydrolyzed yielding a reactive electrophilic species (58). The alternative metabolic pathways discussed above may be important in activating AAF to a reactive form in extrahepatic tissues (85).

5. Interactions of AAF with DNA

The assumption that DNA is a critical target for AAF-induced carcinogenesis had led many investigators to study the nature of the interaction of ultimate reactive form of AAF with DNA. Initial studies revealed that guanines within DNA were the primary sites for adduct formation following AAF treatment (see ref. 69, 85 and 85 for review). AAF reacted with DNA to yield 2 types of adducts: 1) an arylamidation product as the result of addition of a nitrenium ion to the C8 of guanine (N-(guanin-8-yl)acetylaminofluorene or N-(guanin-8-yl)-aminofluorene), and 2) an arylation product as the result of addition of a carbon cation to the 2-amino group of guanine (3-(deoxyguanosin-N^2-yl)-N-acetylaminofluorene) (40). Approximately 84% of the reaction products with native DNA are arylamidation products, whereas only 16% of the products are derived from an arylation reaction (40,67,69). In vivo, approximately 65-70% of the arylamidation product was found in a deacetylated form (54,64,69). The arylation product, 3-(dexoyguanosin-N^2-yl)-N-acetylaminofluorene, does not occur in RNA (65,69). N-(deoxyguanosin-8-yl)-AAF is the predominant adduct in RNA (the acetylated form predominates) (54,69). The half-life of adducts in RNA is approximately 3 days (54), while the half-life for the C8 adduct in DNA is approximately 7-10 days (54,69). The N^2-gua-AAF adduct in DNA has been found to be persistant in DNA (65). Percentages of persisting adducts may vary depending on the strain of rat used for investigations (136). Furthermore, adducts may accumulate and persist in nontarget tissues for a carcinogen. In male rat liver, following carcinogen treatment, the N^2-gua-AAF adduct accumulated and

persisted for up to 2 weeks of carcinogen treatment (10). However, the C8-gua-AF adduct accumulated and persisted in the liver (target tissue) and kidney (nontarget tissue) of male rats (10). These results suggest accumulation and persistance of adducts alone is not sufficient for tumor induction.

The identification of products of carcinogen reaction with nucleic acids led investigators to question the consequences of such interactions. Through biochemical and physical analyses, it was found that the 3-dimensional confirmation of nucleic acids was modified by AAF (72). These studies led to proposal of the "base displacement model" in which the attachment of the reactive form of AAF to the C-8 position of guanosine results in rotation of the guanine base around the glycosidic bond from the anti (normal Watson-Crick orientation) to the syn conformation (72). The fluorene residue swings into the helix and is stacking in a coplanar manner with adjacent bases (72). It has been found that base modifications by carcinogens can alter base-pairing ability, resulting in reduction of recognition of tRNA codons (45), and reduction in DNA template capacity, probably due to an apparent decrease in RNA chain size (116). These interactions result in local regions of denaturation, as shown by a decreased thermal stability and intrinsic viscosity of AAF-reacted DNA, as well as elution from a hydroxyapatite column at lower salt concentrations (72). Analysis of carcinogen adducts released from digestion by a single-strand-specific nuclease of Neurospora crassa of duck reticulocyte DNA reacted in vitro with [9-^{14}C]-acetoxy-AAF revealed that the areas containing the C8-gua-AAF adduct were selectively

susceptible to the single-strand-specific nuclease digestion (the N^2 adduct remained in undigested DNA) (149). These results strengthened the contention that the C8-gua-AAF adduct caused major conformation distortions in the double helix, while the N^2-gua-AAF adduct does not (149). Kriek and Spelt (68) demonstrated that calf-thymus DNA containing C8-gua-AAF reaction products from N-OH-AAF treatment is hydrolyzed 3 times more slowly by nuclease S_1 of Aspergillus oryzae compared to DNA containing C8-gua-AAF adducts following reaction with N-acetoxy-AAF. These results indicate that C8-gua-AAF residues result in smaller regions of denaturation in DNA than do C8-gua-AAF residues (68).

Further studies indicate that carcinogen residues in DNA following reaction with N-OH-AAF in vitro are less accessible to adduct-specific antibodies in native DNA than in denatured DNA lending support to the base-displacement model (126). Very recently, it has been shown that AAF adducts present in poly(dG-dC) DNA can cause this polymer to change from the standard B form at the helix to a newly discovered Z form, which is a left-handed helix (111). Circular dichroism studies indicate that, in this form, AAF-guanine residues are in the syn conformation and may remain paired with cytosine residues (111). In this form, guanine is more accessible to attack by carcinogens, and because there is no base displacement, and therefore, no denaturation, this conformation of carcinogen-modified DNA may be more stable (111). The biological consequences of this observation remain to be investigated.

From the extensive work that has been done on binding and persistance of adducts following AAF treatment, several conclusions can be drawn concerning AAF adducts. The acetylated adducts, C8-gua-AAF and N^2-gua-AAF, are probably formed through N,O-sulfation of N-OH-AAF *in vivo* (82). However, the deacetylated adduct, C8-guaAF, may be formed through an alternative activating pathway (82). The persistant adducts include C8-guaAF and N^2-gua-AAF (the latter persists for the longest periods) (10). The C8-gua-AAF adduct was lost rapidly from liver, while the C8-gua-AF adduct accumulated in target and nontarget tissue with progressive carcinogen treatment (10). However, recent evidence suggests that prolonged carcinogen administration (28 days) results in a decreased ability for removal of acetylated and deacetylated C-8 adducts (99). Lastly, the C8-gua-AAF adduct causes the greatest DNA conformational distortion, while the C8-gua-AF adduct causes less distortion (68), and the N^2-gua-AAF adduct results in even less conformation distortion of the DNA double helix (149). In view of the unique characteristics of each AAF adduct, it is possible that AAF-induced carcinogenesis may depend on effects caused by all three adducts, rather than any 1 or 2 of the individual adducts.

6. Experimental Objectives

The objective of this thesis project has been to discern initial molecular events critical to target-specific carcinogenesis induced by chemicals. AAF and its N-hydroxy metabolite were used as model hepatocarcinogens to investigate events related to carcinogenesis targeted to hepatic parenchymal cells of the liver. These studies included a determination of the binding of the parent carcinogen, AAF,

and its N-hydroxy metabolite, to DNA of target cells, parenchymal cells (PC), and at the nontarget cells, nonparenchymal cells (NPC). DNA was isolated from centrifugally elutriated PC and NPC populations of rat carcinogen treatment of rats. Alternatively, DNA was isolated from nuclei of PC (NI nuclei) or from nuclei of NPC (NII nuclei) following the same carcinogen treatment regimens. In both cases, total carcinogen binding to DNA was assessed to determine any specificity for carcinogen binding to DNA of target and nontarget cell populations.

In view of the apparent nonrandom nature of binding to DNA exhibited by several carcinogens, studies were undertaken to investigate whether or not AAF binding to DNA was specific for regions of the genome in target cells and nontarget cells. DNase I was used as a tool to enable analysis of carcinogen binding to DNA at transcriptionally active and inactive areas of the genome at target parenchymal cell (NI) and nontarget nonparenchymal cell (NII) cell nuclei. Carcinogen binding to DNase I-accessible and inaccessible regions of DNA from NI and NII nuclei was determined at the time of peak binding and 3 days later following treatment of rats with AAF and its N-hydroxy metabolite. The influence of daily exposure to AAF for 1, 3, and 5 days on binding at tracer doses of [ring $^{-3}$H]-AAF to DNase I-accessible and inaccessible regions of DNA of target and nontarget cell nuclei were also investigated.

The DNA of target (NI) and nontarget (NII) nuclei was nick-translated by incorporation of ^{32}P-labelled deoxyribonucleotide

triphosphates into DNase I-sensitive regions of chromatin to determine the degree of transcriptional activity within the nuclei of the 2 liver cell populations.

Lastly, a series of experiments were performed to determine the effect of carcinogen modification of DNA from target and nontarget cells on the ability of several restriction endonucleases, enzymes which cleave at specific base sequences, to recognize and cleave at these sequences. Changes in the molecular weight of particular restriction fragments could be monitored as an indication of impaired restriction enzyme cleavage. These fragments could be identified on the basis of localization of albumin gene sequences within these fragments by hybridization with a radioactively-labelled cDNA probe complementary for the rat albumin gene. DNA from target and nontarget (NI and NII, respectively) rat liver cell nuclei was analyzed in this manner following treatment of rats for 1, 3, 5 or 7 days with AAF. Another set of rats treated for 1, 3, 5 or 7 days was left untreated for 7 days followed by restriction enzyme analysis of DNA from target and nontarget cell nuclei to determine the effect of a period of repair on restriction enzyme recognition of carcinogen-modified DNA.

From these types of experiments, it is hoped that the importance not only of cell target of a carcinogen may be determined, but also, the DNA target region(s) critical to initiation of carcinogenesis in a particular cell population may be elucidated.

MATERIALS AND METHODS

1. Animals: Maintenance and Carcinogen Treatment

Male, Sprague-Dawley rats (150-200 g), purchased from Spartan Farms (Haslett, MI) were used in these studies. Animals were housed 2 per cage in a room with a controlled 12 hour light cycle beginning at 7 p.m. Rats were given food (Lab-Blox, Chicago, IL) and water ad libitum. When indicated, rats were injected intraperitoneally (i.p.) with [ring-^{3}H]-N-2-acetylaminofluorene or [ring-^{3}]-N-hydroxy-N-acetyl-2-aminofluorene, 51.0 mCi/mmole and 50.8 mCi/mmole, respectively (Midwest Research Institute, Kansas City, MO). [ring-^{3}H]-N-hydroxy-acetylaminofluorene was prepared in 0.9% sodium chloride in a concentration of 1.8 μmole/0.5 ml injection volume/100 g body weight. [ring-^{3}H]-N-2-acetylaminofluorene was prepared in corn oil:DMSO (6:1, v/v) containing 14% ethanol in a concentration of 1.8 μmole carcinogen/0.5 ml injection volume/100 g body weight. In some studies rats were injected i.p. with N-2-acetylaminofluorene (Aldrich Chemical, Milwaukee, WI) dissolved in corn oil:DMSO (6:1, v/v) in a dose of 15 mg/0.75 ml injection volume/100 g body weight or corn oil:DMSO (6:1, v/v) in a dose of 0.75 ml injection volume/100 g body weight as vehicle control.

2. Isolation of Nuclei

Liver cell nuclei were isolated from rats by the method of Blobel and Potter (14) as follows. Livers were homogenized in 3 volumes of ice-cold 250 mM sucrose, 50 mM Tris (pH=7.5), 25 mM KCl, 5 mM $MgCl_2$ (STKM) and filtered through cheese cloth. Total liver cell nuclei were isolated by washing twice in 2% Triton X-100 and STKM followed by centrifugation at 750 x g for 10 minutes. The nuclear pellet was washed once in STKM. The method of Bushnell et al. (19) was used to isolate nuclei of hepatic parenchymal cells (class N1 nuclei) and nuclei of hepatic non-parenchymal cells (class NII nuclei). Following filtration of homogenate through cheese cloth, 12.5 ml of filtered homogenate and 12.5 ml of 2.3 M sucrose in TKM were mixed, and underlayed with 10 ml of 2.3 M sucrose in TKM followed by centrifugation at 23,000 rpm at 5°C for 1 hour. The SW27 Rotor (Beckman Instrument Co.) was used for this procedure. Following centrifugation the NI nuclei pellet was isolated and washed once in STKM. The 750 x g supernatant was added to 255 ml of STKM and 5.8 ml 20% Triton X-100, and centrifuged at 5000 rpm at 5°C, 20 minutes. Pelleted NII nuclei were washed twice in STKM followed by centrifugation at 4000 rpm, 5°C, 10 minutes.

3. Isolation of Distinct Liver Cell Populations

This procedure was performed in collaboration with Dr. James Swenberg (Chemical Industry Institute of Toxicology, Research Triangle Park, NC). Populations of hepatic parenchymal (PC) and non-parenchymal (NPC) cells were isolated by a method for centrifugal elutriation detailed elsewhere (Lewis and Swenberg, 74). Livers from rats

were perfused with collagenase through the portal vein. The mixed liver cell suspension was washed twice in Hepes buffered saline solution (HBSS), then slowly injected into a large mixing chamber of the Beckman JE-6 elutriation rotor containing a Sanderson separation chamber in a Beckman J2-21 refrigerated chamber. The rotor speed was held constant at 1,500 rpm and the flow rate varied. The nonparenchymal cells were separated at 18 ml/minute, and the hepatocytes were isolated at 100 ml/minute. Elutriated nonparenchymal cells (NPC) were composed of Kupffer and endothelial cells, in contrast to the population of NPC from which NII nuclei were derived. NII nuclei were obtained from Kupffer and endothelial cells as well as from bile duct cells and fat-storing cells.

4. Nuclease Digestion of DNA from Nuclei

DNase I was used as a tool to delineate transcriptionally active DNA from inactive DNA. Parenchymal (NI) and nonparenchymal cell nuclei (NII) were digested by pancreatic deoxyribonuclease I according to a method of Weintraub and Groudine (140). Nuclei were washed twice in 10 mM Tris (pH=7.4), 10 mM NaCl, 3 mM $MgCl_2$ (TNM). Nuclei were suspended by gentle homogenization in TNM such that 1 ml suspension contained 1 mg DNA. DNase I was added (20 µg/125 units/mg DNA) followed by incubation at 0, 5 and 10 minutes at 37°C with mild shaking. The reaction was terminated by addition of ice-cold trichloroacetic acid to a final concentration of 10%. Suspensions were centrifuged at 2500 rpm, 5°C, 5 min. Supernatants were boiled for 5 minutes and cooled on ice to preferentially solubilize DNA digested by DNase

I. Supernatants were centrifuged at 5000 rpm, 5°C, 10 minutes. Supernatants were removed for DNA analysis.

5. Nick Translation of Transcriptionally Active DNA from Nuclei of Parenchymal and Nonparenchymal Liver Cells

It has been shown recently that DNA of hen oviduct cell nuclei can be nicked by the endonuclease DNase I and translated with E. coli DNA polymerase I using ^{32}P-labelled deoxyribonucleotide triphosphates resulting in radioactive labelling in regions of transcriptionally active chromatin (73). This procedure was adapted to nick translate DNA of target (PC) and nontarget (NPC) liver cells. DNase I was added in a sufficiently low concentration so as to introduce single-strand nicks into active regions of chromatin. Subsequent addition of DNA polymerase I results in incorporation of ^{32}P-labelled deoxyribonucleotide triphosphates in nicked areas opposite an intact template.

Hepatic NI and NII nuclei were isolated by discontinuous sucrose gradient centrifugation and suspended in nick-translation buffer (50 mM Tris, pH=7.9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 µg/ml BSA) in a concentration of 1 mg nuclei DNA/ml. DNase I was added (0.3 µg/ml) and the suspensions were incubated at 37°C for 5 minutes. Deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) were added to a final concentration of 4 µm each and 26 pmoles (769 mCi/µmole) of each ^{32}P-nucleotide triphosphate was added. E. coli DNA polymerase I was added (10 units/ml) and incubation proceeded for 5 minutes at 15°C. Following termination of the reaction on ice, nuclei were centrifuged at 4000 rpm for 10 minutes at 5°C. Nuclei were washed 3 times in nick translation buffer to remove unincorporated nucleotides.

^{32}P-labelled DNA from NI and NII nuclei was isolated by Marmur extraction (6,49,80). DNA was digested by the restriction endonuclease *Eco* R1 (0.15 units/µg DNA) and applied to a 0.9% agarose gel and run at 50 V overnight. DNA in the gel was stained with ethidium bromide (0.5 µg/ml) and photographed while being illuminated with a UV transilluminator (Ultra-Violet Products, Inc., San Gabriel, CA). The gel was dried under vacuum onto gel backing paper (Bio-Rad). The dried gel was exposed to X-ray film (Kodak X-OMat AR) for 4-24 hr, then developed.

6. DNA Isolation

A. Hydroxyapatite Column Chromatography

The method of Beland et al. (9) was used to isolate DNA from parenchymal and nonparenchymal cells or nuclei with the following modifications. Hydroxyapatite (Biogel DNA-Grade HTP, Bio-Rad) was prepared in 1 g portions/mg DNA. Hydroxyapatite was washed and fines were decanted twice in 0.014 M Na_2PO_4 (pH=6.8) at 25°C. The hydroxyapatite slurries were poured into glass wool-plugged plastic spin timbles (Reeve-Angel) and placed in centrifuge tubes. Columns were packed by centrifugation at 1000 rpm (500 x g). Columns were equilibrated in 8 M urea, 1% SDS, 10 mM EDTA, 0.24 M Na_2PO_4 (pH=6.8) (lysing solution). Cells or nuclei were suspended in lysing solution and applied to columns. Columns were centrifuged at 1000 rpm following a 15-30 minute equilibration period to allow for DNA to bind to the hydroxyapatite. Columns were washed twice with 8 M urea and 0.24 M Na_2PO_4, pH=6.8 (MUP) to remove RNA and protein. Residual SDS and

urea were removed from the column with 1 wash of 0.014 M Na_2PO_4, pH=6.8. DNA was eluted from the column in 0.48 M Na_2PO_4, pH=6.8. Each was obtained by centrifugation at 1000 rpm for 5 minutes. The 0.48 M Na_2PO_4 eluant was dialyzed overnight at 5°C against 5 mM Bis-Tris (pH=7.1) and 0.1 mM EDTA.

B. Isolation of DNA for Agarose Gel Electrophoresis

The method of Marmur (6,49,80) was used to isolate high molecular weight DNA free of protein and RNA. Nuclei were gently homogenized (1 stroke) in 10 mM Tris-HCl (pH=7.9), 0.1 M NaCl, 5.0 mM EDTA, 0.5 M $NaClO_4$ and 1% sodium dodecyl sulfate (nuclei from 6.25 g liver in 15 ml suspension). The suspension was incubated with constant shaking at 37°C for 40 minutes. The suspension was deproteinized with an equal volume of chloroform:3% isoamylalcohol after incubation. The wash was repeated once. Layers were separated by centrifugation at 400 x g for 10 min. The DNA from the upper, aqueous layer was precipitated with 2 volumes of ice-cold 95% ethanol and centrifuged at 12,100 x g for 10 min at 5°C. Following removal of the supernatant, the precipitated DNA was resuspended in 10 mM Tris (pH=7.9), 5 mM EDTA (DNA from 6.25 g liver in 10 ml). Ribonuclease A (Sigma) (200 µg enzyme/ml) which was treated at 80°C for 10 min to destroy DNase contamination, was added and the samples were incubated at 37°C for 40 minutes.

NaCl was added to a final concentration of 0.1 M and protease (Sigma Chemical Co.) which was heat treated at 80°C for 10 min was added (3 mg/ml). Incubation proceeded for 10 minutes at 37°C. The solution was deproteinized by 2 washes of chloroform:3% isoamyl

alcohol and one wash of redistilled phenol. Layers were separated by centrifugation at 400 x g for 10 minutes. Purified DNA in the aqueous layer was precipitated with 2 volumes of ice-cold 95% ethanol by spooling on a glass rod, then dissolving in 5 mM Tris (pH=7.8) and 0.5 mM EDTA. DNA concentration was determined by the absorbance at 260 nm assuming 1 A_{260} is equal to 50 μg DNA/ml.

7. Isolation of Plasmid Containing Rat Albumin cDNA

In order to amplify and isolate the plasmid pBR322 which contained the pRSA13 cDNA from the rat albumin gene (see Sargent et al., 11]), the following experiments were done. Ten milliliters of M-9 glucose medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.5% glucose 0.5% Casamino acids, .002% thiamine-HCl, 1 mM $MgCl_2$) was innoculated with E. coli strain (HB101) containing the plasmid pBR322 into which the cDNA pRSA13 from rat albumin gene had been incorporated (a gift from Dr. James Bonner). The innoculum was incubated overnight at 37°C with constant shaking. Following incubation, the entire 10 ml were added to 1 liter of M-9 medium, and the mixture was incubated and shaken at 37°C until the absorbance at 600 nm was 0.5-0.6 at that point, 150 mg of chloramphenicol (Sigma Chemical) was added per liter of culture. The culture was allowed to amplify overnight. Cells were chilled on ice for 5 minutes, then centrifuged at 5,000 rpm, 10 minutes at 5°C. Cells were suspended in 40 ml of 10 mM Tris (pH=8.0) and 1 mM EDTA (washing buffer) and centrifuged at 5000 rpm, 10 minutes, 5°C. Cells were resuspended in 4 ml freshly prepared lysozyme (2 mg/ml), 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH=8.0). The suspension was incubated at 0°C for 30 minutes,

followed by the addition of 2 volumes of alkaline SDS (0.2 N NaOH, 1% SDS) and incubated at 0°C for 5 additional minutes. The suspension was mixed with 1.5 volumes of 3 M sodium acetate (pH=4.8) and incubated at 0°C for 1 hour followed by centrifugation at 15,000 rpm for 20 minutes. The supernatant was precipiated with 2 volumes of ethanol overnight at -20°C followed by centrifugation at 10,000 rpm for 15 minutes. The pellet was resuspended in 15 ml of sterile 10 mM Tris (pH=7.4) and 1 mM EDTA. Cesium chloride (15.8 ml) was added along with ethidium bromide (7.5 mg). The suspension was centrifuged in a type 50.1 TI fixed angle rotor at 22°C, 37,000 rpm for 48 hours. The lower UV-visible band which represented supercoiled plasmid DNA was removed. The ethidium bromide was extracted with an equal volume of water saturated butanol until the pink color of the top layer disappeared. The top layer was dialyzed overnight against 1 liter of 10 mM Tris, 0.1 mM EDTA. Dialysate was centrifuged at 10,000 rpm for 20 minutes, 5°C. The pellet was visualized with UV light (360 nm) and the supernatant removed. The pellet was solubilized in 500 µl of 10 mM Tris, 0.1 mM EDTA and stored at 20°C.

8. Agarose Gel Electrophoresis

DNA fragments produced by restriction enzyme digestion of rat liver genomic DNA were separated on the basis of molecular weight by agarose gel electrophoresis. DNA of nuclei from parenchymal and nonparenchymal liver cells of AAF- and vehicle-injected rats were isolated as previously described. DNA of parenchymal and nonparenchymal cells from treated and control rats was digested with Eco Rl or Kpn 1

restriction endonucleases. Samples for agarose gel electrophoresis were prepared in a total well volume of 120 µl containing 50 µg rat DNA, Eco Rl (1.5 units enzyme/µg DNA), Eco Rl reaction buffer (100 mM Tris, pH=7.2), 5 mM $MgCl_2$, 50 mM NaCl and 2 mM 2-mercaptoethanol), and electrophoresis buffer (89 mM Tris, pH=8.3, 89 mM boric acid, 2.5 mM EDTA) to bring the total volume to 120 µl. Alternatively, samples digested with Kpn 1 (8 units enzyme/1 µg DNA) and Kpn 1 reaction buffer (6 mM Tris, pH=7.5, 6 mM $MgCl_2$, 6 mM NaCl and 6 mM 2-mercaptoethanol). Enzyme digestions were carried out for 2 hours at 37°C with mild shaking to allow for complete digestion of rat genomic DNA. Following digestion, 10 µl of a marker dye solution (0.25% bromophenol blue and 0.25% xylene cyanole FF in 50% glycerol) was mixed into each sample before loading onto the agarose gel.

Agarose gels were prepared in a model HO/Hl horizontal gel electrophoresis apparatus (Bethesda Research Laboratories, Gaithersburg, MD). Wick gels consisting of 1.4% agarose (Bethesda Research Laboratories) in electrophoresis buffer were poured and reused for up to 2 months. For electrophoresis of DNA samples, a gel consisting of 0.9% agarose in electrophoresis buffer was prepared by heating the solution to 100°C to dissolve agarose, and cooling slowly with stirring to 55°C. Edges of the tray support for the gel were sealed with 55°C agarose solution using a Pasteur pipet and allowed to solidify. The gel was poured slowly so as to prevent bubble formation. The well-forming comb was inserted, and the gel allowed to solidify for 30 minutes. The comb was carefully removed, and digested DNA samples were applied with a Pasteur pipet. Unused wells were filled with

electrophoresis buffer. The buffer chambers were filled to 2 cm above lower wick surface before covering and connecting the apparatus to a power supply (Pharmacia). Electrophoresis was started at 100 V until the dye left the wells and was 7-10 mm into the gel. Power was turned off, and the wells were refilled with electrophoresis buffer. Electrophoresis proceeded overnight (16 hours) at 50 V. The power was disconnected, and the gel was cut along the edges of the support tray and placed in a solution of ethidium bromide (0.5 μg/ml glass-distilled water) for staining 15-20 minutes. The gel was rinsed and destained in glass-distilled water (GDW) for 5 minutes. The gel was photographed through a Kodak No. 9 Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) at an aperture of 5.6 for 0.5 seconds exposure time using Polaroid Type 667 black and white film (Polaroid Corp., Cambridge, MA). A ruler was placed along side the gel to measure band distances for molecular weight determinations. Molecular weights of markers ranged from 2.2 kilobases (kb) to 17.5 kb. Known molecular weights of markers were plotted on the ordinate of semilog paper as a function of distance (cm).

9. Southern Transfer of DNA

Transfer of DNA from agarose gels to nitrocellulose filters was performed according to a method of Southern et al. (127). Following ethidium bromide staining and photography, the agarose gel was soaked in 0.25 M HCl at 25°C for 30-60 minutes to randomly nick DNA in the gel allowing for a more complete transfer of DNA from the gel to the nitrocellulose filter (130,137). The gel was transferred to a denaturing solution (1.5 M NaCl and 0.5 M NaOH) and soaked for 2 hours

with occasional agitation. The gel was finally transferred to a neutralizing solution (1 M Tris, pH=8 and 1.5 M NaCl) and soaked for 2 hours. The gel was placed on a sheet of 3 mm filter paper (Whatman) wetted in 10XSSC (1.5 M NaCl and 0.15 M sodium citrate, pH=7) such that edges of filter paper are immersed in a tray containing 10XSSC. A nitrocellulose filter (BRL, Gaithersburg, MD) was cut to fit the gel, and air bubbles were removed. Two pieces of 3 mm paper wetted in 10XSSC and cut to fit the gel were placed on top the nitrocellulose filter. Blotting pads (BRL) were placed on top, along with a stack of paper towels to wick the 10XSSC solution through the gel and filter, allowing for transfer of DNA from the gel to the nitrocellulose. Transfer proceeded for 48 hours, carefully removing and replacing blotting pads and paper towels as they became wet. Following transfer, the blotters and 3 mm filter paper were removed, and the nitrocellulose filter was taken off the gel and air-dried. The nitrocellulose filter was baked in a vacuum oven (Cole-Parmer, Chicago, IL) at 80°C for 2 hours to fix the DNA onto the filter.

10. ^{32}P Nick Translation of cDNA Probe

Radioactively-labelled albumin gene probe was synthesized by nick translation of the cDNA corresponding to the pRSA13 restriction fragment (approximately 1.2 Kb) of the rat albumin gene described by Sargent *et al.* (112,113) (pRSA13 incorporated into pBR322 plasmid DNA contained in *E. coli* strain HB101 was a generous gift of Dr. James Bonner. The reaction mixture contained 1 μg pRSA13 cDNA, DNase I (110 ng in 1.1 μl glass-distilled water), *E. coli* DNA polymerase I (Sigma Chemical), 10-concentrated nick translation buffer to yield a final

concentration of 50 mM Tris, pH=7.5, 10 mM $MgSO_4$, 1 mM dithiothreitol and 50 μg/ml BSA. Glass distilled water was added to a final volume of 25 μl followed by addition of four ^{32}P-labelled deoxyribonucleotide triphosphates (New England Nuclear, Boston, MA), each in a final concentration of 2 μm for a total volume of 50 μl. The reaction mixture was incubated at 16°C for 1 hour, and the reaction was terminated on ice. Separation of newly synthesized probe from unincorporated nucleotides was achieved on a Sephadex G-50 column (15 x 10 cm) in column buffer (0.24 M NaCl, 1.6 mM EDTA, 16 mM Tris, pH=8.0, and 0.16% SDS). The reaction mixture was mixed with 50 μl of bromophenol blue in 5% glycerol, and carefully layered onto the column. Fractions of 0.7 ml portions were collected, counted in the ^{3}H channel for Cerenkov counts, and counts per fraction were plotted (Figure 1). The first radioactive peak represented the newly synthesized probe, while the second peak represented unincorporated nucleotides. First peak fractions were pooled, and washed with an equal volume of redistilled phenol and centrifuged at 5 for 9 minutes. The supernatant was similarly washed with an equal volume of chloroform to extract proteins. Ethanol (95%) was added in a volume twice that of the supernatant, and stored at -20°C overnight for precipitation of DNA. The nick translated probe was pelleted by centrifugation at 7,500 rpm (7000 x g) at 5°C for 40 minutes. The pellet was solubilized in 10 mM Tris, pH=7.5 and 0.1 mM EDTA. Radioactivity concentration of the probe was determined by counting a small volume in the ^{32}P channel of a Packard Tricarb Model 460C Scintillation Counter (Packard Instruments).

Figure 1. Isolation of ^{32}P-labelled nick-translated cDNA probe from pRSA-13 cDNA. pRSA-13 cDNA was isolated from E. coli strain HB101 as described in Methods. The cDNA probe (1 μg) was incubated with DNase I (0.21 units), E. coli DNA polymerase I (1 unit) and 2 μm of each ^{32}P-nucleotide triphosphate (769 mCi/μmole) as described in Methods. Newly synthesized ^{32}P-labelled cDNA probe was separated from unincorporated nucleotides on a Sephadex G-50 column. Cerenkov radiation (cpm from ^{3}H channel) is plotted per fraction (0.7 ml). Fractions under the first peak (18-26) were pooled, and pelleted in absolute ethanol as newly synthesized probe. Fractions 19-22 contained greater than 10×10^{6} cpm.

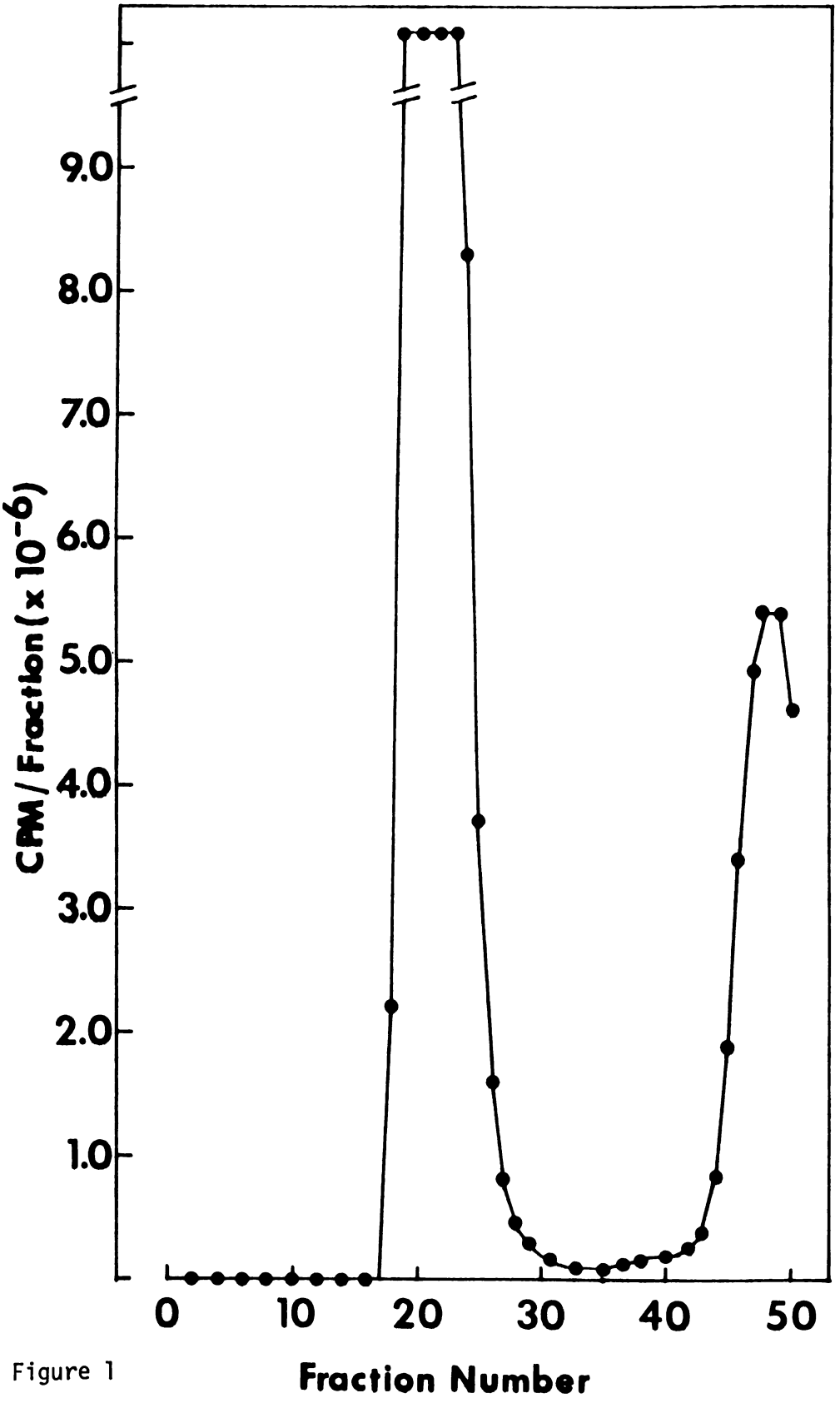

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
CPM/Fraction (x 10⁻⁶)
0
10
20
30
40
50
Fraction Number

Figure 1

11. Hybridization of Rat Liver DNA with ^{32}P-labelled cDNA Probe

The ^{32}P-labelled cDNA probe for the rat albumin gene was hybridized to restriction fragments containing complementary sequences from rat liver DNA in the following manner. The vacuum-dried nitrocellulose filter was wrapped in plastic wrap following wetting with 2 ml of annealing buffer (50% formamide, 10 mM Hepes, pH=7.4, 1 mg/ml yeast transfer RNA, 100 µg/ml denatured salmon sperm DNA, 3XSSC, and 1X Denhardt's buffer). The wrapped blot was sealed in foil and prehybridized at 42°C for 6-24 hours to allow for saturation of non-specific binding sites on the nitrocellulose filter. The ^{32}P-labelled probe was denatured by heating in a boiling water bath for 5 minutes immediately before hybridization. Probe ($10x10^6$ cpm) was added to 2 ml of annealing buffer, and placed on the plastic wrap adjacent to the blot. The blot was placed on the buffer containing probe and wrapped again. Probe was smoothed over the entire blot surface. The wrapped blot was again sealed in foil and hybridized at 42°C for 48 hours.

Following hybridization, the blot was unwrapped and washed in approximately 500 ml of 2XSSC, 0.1% sodium dodeceyl sulfate (SDS) at room temperature, 5-10 minutes, for each of 3 washes. Wash stringency was increased to 0.1XSSC-0.1% SDS and the blot was washed at 55°C for 2 hours with mild agitation, changing the buffer several times during the wash procedures. Removal of non-specifically-associated probe was achieved by this procedure. The blot was then air-dried in preparation for autoradiography.

12. Autoradiography

Restriction fragments containing albumin gene sequences could be visualized following autoradiography of probe-hybridized blots of rat liver parenchymal and nonparenchymal cell DNA. The hybridized blot wrapped in a single layer of plastic wrap, was placed against a sheet of Kodak X-OMat AR film and placed between two intensifying screens. The assembly was placed in an X-ray film holder and wrapped well to prevent light exposure. The assembly was carried out under low yellow light in the dark room, and secured to a clipboard to keep assembly flat. Autoradiography proceeded at -90°C for 24-72 hours. The film was then removed in the dark room and developed for 6 minutes washed in water, and fixed for 6 minutes.

Molecular weight of the restriction fragments containing albumin gene sequences was determined in the following manner. The molecular weight (in kilobases) of the plasmid DNA molecular weight markers was plotted against the distance each marker had migrated as determined from a photograph of the ethidium bromide-stained gel. The points were plotted on semi-log paper, and a curve was drawn through points. From the autoradiograph the migration distance of each band was determined, and the appropriate molecular weight could be determined from the standard molecular weight plot.

13. Other Methods

DNA was determined colorimetrically by the method of Ceriotti (22). RNA was determined by the method of Lin and Schjeide (55). Protein was determined by the method of Lowry et al. (77). Radioactivity was determined by liquid scintillation in a Tricarb Model

460C scintillation counter (Packard Instruments) using NEN 963 scintillation fluor purchased from New England Nuclear (Boston, MA). Dissintegrations per minute were calculated from counts per minute based on a quench curve developed from a series of ^{3}H-water standards.

Purity of DNA samples isolated by the procedure of Marmur (6,49, 80) for agarose gel electrophoresis was determined by the ratio of absorbance at 260 nm to the absorbance at 280 nm (A_{260}/A_{280}). In all cases, this ratio ranged between 1.6-1.9 indicating isolation of highly purified DNA.

RESULTS

1. Hydroxyapatite Column Chromatographic Isolation of DNA Elution by Means of Centrifugal Force

DNA can be selectively isolated from RNA and protein by hydroxyapatite column chromatography (9). Previous studies employed a limited number of columns having long elution times. We have modified these methods by employing centrifugal force other than a peristaltic pump to elute columns containing 1 g hydroxyapatite per column. This modification allows us to elute many columns simultaneously in a much shorter period of time than allowed by previous methods. To be certain that our modifications of our earlier procedure did not alter the purity and yield of DNA, the following experiments were performed.

Known amounts of hepatic chromatin from 6 separate experiments were applied to hydroxyapatite columns. Each 0.48 M sodium phosphate buffer eluate was then assessed for DNA content. As shown in Table 1, a quantitative recovery of DNA was obtained by hydroxyapatite column centrifugation. Table 2 indicates that RNA and protein contamination of the DNA-containing 0.48 M sodium phosphate buffer eluate was less than 1% of the total macromolecular content. Beland *et al.* (9) found only carcinogen adduct peaks that corresponded to those of DNA adduct standards upon HPLC analysis of the 0.48 M sodium phosphate buffer fraction. These results indicate that employing centrifugal force to

TABLE 1

Recovery of DNA Following Hydroxyapatite Column Chromatography

Experiment No.[a]	µg DNA Applied to the Column[b]	µg DNA Recovered[c]
1	202	195
2	334	335
3	350	340
4	472	462
5	532	530
6	819	790

[a]Each experiment represents a separate hepatic chromatin sample subjected to hydroxyapatite chromatography.

[b]Portions of hepatic chromatin were incubated at 37°C for 1 hour to remove RNA, followed by boiling samples in 5% TCA for 15 min to selectively hydrolyze and solubilize DNA. DNA concentration determined by the method of Ceriotti (22).

[c]Remaining portions of hepatic chromatin were applied to hydroxyapatite columns as described in Methods. Following dialysis of the 0.48 M phosphate fraction against 5 mM Bis-Tris (pH=7.1), 0.1 mM EDTA, DNA concentration was determined by the method of Ceriotti (22).

TABLE 2

Assessment of RNA and Protein Contamination of DNA Eluate from Hydroxyapatite Columns[a]

DNA[b] (μg)	RNA[c] (μg)	Protein[d] (ng)
493 ± 58	3 ± 2	23 ± 5

[a]Nuclei from 0.28 g rat liver were dissolved in 8 M urea, 1% SDS, 10 mM EDTA, 0.24 M Na_2PO_4 (pH=6.8) (lysing solution) and applied to hydroxyapatite columns, and the 0.48 M sodium phosphate eluate was analyzed for DNA, RNA and protein content. Results are expressed in terms of mean ± SEM from 6 rats.

[b]As determined by method of Ceriotti (22).

[c]As determined by method of Lin and Schjeide (55).

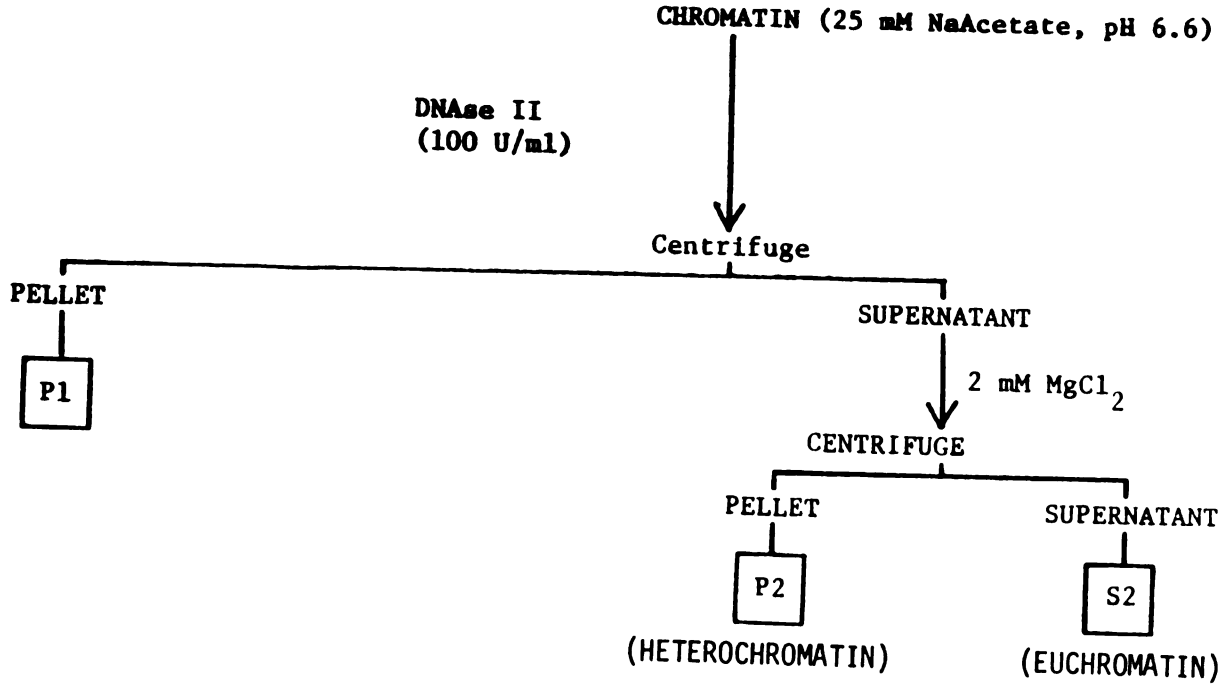
[d]As determined by method of Lowry *et al.* (77).

elute DNA from a hydroxyapatite column results in a quantitative recovery of DNA relatively free of RNA and protein contamination.

To assess elution of carcinogen-modified DNA from hydroxyapatite column, the following experiment was done. Rats were injected i.p. with [ring-^{3}H]-N-OH-AAF (1.8 μmoles/100 g) 2 and 72 hours prior to sacrifice. Chromatin was fractionated by DNase II followed by $MgCl_2$ precipitation into a putative transcriptionally active fraction (S2), a putative transcriptionally inactive fraction (P2) and a nuclease-resistant fraction (P1) as diagrammed in Figure 2. At 2 hours following injection (the time of peak binding of N-OH-AAF to DNA), there was significantly more carcinogen bound to the DNA of the putative transcriptionally active than repressed chromatin. However, 3 days following carcinogen administration there was no difference in the amount of carcinogen bound to DNA of either fraction (Figure 3). These results are in agreement with similar studies employing a different method for DNA isolation, suggesting that the hydroxyapatite method does not alter the ability to determine the extent of carcinogen modification of DNA from various chromatin fractions.

To verify further that hydroxyapatite column centrifugation does not result in loss of adducts from the DNA, samples of DNA containing known amounts of [ring-^{3}H]-N-OH-AFF adducts were applied to hydroxyapatite columns. The results in Table 3 indicate that when radioactivity in each column eluate was determined and summed, a quantitative recovery of radioactivity was obtained. These results indicate that employing centrifugal force to elute simultaneously large numbers of samples at room temperature results in at most minimal degradation of carcinogen-DNA adducts during the isolation procedure.

Figure 2. Fractionation of chromatin by DNase II digestion and selective $MgCl_2$ precipitation. Chromatin (0.5 mg) in 25 mM sodium acetate (pH=6.6) was digested with DNase II (100 units per 500 mg chromatin DNA, and the reaction was terminated in 50 mM Tris (pH=11). Nuclease resistant chromatin (PI) was removed by centrifugation at 9000 x g for 15 min. $MgCl_2$ was added to the supernatant for a final concentration of 2 mM. Heterochromatin (P2) was pelleted (9,000 x g for 15 min) and euchromatin (S2) remained in the supernatant.



Diagramatic representation of chromatin fractionation by DNAse II-$MgCl_2$.

Figure 2

Figure 3. Binding of carcinogen to DNA of rat liver following administration of [ring-^{3}H]-N-hydroxyacetylaminofluorene. Rats were injected i.p. with 1.8 μmoles/100 g [ring-^{3}H]-N-OH-AAF (51.0 mCi/mmole) and sacrificed 2 and 72 hrs after injection. Chromatin was prepared and fractionated from whole rat liver as described above (see legend, Figure 2). Carcinogen binding to DNA of the S2 fraction (putative transcriptionally active regions, ●) and the P2 fraction (putative transcriptionally inactive regions, ○) is expressed in terms of DPM/mg DNA x 10^{2} (8.8×10^{-9} μmoles carcinogen/DPM). Points represent means of 3-6 rats.

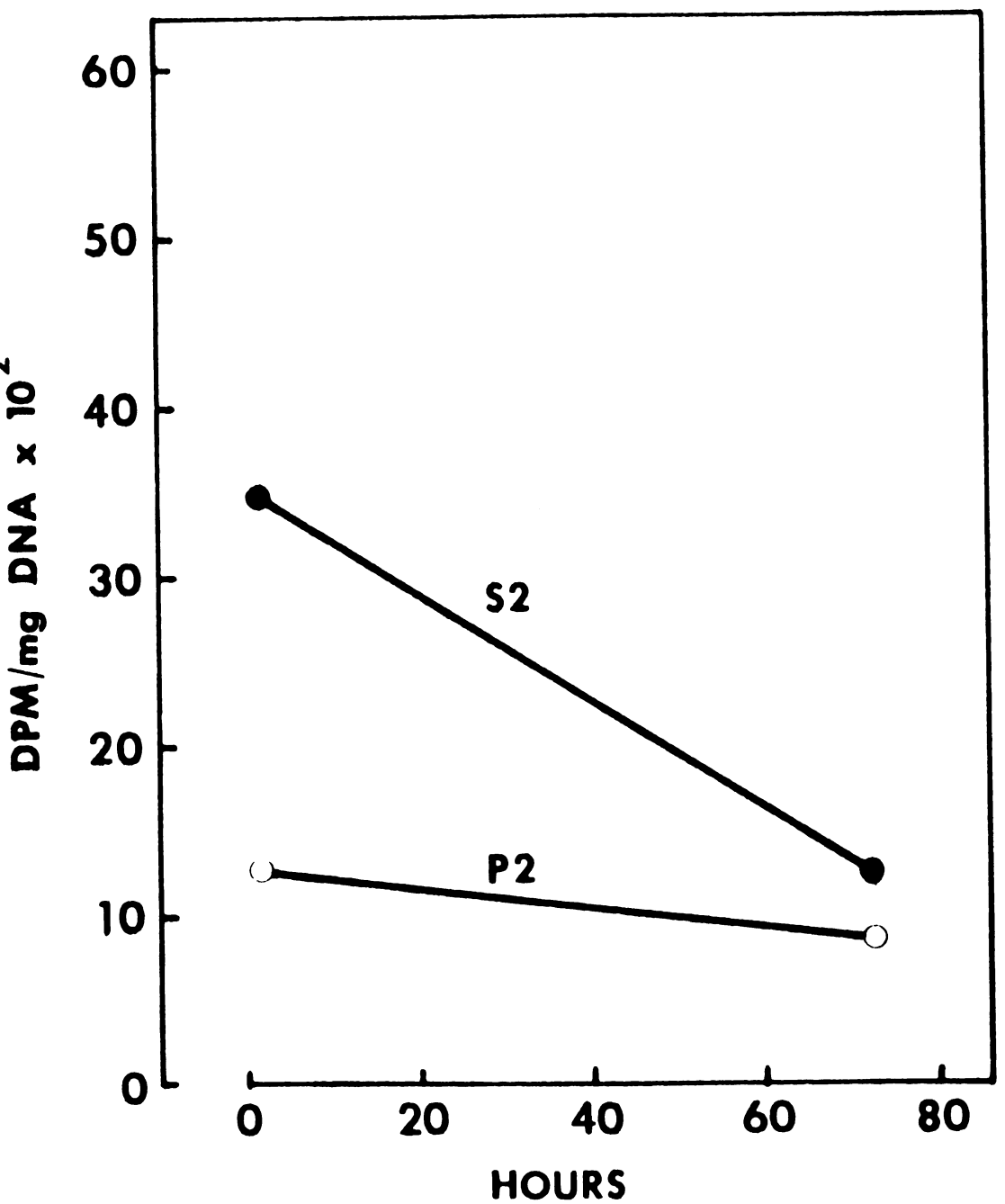


Figure 3

TABLE 3

Recovery of Radioactivity Applied to the Hydroxyapatite Column[a]

Fraction	Radioactivity Recovered	Percent of Total Radioactivity Applied to the Column
RNA and Protein	1220[b]	77%
0.014 M Phosphate Buffer Wash	70	4%
DNA	251	16%
Total	1541	97%

[a]1.5 ml Total chromatin lysate was applied to the hydroxyapatite column (1x2.5 cm); 1.5 ml lysate contained 192 µg DNA and 1,581 dpm.

[b]Total dpm recovered.

2. Carcinogen Binding to Hepatic Macromolecules

Following a single i.p. injection of carcinogen (either AAF or N-OH-AAF), only a very small portion of the injected dose binds to DNA of liver cells (0.004%) as illustrated in Table 4. At 2 hours following injection of [ring-^{3}H]-N-OH-AAF, approximately 1.5% of the injected dose binds to total acid-precipitable hepatic macromolecules, i.e., RNA, DNA, protein, lipid carbohydrate, etc., when a portion of liver homogenate is precipitated and washed once with 5% trichloroacetic acid (TCA). However, only 0.08% of the injected dose binds to acid-insoluble material derived from liver cell nuclei. Three days following injection, approximately 50% of the adducts are lost from total hepatic and hepatic nuclear acid-precipitable material, but 78% of the carcinogen adducts remain in total liver DNA. Table 5 illustrates carcinogen binding to hepatic tissue and hepatic DNA in terms of μmoles carcinogen adduct per g liver. The ratio of carcinogen bound to acid-precipitable hepatic material to that bound to hepatic nuclei DNA greatly decreases 3 days following a single injection of either AAF or N-OH-AAF. From data in the table, it is obvious that this decrease is due to rapid loss of carcinogen from hepatic macromolecules other than DNA. In addition, results from the table indicate that, following injection of equimolar doses of AAF or its N-hydroxy metabolite, more adducts are found in total acid precipitable material following injection with N-OH-AAF, suggesting that, following N-hydroxylation, cells are able to rapidly metabolize carcinogen to a binding form.

TABLE 4

Binding of N-Hydroxy-N-Acetylaminofluorene (N-OH-AAF) to Hepatic Macromolecules[a]

	Time After Injection of N-OH-AAF	
	2 hrs (dpm/g liver)[b]	72 hrs
Binding to Total Acid-insoluble Material[c]	747,606	379,750
Binding to Nuclear Acid-insoluble Material[d]	41,454	20,201
Binding to DNA[e]	2,223	1,751

[a]Male Sprague-Dawley rats weighing 200 g were injected, i.p., with N-OH-AAF 1.8 μmoles, 100 μCi/100 g.

[b]8.18×10^{-9} μmole carcinogen/dpm.

[c]0.5 ml liver homogenate (25% w/v) was precipitated and washed once in 5 ml 5% TCA. The resulting pellet following centrifugation at 900 x g was solubilized in 0.5 ml 88% formic acid.

[d]Nuclei from 0.25 g liver were precipitated and washed once in 5% TCA. The resulting pellet following centrifugation at 900 x g was solubilized in 0.5 ml 88% formic acid.

[e]DNA isolated by hydroxyapatite column centrifugation as described in Methods.

TABLE 5

Binding of Carcinogen to Acid-Precipitable Hepatic Tissue and Hepatic DNA

Carcinogen Treatment[a]	Acid-Precipitable Hepatic Material[b]	Hepatic Nuclei DNA	Ratio
AAF			
18 h	1346±43[c]	16±2[d]	84
90 h	259±34	6±2	43
N-OH-AAF			
2 h	4200±1712	11±5	382
72 h	640±27	6±1	107

[a]Male Sprague-Dawley rats (200 g) were injected i.p. with [ring-^{3}H]-AAF, or [ring-^{3}H]-N-OH-AAF (1.8 μmoles/100 g) and were sacrificed at the time of peak binding (18 and 2 hr for AAF and N-OH-AAF, respectively) and 3 days later.

[b]Liver homogenate (25% w/v) precipitated and washed with 5% trichloroacetic acid.

[c]μmoles adduct/g liver.

[d]μmoles adduct/DNA from 1 g liver.

3. Carcinogen Binding to DNA of Parenchymal and Nonparenchymal Liver Cells

The following experiments were carried out in order to determine if there are differences in the binding of the hepatocarcinogens AAF and its N-hydroxy metabolite to DNA of target (parenchymal) and non-target (nonparenchymal) cells. The method of centrifugal elutriation has been used to separate whole liver cell suspensions into distinct populations of cells (74,139). This procedure was employed to isolate a population of liver parenchymal cells, and a population of non-parenchymal cells consisting primarily of Kupffer and endothelial cells (sinusoidal lining cells).

Results presented in Figure 4 indicate that following a single i.p. injection of [ring-^{3}H]-AAF (1.8 μmoles/100 g), there is significantly more carcinogen bound to the DNA of the parenchymal cells (PC) (Student's t test, $p<0.05$) at 18 hours following injection (time of peak binding). This difference is statistically significant ($p<0.05$) at 3 days following the time of peak binding as well. However, carcinogen adducts appear to be lost from DNA of both cell populations to a similar extent, i.e., 41% of the adducts remained in the DNA of PC, and 36% remained in the DNA of nonparenchymal cells (NPC).

An analogus series of studies were carried out using [ring-^{3}H]-N-OH-AAF in an equimolar dose. This carcinogen is one metabolic step closer to the presumed carcinogenic form (50,85,86). Results illustrated in Figure 5 indicate that there was no significant difference in the amount of carcinogen bound to DNA of PC compared to NPC either

Figure 4. Binding of [ring-^{3}H]-acetylaminofluorene to DNA of hepatic parenchymal (PC) and nonparenchymal (NPC) cells (open and hatched bars, respectively). Following a single i.p. injection of [ring-^{3}H]AAF (1.8 μmoles/100 g), the amount of carcinogen bound per mg DNA was determined at the time of peak binding (18 hr post-injection) and 3 days later. Results are expressed as the mean value from 6 rats. The standard error is represented by a bar. At 18 hr and 90 hr post-injection the amount of carcinogen bound to NPC DNA was significantly different ($p<0.05$) than to that of PC, as determined by Student's t-test.

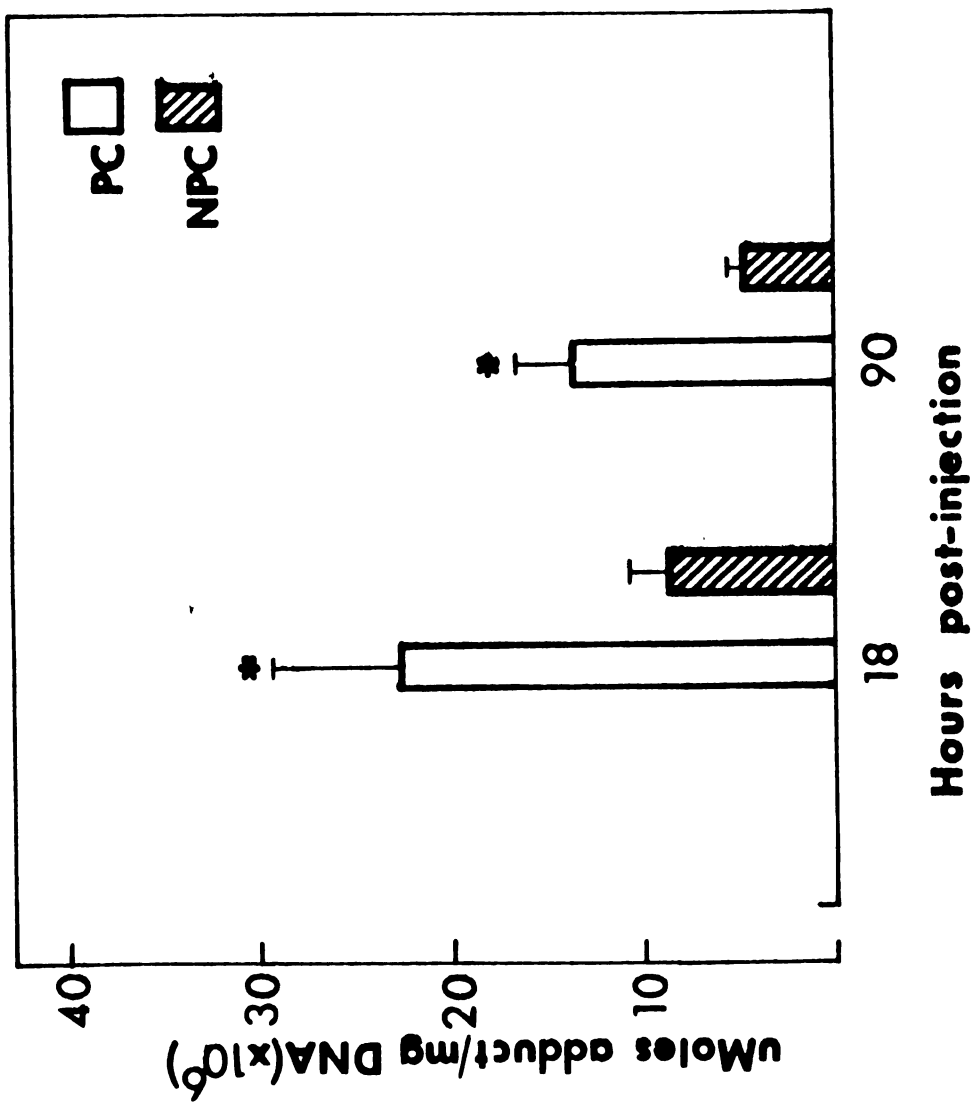

PC
NPC
40
30
20
10
umoles adduct/mg DNA(x10^6)
18
90
Hours post-injection

Figure 4

Figure 5. Binding of [ring-^{3}H]-N-hydroxyacetylaminofluorene to DNA of hepatic parenchymal (PC) and non-parenchymal (NPC) cells (open and hatched bars, respectively). Following a single i.p. injection of [ring-^{3}H]-N-OH-AAF (1.8 μmoles/100 g), the amount of carcinogen bound per mg DNA was determined at the time of peak binding (2 hr post-injection), and 3 days later. Results are expressed as the mean value from 6 rats. Standard error is represented by a bar.

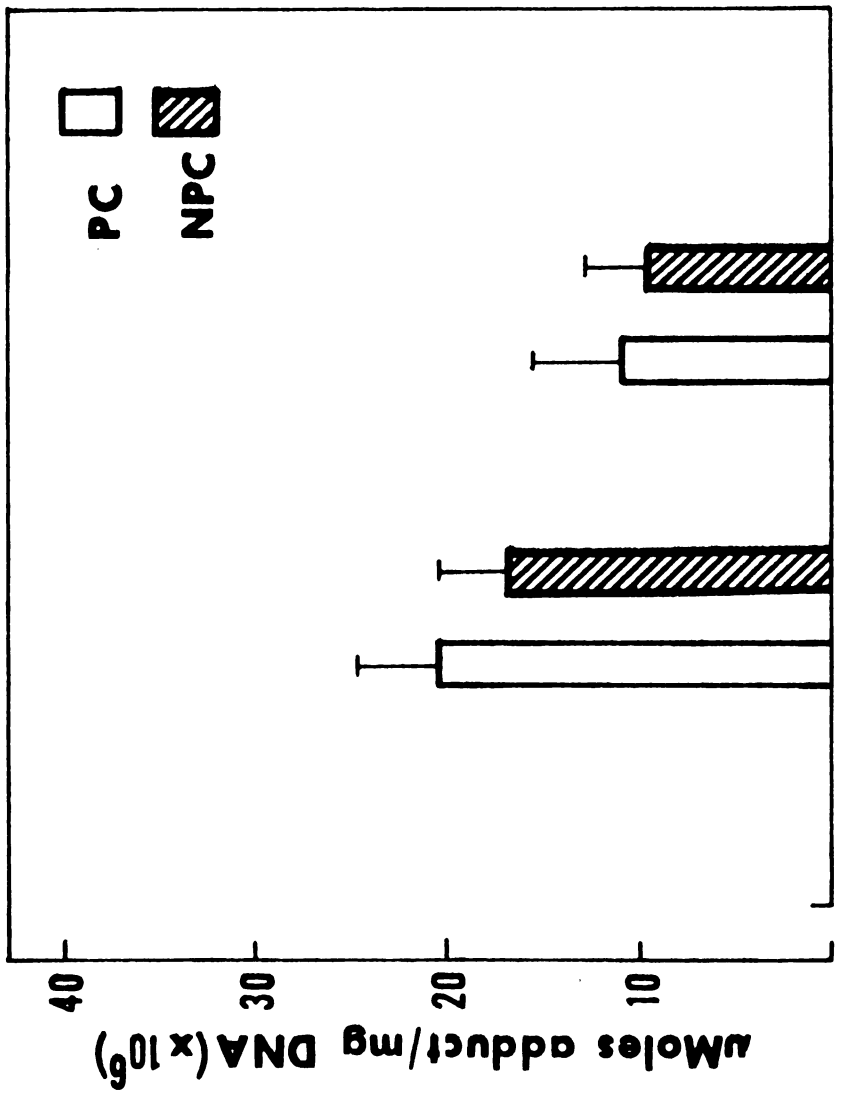

PC
NPC
40
30
20
10
μMoles adduct/mg DNA (x 10⁶)
2
72
Hours post-injection

Figure 5

at the time of peak binding (2 hr for N-OH-AAF), or 3 days later. As with AAF, at 3 days after peak binding, there was a similar extent of loss of adducts from DNA of both cell populations, i.e., 54% of the initially-bound adducts remained in DNA of PC, and 57% remained in the DNA of NPC at 3 days after peak binding.

When these data are adjusted for the total amount of DNA within these cell populations of the whole liver, there is 19 and 24 times the amount of carcinogen bound to the DNA of target cells (PC) compared to DNA of nontarget cells (NPC) as shown in Table 6, at the time of peak binding as well as 3 days later, respectively. In addition, this increased concentration of carcinogen adducts in DNA of PC compared to that of NPC results following N-OH-AAF administration, though not as great as following AAF treatment.

4. Carcinogen Binding to DNA of Nuclei Isolated from Parenchymal and Nonparenchymal Liver Cells

A procedure developed by Bushnell et al. (19) was used to isolate nuclei derived from PC and NPC by sedimentation through a discontinuous sucrose gradient. Nuclei isolated in this manner were used for preparation of highly purified DNA following carcinogen treatment of rats in vivo.

Experiments carried out on the binding of AAF and its N-hydroxy metabolite to DNA of PC and NPC were repeated on isolated DNA of PC and NPC nuclei populations (NI and NII nuclei, respectively) following treatment of rats with 1.8 μmoles/100 g of either carcinogen. Figure 6 shows there was no significant difference in binding of carcinogen to DNA isolated from NI or NII nuclei at 18 or 90 hours following

Table 6

Total Binding of AAF and N-OH-AAF to Liver Cell DNA

Carcinogen/Time	DNA Binding (pmol/total DNA)		Hepatocyte/Sinusoidal Cell Ratio
	Hepatocyte	Sinusoidal Cell	
AAF			
18 hr	230±43	12±4	19.2
90 hr	95±21	4±1	23.8
N-OH-AAF			
2 hr	141±32	17±3	8.3
72 hr	76±31	10±3	7.6

Figure 6. Binding of [ring-^{3}H]acetylaminofluorene to DNA of hepatic parenchymal (NI) and nonparenchymal cell (NII) nuclei (open and hatched bars, respectively). Following a single i.p. injection of [ring-^{3}H]AAF (1.8 μmoles/100 g), NI and NII nuclei populations were isolated from whole liver homogenate (25% w/v) by discontinuous sucrose gradient sedimentation as described in Methods. The amount of carcinogen bound per mg DNA was determined at the time of peak binding (18 hr post-injection), and 3 days later. Results are expressed as the mean value from 6 rats. Standard error is represented by a bar.

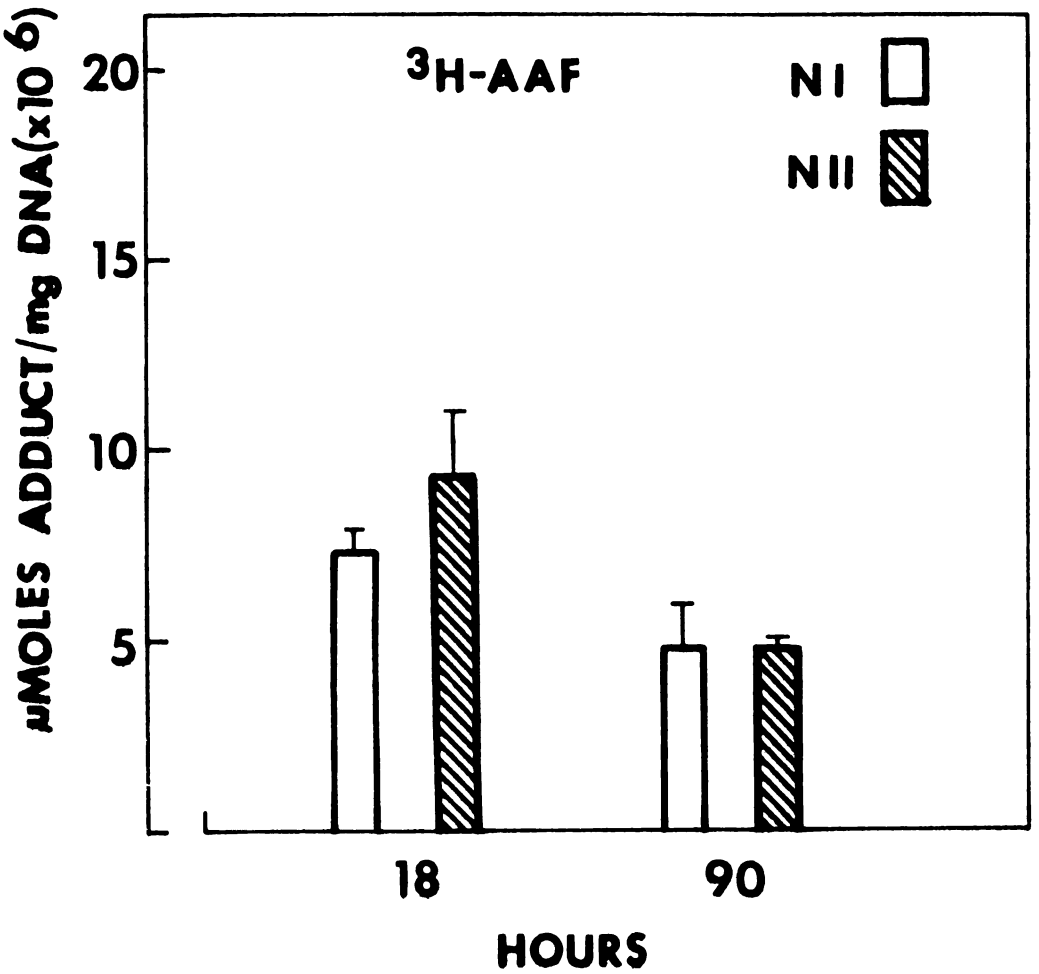


Figure 6

injection of [ring-^{3}H]-AAF. Three days following carcinogen treatment 67% and 52% of the initially bound carcinogen remained bound to DNA of NI and NII nuclei, respectively. Similarly, following an equimolar dose of [ring-^{3}H]-N-OH-AAH, there was no significant difference in binding of carcinogen at the time of peak binding (2 hr) or 3 days following (Figure 7). By 3 days after carcinogen injection, 58% and 41% of the initially bound carcinogen remained in the DNA of PC and NPC nuclei, respectively. As in the previous studies repair appeared to occur to a similar extent in the DNA derived from NI and NII nuclei.

Table 7 illustrates a comparison of data on carcinogen binding to DNA of rat liver cell populations isolated by centrifugal elutriation and to DNA of rat liver cell nuclei populations isolated on a discontinuous sucrose gradient. DNA from centrifugally elutriated PC and from NI nuclei contained approximately the same amount of adduct per mg DNA, indicating that we are dealing with a similar population of cells in both procedures. However, DNA of elutriated NPC contained a significantly lower amount of adduct per mg DNA ($p<0.05$) compared to DNA isolated from NII nuclei. Elutriated hepatic NPC are composed of Kupffer and endothelial cells only, while NII nuclei are derived from all the NPC of the liver, including bile duct cells and fat storing cells. Data on carcinogen concentration in DNA of NPC and NII nuclei cannot be meaningfully compared due to differences in the cell populations from which the DNA is derived by these procedures.

Figure 7. Binding of [ring-^{3}H]-N-hydroxyacetylaminofluorene to DNA of hepatic parenchymal (NI) and nonparenchymal cell (NII) nucleic (open hatched bars, respectively). Following a single i.p. injection of [ring-^{3}H]-N-OH-AAF (1.8 μmoles/100 g), NI and NII nuclei populations were isolated from whole liver homogenate (25% w/v) by discontinuous sucrose gradient sedimentation as described in Methods. The amount of carcinogen bound per mg DNA was determined at the time of peak binding (2 hrs post-injection) and 3 days later. Results are expressed as the mean value from 6 rats. Standard error is represented by a bar.

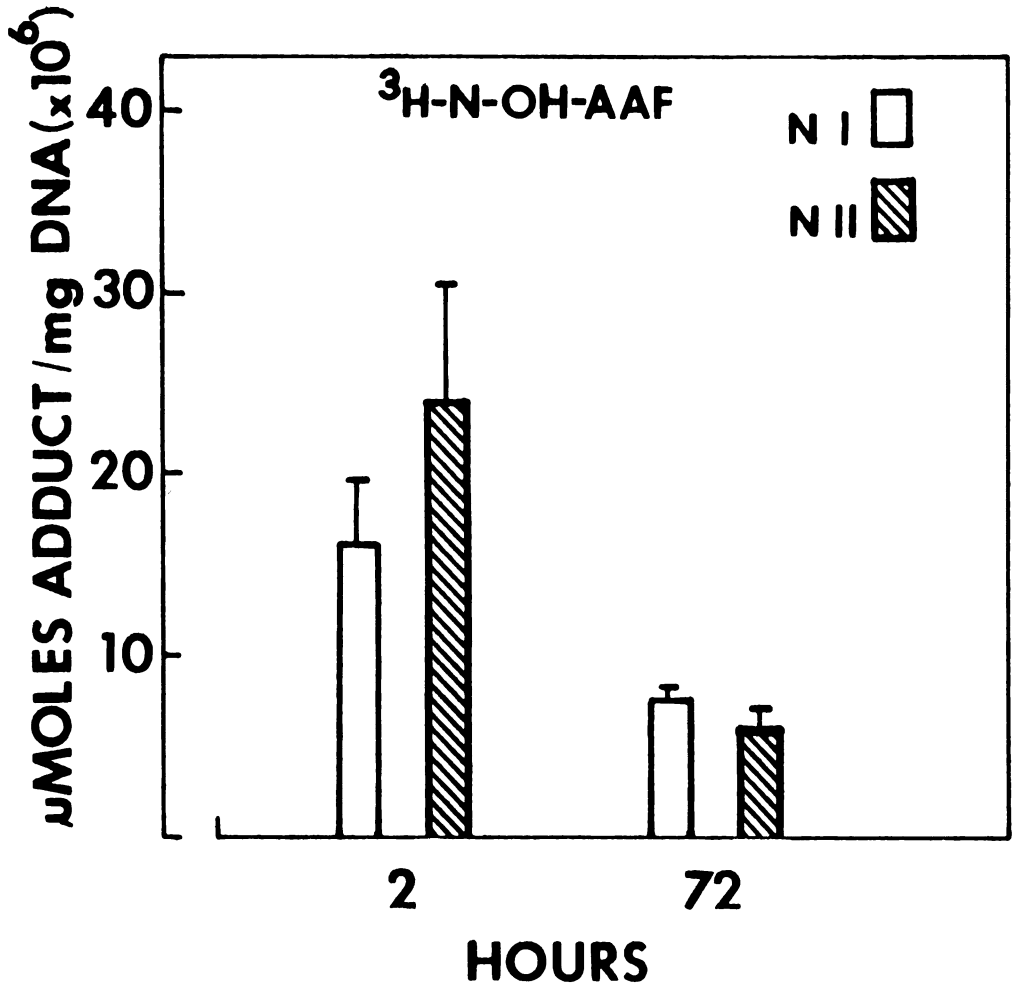

μMOLES ADDUCT/mg DNA(x10^6)
40
30
20
10
3H-N-OH-AAF
N I
N II
2
72
HOURS

Figure 7

TABLE 7

Binding of ^{3}H-N-Hydroxy-Acetylaminofluorene DNA of Rat Liver[a]

	$\frac{\mu\text{Moles Adduct}}{\text{mg DNA}}(\times 10^{5})$
Elutriation Centrifugation[b]	
Hepatocytes	2.01±0.45
Nonparenchymal Cells	1.68±0.33
Discontinuous Sucrose Gradient Centrifugation[c]	
Class NI	1.60±0.36
Class NII	2.37±0.65

[a]DNA isolated from rat liver by hydroxyapatite chromatography 2 hours after a single injection of ^{3}H-N-OH-AAF (100 μCi/1.8 μmole/100 g). Values represent means ± SEM of 3-6 rats.

[b]Livers were perfused with collagenase through the portal vein. The mixed liver cell suspension was washed twice in Hepes buffered saline solution, then slowly injected into a large mixing chamber of the Beckman JE-6 elutriation rotor containing a Sanderson separation chamber in a Beckman J2-21 refrigerated chamber. Elutriation proceeded as described in Methods.

[c]25% (w/v) liver homogenate in 1.5 M sucrose was layered over 2.3 M sucrose and centrifuged at 23,000 rpm (x g) for 60 min in a Beckman SW27 rotor in a Beckman chamber at 5°C.

5. Binding of Carcinogen to DNase-I Accessible and Inaccessible Regions of Chromatin Derived from Parenchymal and Nonparenchymal Cell Nuclei

The endonuclease pancreatic deoxyribonuclease I (DNase I) appears to digest selectively chromatin which is transcriptionally active (42, 140). This endonuclease was used as a tool to enable analysis of carcinogen binding to DNA of transcriptionally active and inactive areas of the genome of target cells (PC) and nontarget cells (NPC).

DNase I has been shown to digest selectively active ovalbumin gene sequences in the DNA of hen oviduct cell nuclei (140), and globin gene sequences in the DNA of chicken erythrocyte nuclei (140). In both cases, when only 10% of the genome was digested with DNase I, 70-75% of the active gene sequences were no longer present. Figure 8 diagrams the time course for digestion of DNA from nuclei obtained from rat whole liver. When DNase I (125 units enzymes/mg nuclear DNA) was incubated with nuclei for up to 60 minutes, not more than 20% of the total nuclear DNA was digested. These results are consistent with those of other investigators (42). By 5-10 minutes, DNase I activity began to plateau (8-10% of the total DNA was digested at these times). For subsequent studies, 5 and 10 minute digestion times were chosen.

Table 8 represents a summary of values for the percent of total parenchymal cell nuclear DNA digested by DNase I from control and AAF or N-OH-AAF treated rats. After treatment with either carcinogen, there was no significant difference from control in the amount of DNA that was nuclease-accessible. The high percentage of nuclease-accessible DNA may be due to a relatively higher degree of transcriptionally

Figure 8. Digestion of rat liver cell nuclei with pancreatic deoxyribonuclease I. Total nuclei were isolated from a 25% (w/v) liver homogenate according to a method of Blobel and Potter, as described in Methods. Nuclei were suspended in 10 mM Tris (pH=7.4), 10 mM NaCl, 3 mM $MgCl_2$ (TNM buffer) in a concentration of approximately 1 mg DNA/ml. Pancreatic DNase I (125 units/mg DNA) was added, and the incubation proceeded at 37°C for appropriate times. The reaction was terminated on ice by the addition of TCA to a final concentration of 10%. Suspensions were centrifuged at 2500 rpm for 5 min, 5°C. The supernatant was removed and boiled for 5 min, followed by centrifugation at 5,000 rpm, 5°C. The 5000 rpm supernatant was analyzed for DNA concentration by the method of Ceriotti (22).

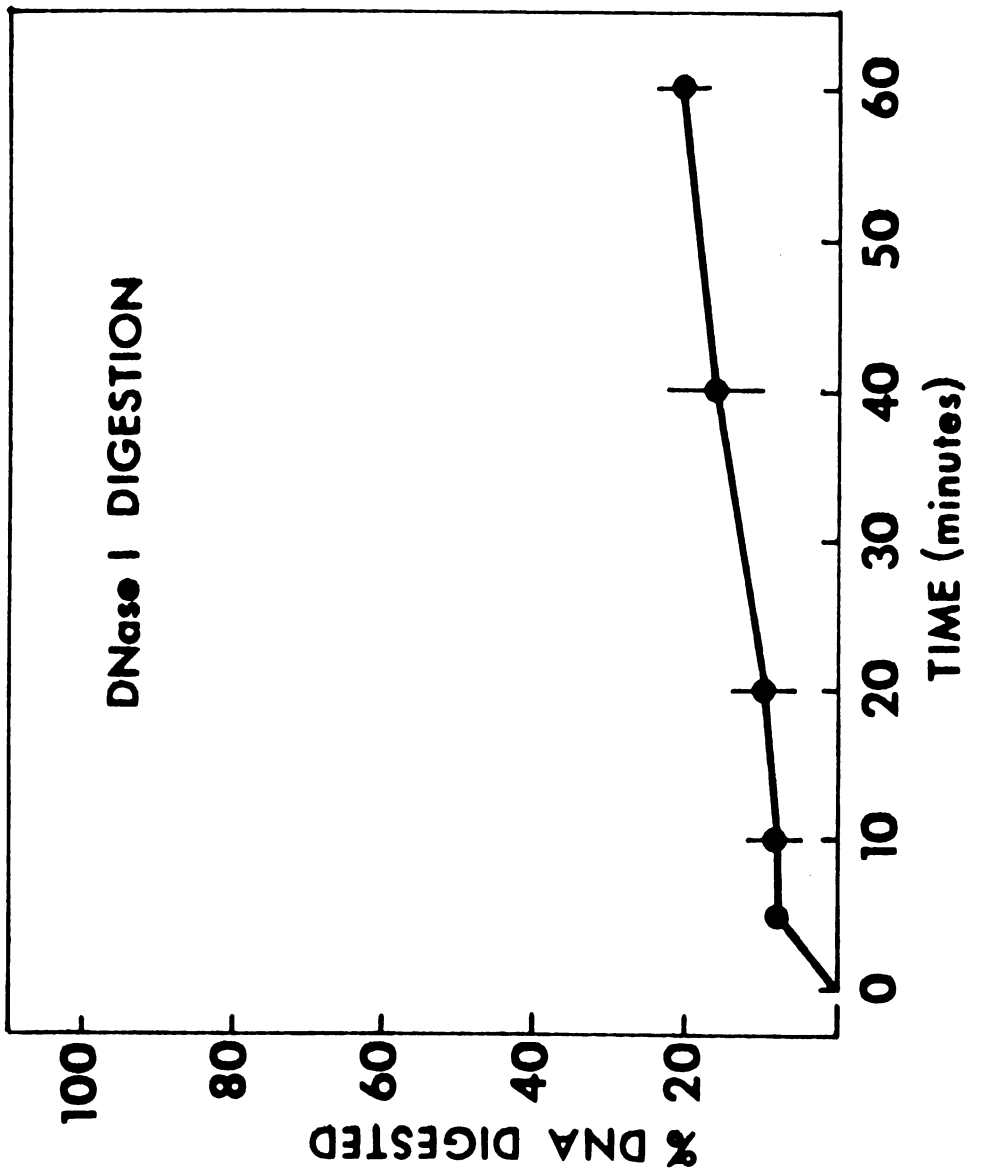

DNase I DIGESTION
% DNA DIGESTED
100
80
60
40
20
0
10
20
30
40
50
60
TIME (minutes)

Figure 8

TABLE 8

DNA Digested from Parenchymal Cell Nuclei by DNase I[a]

	Digestion Time 5 Minutes	10 Minutes
Control	45±20[b]	68±27
^{3}H-AAF[c]		
18 hr	38±11	58±13
90 hr	68±33	81±36
^{3}H-N-OH-AAF[c]		
2 hr	33± 8	64±10
72 hr	56±26	66±14

[a]PC nuclei (Class NI) were incubated with DNase I (125 units/mg DNA) for 5 or 10 minutes at 37°C. Incubation was terminated by the addition of ice-cold TCA, final concentration 10% w/v.

[b]Percent of total PC DNA. Data represent the mean ± S.E. of the results obtained from 4-6 rats.

[c]Male Sprague-Dawley rats were injected i.p. with 1.8 μmoles/100 g ^{3}H-AAF (35.5 mCi/mMole) or ^{3}H-N-OH-AAF (55.6 mCi/mMole).

active DNA in hepatic parenchymal cells as compared to nonparenchymal cells. Alternatively, parenchymal cell nuclei (NI) may be more permeable to DNase I than are NII nuclei.

Similar results for nuclease digestion of DNA from nonparenchymal cells are summarized in Table 9. Following treatments of rats with AAF or N-hydroxy AAF, there was no significant difference in the amount of DNA digested as compared to control except at 5 minutes digestion of DNA from rats 90 hours following treatment with AAF, which was significantly different from control ($p<0.05$).

These results from studies on DNase I digestion of DNA from target (NI) and nontarget (NII) nuclei suggest that the initial presence of adducts in DNA of nontarget cells and subsequent repair of adducts does not influence the ability of DNase I to cleave DNA, i.e., does not enhance or inhibit enzyme activity.

Following treatment of rats with [ring-^{3}H]-AAF or -N-OH-AAF (1.8 μmole/100 g), the DNA from NI and NII chromatin which was nuclease-accessible and nuclease-resistant was analyzed for carcinogen binding. As shown in Figure 9, there is significantly less carcinogen bound per mg DNA to DNase I-accessible DNA compared to that bound per mg DNA of total undigested NI DNA at the time of peak binding as well as 3 days following. This trend is noted following treatment of rats with N-OH-AAF, although this difference is not statistically significant (Figure 9). These results suggest that regions of the target cell DNA that are transcriptionally active (DNase-I sensitive) are not necessarily the same regions to which carcinogen selectively binds.

TABLE 9

DNA Digested from Nonparenchymal Cell Nuclei by DNase I[a]

	Digestion Time	
	5 Minutes	10 Minutes
Control	9±4	27±16
^{3}H-AAF[c]		
18 hr	16±8[b]	19± 7
90 hr	34±6[d]	33± 6
^{3}H-N-OH-AAF[c]		
2 hr	12±3	17± 6
72 hr	9±2	16± 5

[a]NPC nuclei were incubated with DNase I (125 units/mg DNA) for 5 or 10 minutes at 37°C. Incubation was terminated by the addition of ice-cold TCA, final concentration 10% w/v.

[b]Percent of total NPC DNA. Data represent mean ± S.E. of the results obtained from 4-6 rats.

[c]Male Sprague-Dawley rats were injected i.p. with 1.8 μmoles/100 g ^{3}H-AAF (35.5 mCi/mmole) or ^{3}H-N-OH-AAF (55.6 mCi/mmole).

[d]Significantly different from control as determined by Student's t test, $p<0.05$.

Figure 9. Binding of [ring-^{3}H]-acetylaminofluorene and [ring-^{3}H]-N-hydroxyacetylaminofluorene to total DNA and DNase I-digested DNA from parenchymal cell nuclei. Solid bars represent binding to DNase I-digested DNA at the time of peak binding (18 or 2 hr after ^{3}H-AAF or ^{3}H-N-OH-AAF treatments, respectively) and 3 days later. Open bars represent binding to total DNA at the indicated time periods. The data shown represent the mean ± SEM of the values obtained from 3-6 rats. The standard error for the binding to DNase I-digested DNA overlapped that of the binding to total DNA for the ^{3}H-N-OH-AAF treatment group at 3 days after peak binding. *Significant at $p<0.05$ as determined by Student's t-test.

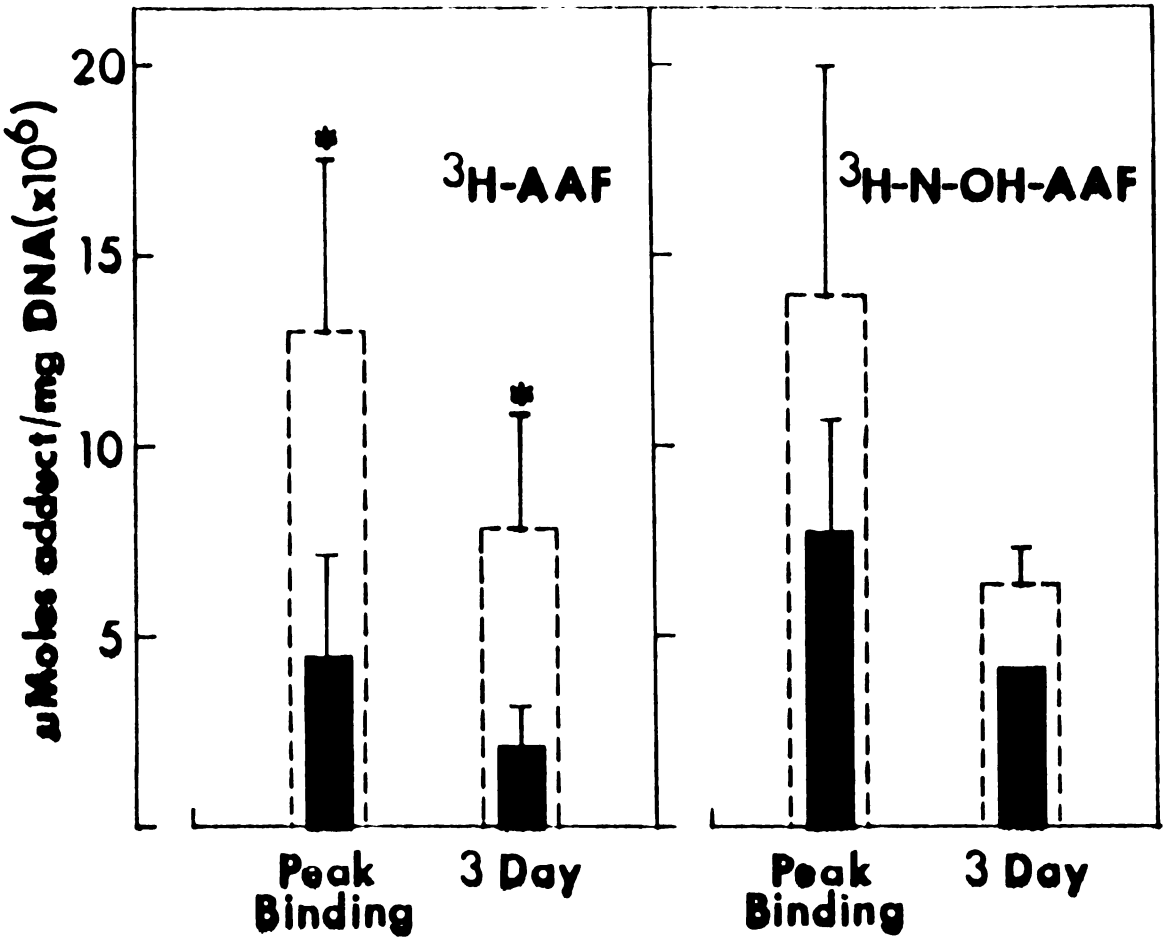

20
15
10
5
μMoles adduct/mg DNA(x10⁶)
³H-AAF
³H-N-OH-AAF
Peak Binding
3 Day
Peak Binding
3 Day

Figure 9

Binding of carcinogen to DNase I-accessible regions of nontarget cell NII DNA compared to that of total, undigested NII DNA is represented in Figure 10. At the time of peak binding for AAF or its N-hydroxy metabolite, there is a greater quantity of carcinogen adducts per mg nuclease-accessible DNA as compared to the amount bound per mg undigested DNA. However, 3 days following AAF treatment, no detectable carcinogen remained bound to DNase I-accessible regions of DNA. Approximately 42% of the initially bound carcinogen remained in total, undigested NII DNA 3 days following AAF treatment. Three days following treatment of rats with N-OH-AAF, carcinogen bound to DNase I-accessible regions of NII DNA was detectable (13% of that initially bound). Approximately 28% of the initial carcinogen adducts remained in the DNA of total, undigested NPC genome. Thus, transcriptionally active (DNase I-accessible) areas of nontarget cell (NII) DNA are highly sensitive to carcinogen attack initially, however, damage within these regions is rapidly repaired.

Data from studies on AAF binding to DNase I-accessible and inaccessible regions of DNA from NI and NII nuclei populations are compiled in Table 10. Carcinogen adducts accumulate in nuclease-resistant regions of target cell nuclei (NI) DNA at the time of peak binding following AAF treatment, as well as at 3 days following. However, AAF preferentially attacked nuclease-accessible regions of nontarget cell nuclei (NII) cell DNA at the time of peak binding. The carcinogen adducts in these nuclease-accessible regions were rapidly repaired, while carcinogen adducts remained in DNA of DNase I-inaccessible regions of DNA from NII nuclei.

Figure 10. Binding of [ring-^{3}H]-acetylaminofluorene and [ring-^{3}H]-N-hydroxyacetylaminofluorene to total DNA and DNase I-accessible DNA from nonparenchymal cell nuclei. Solid bars represent binding to DNase I-accessible DNA at the time of peak binding (18 or 2 hr after ^{3}H-AAF or ^{3}H-N-OH-AAF treatments, respectively) and 3 days later. Open bars represent binding to total DNA at the indicated time periods. The data shown represent the mean ± S.E. of the values obtained from 3-6 rats.

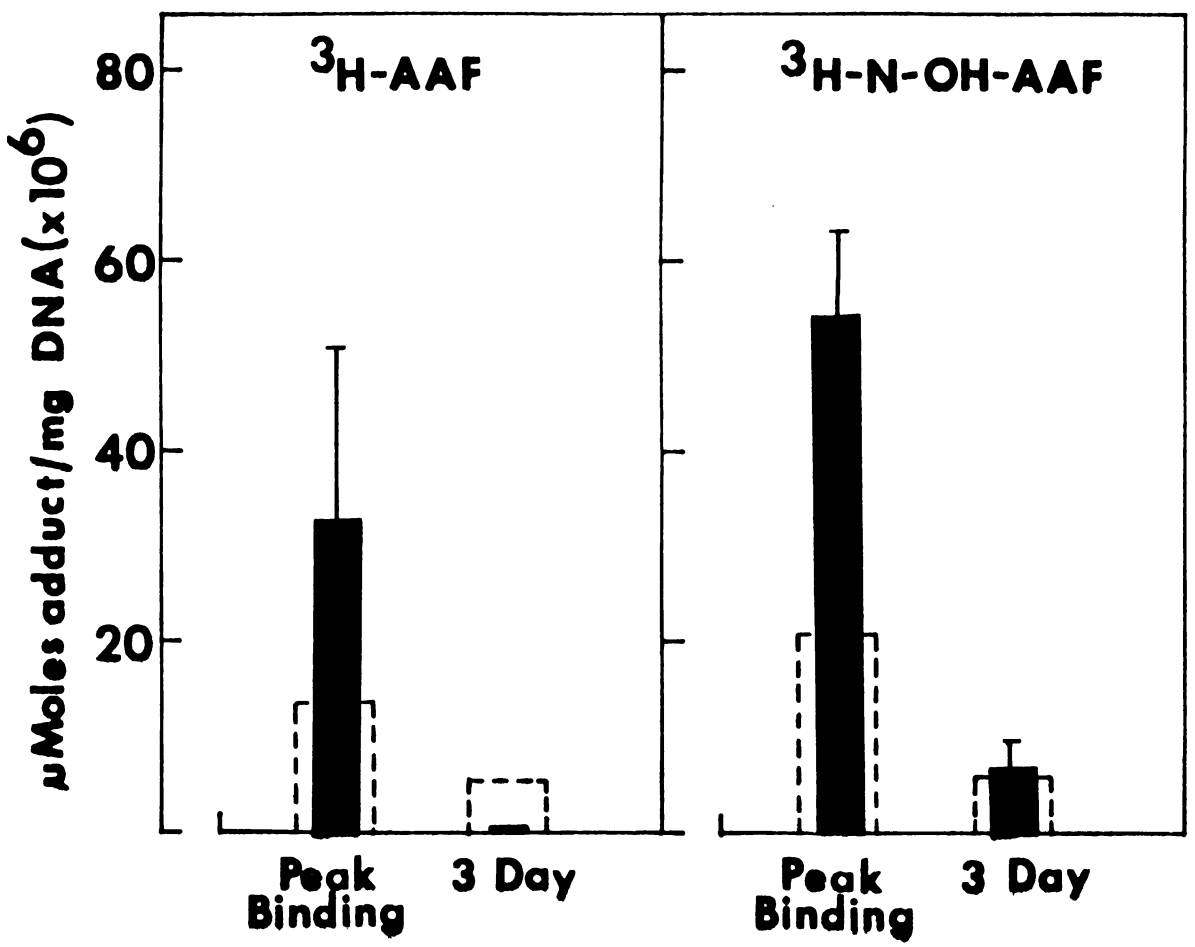

3H-AAF
3H-N-OH-AAF
80
60
40
20
μMoles adduct/mg DNA(x10^6)
Peak Binding
3 Day
Peak Binding
3 Day

Figure 10

TABLE 10

N-2-Acetylaminofluorene Adducts in Specific Regions of Chromatin DNA of Different Hepatic Nuclei Populations

Nuclei Population	Nuclease Accessible[a] (μmoles/mg DNA)	Nuclease Inaccessible (μmoles/mg DNA)
Parenchymal		
18 h[b]	4.53± 2.7[c]	26.24±8.03
90 h	2.05± 1.2	9.81
Nonparenchymal		
18 h	33.70±17.4	5.51±2.77
90 h	0.00	9.24

[a]Nuclei were incubated with DNase I (125 units/mg DNA) for 10 min at 37°C. Nuclease-accessible DNA remained soluble in a supernatant. Nuclease-inaccessible DNA was TCA-insoluble, pelleted at 5000 rpm.

[b]Rats were injected i.p. with 1.8 μmoles [ring-^{3}H]-N-2-acetylaminofluorene/100 g and sacrificed 18 and 90 hr following injection.

[c]Mean value of 4-6 rats ± SEM.

Similar results following treatment of rats with N-OH-AAF are summarized in Table 11. At 2 and 72 hours after treatment of rats with N-OH-AAF, there was significantly more carcinogen bound to nuclease inaccessible regions of DNA from NI nuclei ($p<0.05$). Carcinogen adducts appeared to concentrate in nuclease accessible areas (the putative transcriptionally active regions) of DNA from NII nuclei at 72 hours following injection, there was a 7.5-fold loss of carcinogen adducts in the nuclease accessible regions of NII nuclei DNA, while there was a 3-fold loss of the adducts in nuclease-inaccessible regions of NII DNA.

The amount of carcinogen bound per mg DNA of nuclease-accessible and inaccessible regions of NI and NII nuclear DNA is higher in every case following N-OH-AAF treatment in comparison to AAF treatment (Tables 10 and 11). N-OH-AAF it is one metabolic step closer to the ultimate reactive species (85,86).

To determine whether pretreatment of rats with daily doses of AAF alters the pattern of binding for AAF to DNase I-accessible and -inaccessible regions of DNA derived from NI and NII nuclei populations studies were done in which rats were given daily i.p. injections of AAF in corn oil:DMSO (15 mg/0.75 ml/100 g) for 1, 3 or 5 days. At 24 hours following the last dose of AAF, a tracer dose of [ring-^{3}H]-AAF (91.8 μCi/1.8 μmole/100 g) was given 18 hours (time of peak binding) prior to sacrifice. Results presented in Table 12 indicate that rats treated for 3 and 5 days with AAF contained significantly fewer carcinogen residues bound to hepatic macromoles ($p<0.05$), suggesting that

TABLE 11

N-Hydroxy-Acetylaminofluorene Adducts in Specific Regions of Chromatin DNA of Different Hepatic Nuclei Populations

Nuclei Population	Nuclease Accessible[a] (μmoles/mg DNA)	Nuclease Inaccessible (μmoles/mg DNA)
Parenchymal		
2 h[b]	7.16±2.5[c]	22.14±12.8[d]
72 h	4.91±1.2	13.89± 4.0[d]
Nonparenchymal		
2 h	53.91±8.9	21.19
72 h	7.15±3.29	6.85± 2.1

[a]Nuclei were incubated with DNase I (125 units/mg DNA) for 10 min at 37°C. Nuclease-accessible DNA remained soluble in a 5000 rpm supernatant. Nuclease-inaccessible DNA was TCA-insoluble, pelleted at 5000 rpm.

[b]Rats were injected i.p. with 1.8 μmoles [ring-^{3}H]-N-hydroxy-acetylaminofluorene/100 g and sacrificed 18 and 90 hr following injection.

[c]Mean value of 4-6 rats ± SEM.

[d]Significantly different from nuclease accessible, $p<0.05$ as determined by Student's t test for 2 means.

TABLE 12

Analysis of Carcinogen Binding to Hepatic Macromolecules Following Pretreatment with AAF or Vehicle-Control[a]

Days Pretreatment	Treated[b] (μmoles adduct/mg DNA)	Control[c]
3	869±124[d]	3,993±808
5	1,008± 81[d]	3,945±444

[a]Male Sprague-Dawley rats were injected i.p. with a tracer dose (91.86 μCi/1.8 μmole/100 g) [ring-^{3}H]-AAF following 3 or 5 days pretreatment with AAF or vehicle. Portions of 25% w/v liver homogenate (0.5 ml) were precipitated with 5% TCA and washed once, followed by solubilization of pellet in 88% formic acid.

[b]Rats were injected i.p. with 15 mg/0.75 ml injection solution/100 g AAF in corn oil:DMSO (6:1, v/v) daily for 3 or 5 days.

[c]Rats were injected i.p. with 0.75 ml corn oil: DMSO (6:1, v/v)/100 g daily for 3 or 5 days.

[d]Significantly different from control, $p<0.05$, as determined by Student's t test.

pretreatment with AAF results in a decreased ability to metabolically activate [ring-^{3}H]-AAF. This observation has been noted previously (114).

Carcinogen binding to DNase I-accessible regions of DNA from PC and NPC nuclei following carcinogen pretreatment is summarized in Table 13. Following 1, 3 or 5 days pretreatment with AAF (15 mg/100 g), there was no difference from vehicle-injected rats in carcinogen binding to DNase I-accessible regions of DNA from NI or NII nuclei. These results suggest that the presence of carcinogen adducts in DNA of PC or NPC nuclei did not inhibit or enhance the ability of DNase I to recognize and/or cleave at its target sites within DNA.

6. Nick Translation of Transcriptionally Active DNA in Intact PC and NPC Nuclei

DNA of PC and NPC nuclei was nick-translated to determine the degree of transcriptional activity within the nuclei of the 2 liver cell populations. NI and NII nuclei were isolated from untreated rats. DNase I (Sigma Chemical Co.) was used to nick DNA selectively in transcriptionally active regions of DNA. These areas were then filled in with E. coli polymerase I and ^{32}P-labelled deoxyribonucleotide triphosphates. Following agarose gel electrophoresis of equal amounts of DNA derived from NI and NII nuclei digested by the restriction endonuclease, Eco RI, autoradiography after 24 hours reveals a great deal more transcriptional activity in DNA of PC nuclei compared to equal amounts of DNA from NPC nuclei (Figure 11). After only 4 hours autoradiography, labelled transcriptionally active areas of only the NI nuclear DNA were detectable (Figure 12), providing further

TABLE 13

Analysis of Carcinogen Binding to DNase I Accessible DNA of Rat Parenchymal and Nonparenchymal Liver Cell Nuclei Following Pretreatment with AAF or Vehicle-Control[a]

Days Pretreatment[b]	Parenchymal Cell Nuclei (NI)	Nonparenchymal Cell Nuclei (NII)
1	21.1±8.7[d]	22.5± 12.5
Control[c]	19.0±0.9	36.0
3	12.8±4.2	22.8± 5.4
Control	12.7±1.0	26.2± 13.8
5	18.1±7.5	133.9± 27.8
Control	13.8±0.6	267.2±126.9

[a]Male Sprague-Dawley rats were injected i.p. with a tracer dose (91.8 μCi/1.8 μmole/100 g) [ring-^{3}H]-AAF following 3 or 5 days pretreatment with AAF or vehicle. Nuclease-accessible DNA remained soluble in a 5000 rpm supernatant following termination of DNase I digestion at NI and NII nuclei with TCA.

[b]Rats were injected i.p. with 15 mg/0.75 ml injection solution/100 g AAF in corn oil:DMSO (6:1, v/v) daily for 1, 3 or 5 days.

[c]Rats were injected i.p. with 0.75 ml corn oil:DMSO (6:1, v/v)/100 g daily for 1, 3 or 5 days.

[d]moles carcinogen adduct x 10^{-6}/mg DNA. Data represent mean ± SEM of 3 rats.

Figure 11. Incorporation of ^{32}P-labelled deoxyribonucleotide triphosphates into DNase I-accessible regions of DNA from hepatic parenchymal cell and nonparenchymal cell nuclei. Parenchymal cell nuclei (NI) and nonparenchymal cell nuclei. Parenchymal cell nuclei (NI) and nonparenchymal cell nuclei (NII) were isolated as described in Methods. Nuclei were suspended in 50 mM Tris (pH=7.9), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 µg/ml BSA, in a concentration of lung nuclei DNA/ml. DNA was nicked by DNase I (0.65 units/ml), and nucleotide triphosphates (26 pmoles of ^{32}P-labelled nucleotide triphosphates) were incorporated by E. coli DNA polymerase I (10 units/ml) as described in Methods. Purified DNA was digested by Eco R1 (1 unit/µg DNA) and equal amounts (50 and 100 µg) of NI and NII DNA were run on a 0.9% agarose gel. Molecular weight of ^{32}P-labelled fragments ranged from less than 2.2 kilobases to greater than 17.5 kilobases. The dried gel was autoradiographed for 24 hr.

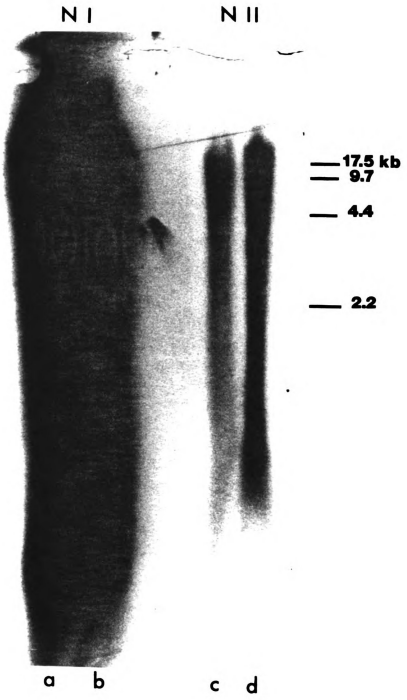


Figure 11

Figure 12. Incorporation of ^{32}P-labelled deoxyribonucleotide triphosphates into DNase I-accessible regions of DNA from hepatic parenchymal cell and nonparenchymal cell nuclei. ^{32}P-labelled DNA prepared from parenchymal cell and nonparenchymal cell nuclei as described above (see legend, Figure 11). The dried agarose gel was autoradiographed for 4 hours.

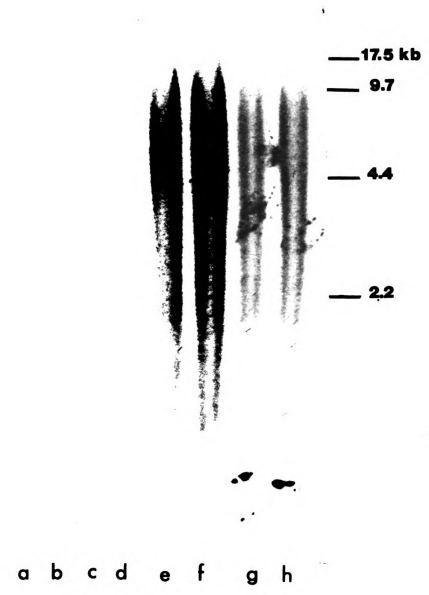


Figure 12

evidence that liver parenchymal cells, the functional cells of the liver, contain a much greater amount of transcriptionally active DNA than do nonparenchymal cells.

7. Effect of Continued AAF Treatment on Restriction Endonuclease Digestion of DNA

The following experiments were performed to determine whether or not restriction endonucleases, enzymes that recognize specific base sequences could recognize and/or cleave DNA that has been modified to various degrees by carcinogen treatment of rats in vivo. The specific endonucleases used to cleave DNA of rat liver were:

1) Eco Rl which cleaves the following base sequence at the point indicated by the arrow: G↓AATTC and

2) Kpn 1 which cleaves the following base sequence at the point indicated by the arrow: GGTAC↓C.

The digestion of DNA by Eco Rl from liver of an untreated rat is illustrated in Figure 13. Following digestion of DNA from rat liver by Eco Rl, fractionated DNA was electrophoresed on an agarose gel to separate restriction fragments on the basis of molecular weight. Lanes a and b of Figure 13 represent the ethidium bromide stained agarose gel. Eco Rl digests rat genomic DNA (b) into a multitude of fragments ranging from less than 2.2 kilobases (Kb) to greater than 17.5 Kb (determined by standard molecular weight markers of Lane a). Eco Rl restriction fragments of rat liver DNA containing albumin gene sequences is illustrated in Lane c of Figure 13. Following Southern transfer of DNA from agarose gels to nitrocellulose filters, ^{32}P-labelled cDNA for the rat albumin gene hybridizes to homologous

Figure 13. Digestion of liver DNA of untreated rats by Eco R1 DNA from liver of untreated rats was purified free of protein and RNA (see Methods). DNA was digested by Eco R1 (0.15 units/10 μg DNA) for 2 hours at 37°C. Digested DNA (40 μg) was electrophoresed in a 0.9% agarose gel, then photographed following staining in ethidium bromide (0.5 μg/ml) to determine the migration distance (cm) of molecular weight standards indicated in Lane a (17.5 Kb, 9.7 Kb, 4.4 Kb, 2.2 Kb) and to observe complete digestion of rat liver DNA (Lane b). Following transfer of DNA from agarose to a nitrocellulose filter, a nick-translated ^{32}P-labelled cDNA probe for the rat albumin gene sequence (pRSA13, gift of Dr. James Bonner) was hybridized to Eco R1 restriction fragments containing homologous sequences (Lane c). The migration distance for each labelled fragment was plotted on the standard curve to determine molecular weights of bands A through E, indicated in kilobases (Kb). Hybridized blots were autoradiographed 3 days at -90°C.

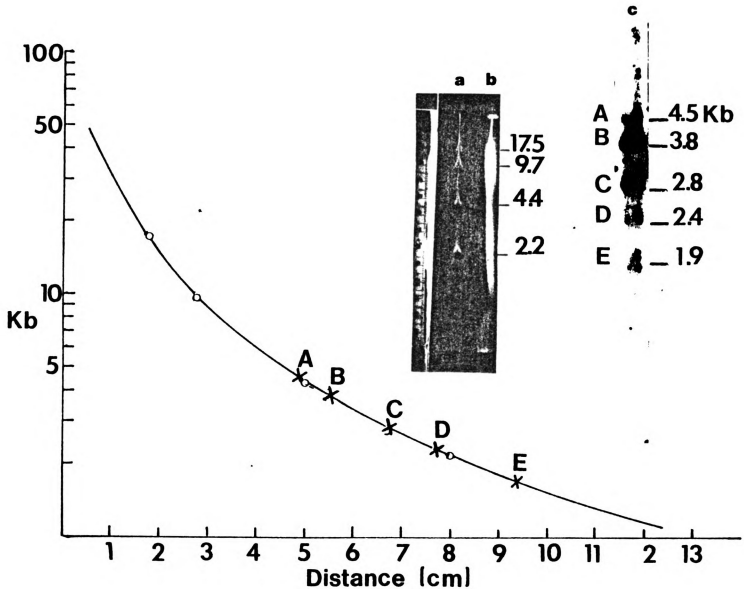

100
50
10
5
Kb
1
2
3
4
5
6
7
8
9
10
11
2
13
Distance (cm)
A
B
C
D
E
a
b
17.5
9.7
4.4
2.2
c
A
B
C
D
E
4.5 Kb
3.8
2.8
2.4
1.9

Figure 13

sequences in restriction fragments of rat genomic DNA covalently bound to the nitrocellulose filter. The distance from the origin to which these fragments have migrated is compared with molecular weight standards, and the molecular weights of albumin gene-containing restriction fragments can be determined on the ordinate. Albumin gene-containing Eco Rl restriction fragments have molecular weights of 1.7, 2.3, 2.8 and 3.8 kilobases (Figure 13).

Digestion of DNA from untreated rat liver by the restriction endonuclease Kpn 1 is represented in Figure 14. Lanes a and b of the agarose gel stained in ethidium bromide represent standard molecular weight markers, the same as those employed for the studies presented in Figure 13, and rat genomic DNA digested by Kpn 1, respectively. Hybridization of ^{32}P-labelled rat albumin gene cDNA to Kpn 1 restriction fragments of rat liver DNA is represented in Lane c of Figure 14, illustrating that Kpn 1 produces albumin gene-containing fragments of DNA having molecular weights of 2.3, 2.9, 3.9, 4.7, 9.7 and 16.0 Kb.

Rats were given daily i.p. injections of 15 mg AAF/100 g or 0.75 ml corn oil:DMSO (6:1, v/v) for 1, 3, 5 or 7 days. The effect of AAF treatment on liver to body weight ratios is illustrated in Table 14. The toxic effect of the carcinogen on rats is noted as early as after 3 days AAF treatment, a time at which the liver/body weight ratio of treated rats is significantly higher than control rats ($p<0.05$). The increasing liver/body weight ratio with progressive AAF treatment is due to a decreasing body weight occurring simultaneously with an increasing liver weight. No change is seen in vehicle-injected rats (Table 14).

Figure 14. Digestion of liver DNA of untreated rats by Kpn 1. DNA from liver of untreated rats was purified free of protein and RNA (see Methods). DNA (40 μg) was digested by Kpn 1 (8 units/1 μg DNA) for 2 hours at 37°C. Digested DNA was electrophoresed in a 0.9% agarose gel, then photographed following staining in ethidium bromide (0.5 μg/ml) to determine the migration distance of molecular weight standards indicated in Lane a (see Figure 13 legend for molecular weights). Complete digestion of rat liver DNA is presented in Lane b. DNA was transferred from agarose to a nitrocellulose filter, then hybridized with a ^{32}P-labelled cDNA probe for rat ablumin gene (pRSA13) (Lane 3). The molecular weights of Kpn 1 restriction fragments containing albumin gene sequences were determined on the molecular weight standard curve (A-F), indicated in kilobases (Kb).

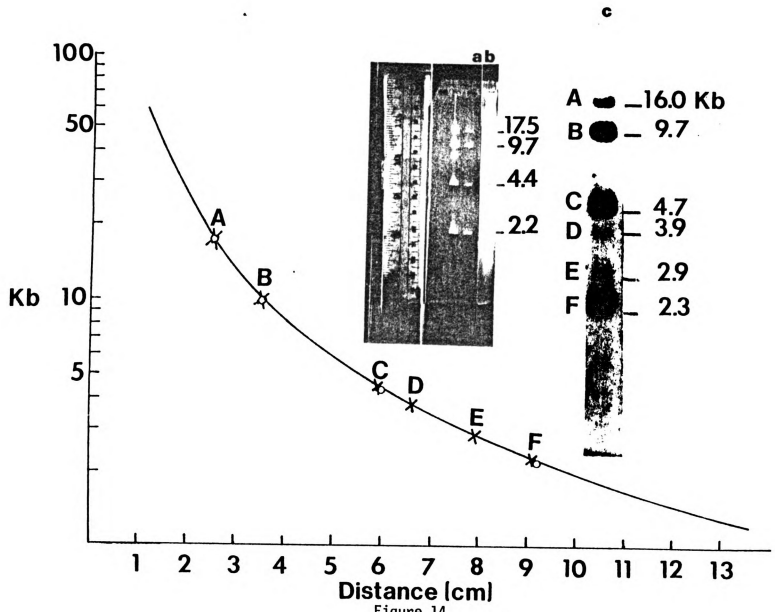

100
50
Kb 10
5
A
B
C
D
E
F
1 2 3 4 5 6 7 8 9 10 11 12 13
Distance (cm)
ab
17.5
9.7
4.4
2.2
c
A 16.0 Kb
B 9.7
C 4.7
D 3.9
E 2.9
F 2.3

Figure 14

TABLE 14

Effect of Daily N-2-Acetylaminofluorene Treatment on Liver/Body Weight Ratios[a]

Day	Treated[b]	Control[c]
1	0.044±0.003	0.046±0.003
3	0.059±0.010[d]	0.047±0.004
5	0.056±0.006[d]	0.042±0.006
7	0.058±0.002[d]	0.047±0.001

[a]Determined as liver weight/body weight on day of sacrifice. Data represent mean ± SEM of 3-6 rats.

[b]Male, Sprague-Dawley rats were injected i.p. with 15 mg/100 g 2-acetylaminofluorene in corn oil:DMSO (6:1, v/v) for 1, 3, 5 or 7 days.

[c]Male, Sprague-Dawley rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) for 1, 3, 5 or 7 days.

[d]Significantly different from control as determined by Student's t test, $p<0.05$.

Following treatment of rats for 1, 3, 5 or 7 days, DNA was isolated, electrophoresed in agarose gels, transferred to nitrocellulose and hybridized with ^{32}P-labelled rat albumin gene cDNA. Autoradiographs of albumin gene-containing Eco R1 and Kpn 1 restriction fragments of rat DNA are depicted in Figures 15-18.

Following treatment of rats with AAF for 1 day, there was no difference in the pattern of Eco R1 restriction fragments containing albumin gene sequences in treated vs. control rats in DNA either from target (NI) or nontarget (NII) nuclei (Figure 15, Table 15). One additional albumin gene-containing restriction fragment was observed in DNA of NII nuclei (Figure 15, Lanes c, d, e).

Following 3 days of treatment with AAF, there was no difference from control in the ability for Eco R1 to recognize and cleave at specific sites in DNA of target (NI) or nontarget (NII) nuclei (Figure 16, Table 16). However, 3 albumin gene-containing Kpn I restriction fragments observed in control rat DNA were not present in DNA of NI and NII nuclei from AAF-treated rats. Control DNA from NII nuclei fractionated by Kpn 1 exhibited one additional labelled restriction fragment of 8.0 Kb not observed in DNA of NI nuclei (Figure 16, Table 16).

Similarly, at 5 days of AAF treatment, the distribution of labelled Eco R1 restriction fragments of DNA from NI and NII nuclei was not influenced by AAF treatment (Figure 17, Table 17). However, 2 fragments (9.7 and 2.2 Kb) and 5 fragments (9.3, 4.4, 3.7, 3.0, and 2.25 Kb) were no longer observed in DNA of NI or NII nuclei, respectively, following carcinogen treatment (Table 17).

Figure 15. Eco R1 digestion of liver DNA of rats treated 1 day with N-2-acetylaminofluorene (AAF) or with vehicle. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) and sacrificed 24 hours later. Nuclei of hepatic parenchymal cells (NI) and nonparenchymal cells (NII) were isolated by discontinuous sucrose gradient centrifugation. DNA was purified free of protein and RNA (see Methods). DNA (40 μg) was digested by Eco R1 and electrophoresed, then transferred and hybridized as indicated (see legend to Figure 13). Lanes a, c and d represent DNA from rats treated 1 day with AAF. Lanes b and e represent DNA from vehicle control rats (0.75 ml corn oil:DMSO per 100 g). Sizes of restriction fragments labelled with ^{32}P-labelled cDNA for rat albumin gene are indicated in kilobases.

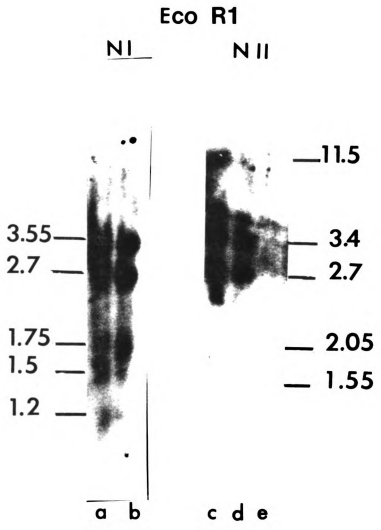

Eco R1
N I
N II
11.5
3.55
3.4
2.7
2.7
1.75
2.05
1.5
1.55
1.2
a b
c d e

TABLE 15

Eco R1 Restriction Endonuclease Fragmentation of DNA[a] from Rats Treated 1 Day with N-2-Acetylaminofluorene[a]

DNA Fragment[b]	NI		NII	
	Treated[c]	Control[d]	Treated	Control
			11.5	11.5
A	3.55[e]	3.55	3.4	3.4
B	2.7	2.7	2.7	2.7
C	1.75	1.75	2.05	2.05
D	1.5	1.5	1.5	1.5
E	1.2	1.2	Not Detected	Not Detected

[a]Purified DNA from rats was digested with Eco R1 (0.15 units/μg DNA) for 2 hours, 37°C.

[b]DNA fragments as noted in Figure 14.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene 24 hours prior to sacrifice.

[d]Rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) 24 hours prior to sacrifice.

[e]Data expressed in terms of kilobases.

Figure 16. Eco R1 and Kpn 1 digestion of liver DNA from rats treated 3 days with N-2-acetylaminofluorene (AAF) or with vehicle. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) for 3 days. Nuclei of hepatic parenchymal cells (NI) and nonparenchymal cells (NII) were isolated, and DNA of each was purified free of RNA and protein (see Methods). DNA was digested by Eco R1 or Kpn 1 and electrophoresed, then transferred and hybridized as indicated (see legends, Figures 13 and 14). Digestion of 40 μg of DNA from NI and NII of treated rats by Eco R1 is represented in Lanes a, b, d and e, and Eco R1 digestion of DNA from vehicle-control rats (0.75 ml corn oil: DMSO, for 3 days) is represented in Lanes c and f. Kpn 1 digestion of DNA from NI and NII of treated rats is represented in Lanes a, c and d. Kpn 1 digestion of control DNA is represented in Lanes b and e. Sizes of restriction fragments labelled with ^{32}P-labelled cDNA for rat albumin gene are indicated in kilobases. Hybridized blots were autoradiographed 3 days at -90°C.

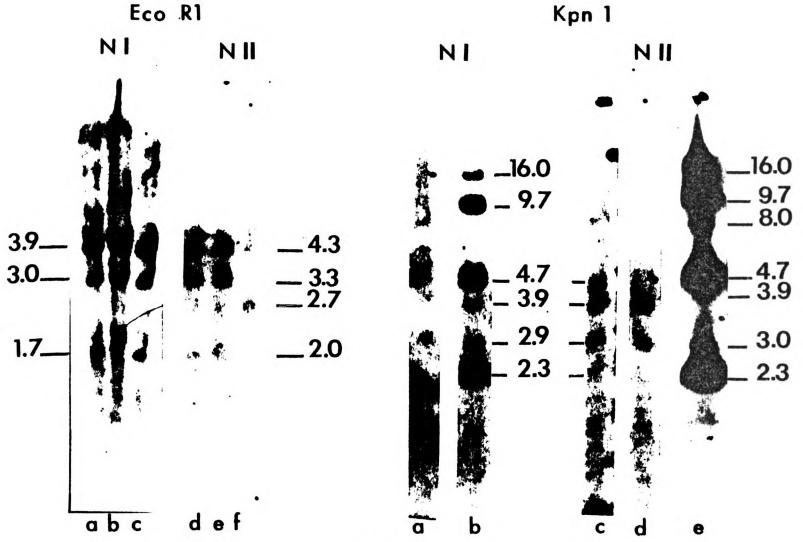

Eco R1
N I
N II
3.9
3.0
1.7
4.3
3.3
2.7
2.0
a b c
d e f
Kpn 1
N I
N II
16.0
9.7
4.7
3.9
2.9
2.3
8.0
a
b
c
d
e

Figure 16

TABLE 16

Eco RI and Kpn I Restriction Endonuclease Fragmentation of DNA from Rats Treated 3 Days with N-2-Acetylaminofluorene

DNA Fragment[b]	NI		NII	
	Treated[c]	Control[d]	Treated	Control
Eco RI				
A	Not detected	Not detected	Not detected	Not detected
B	3.9[e]	3.9	4.3	4.3
C	3.0	3.0	3.3	3.3
D	Not detected	Not detected	2.7	2.7
E	1.7	1.7	2.0	2.0
Kpn I				
A	Not detected	16.0	Not detected	16.0
B	Not detected	9.7	Not detected	9.7
				8.0
C	4.7	4.7	4.7	4.7
D	3.9	3.9	3.9	3.9
E	2.9	2.9	2.9	3.0
F	Not detected	2.3	2.3	2.3

[a]Purified DNA from rats was digested with Eco RI (0.15 units/ 1 μg DNA) or Kpn I (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 3 days prior to sacrifice.

[d]Rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) for 3 days prior to sacrifice.

[e]Data expressed in terms of Kilobases.

Figure 17. Eco R1 and Kpn 1 digestion of liver DNA from rats treated 5 days with N-2-acetylaminofluorene (AAF) or with vehicle. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) or vehicle (0.75 ml/100 g corn oil:DMSO, 6:1 v/v) for 5 days. Nuclei of hepatic parenchymal cells (NI) and nonparenchymal cells (NII) were isolated, and DNA purified (see Methods). DNA was digested by Eco R1 or Kpn 1 and electrophoresed, then transferred and hybridized as indicated (see legends to Figures 13 and 14). Digestion of 40 μg DNA from NI and NII nuclei of treated rat by Eco R1 is represented in Lanes a, b, c and e, f, g. Lanes b and f represent twice the amount of DNA in Lanes a and e, respectively. Lanes d and h represent Eco R1-digested DNA from control rats. Digestion of 40 μg of DNA from NI and NII nuclei from AAF-treated rats by Kpn 1 is represented in Lanes a and c. Lanes b and d represent Kpn 1 digestion of DNA from control rats. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed 3 days at -90°C.

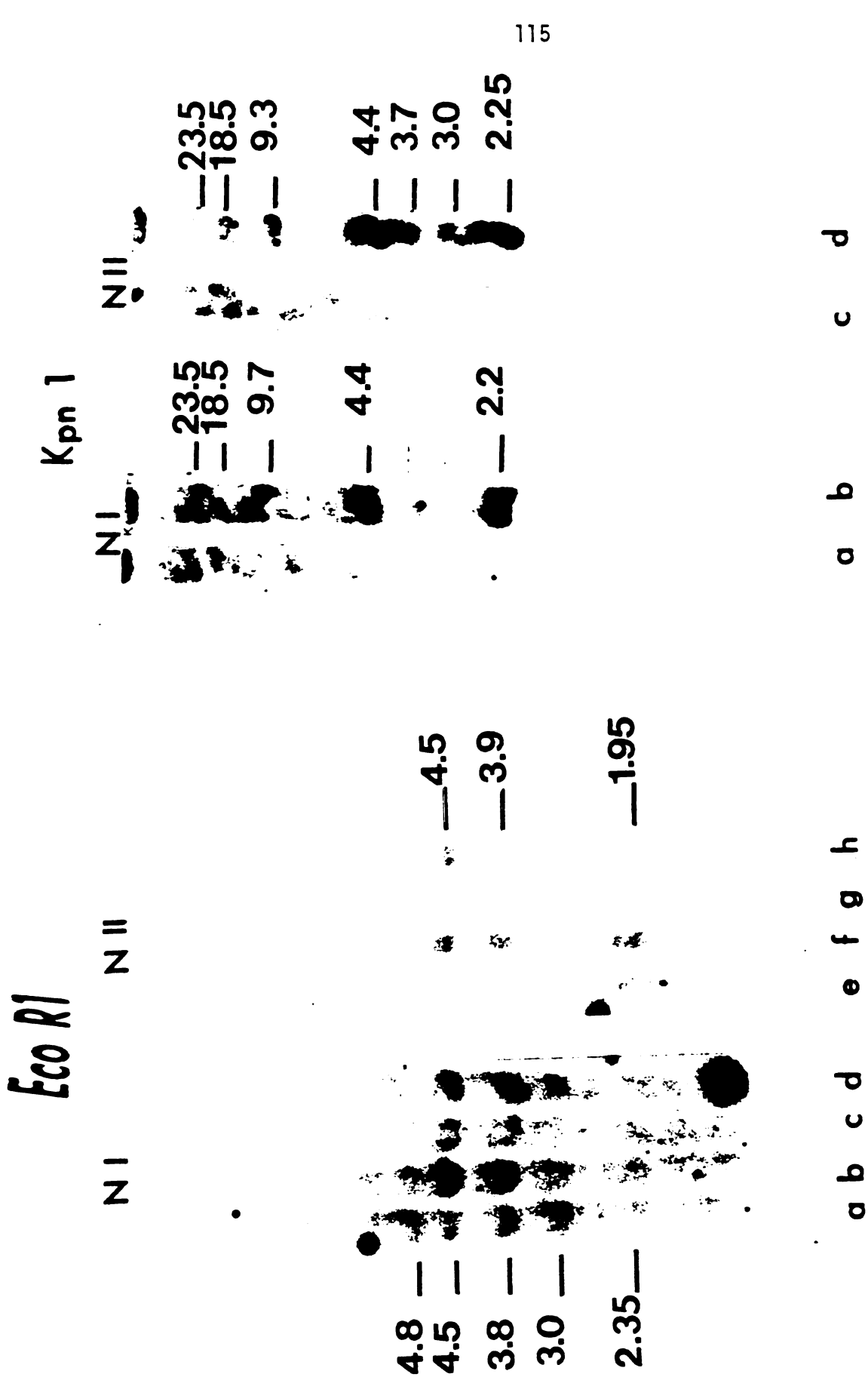

Kpn 1
N I
N II
23.5
18.5
9.7
4.4
2.2
23.5
18.5
9.3
4.4
3.7
3.0
2.25
a b
c d
Eco RI
N I
N II
4.8
4.5
3.8
3.0
2.35
4.5
3.9
1.95
a b c d e f g h

Figure 17

TABLE 17

Eco RI and Kpn I Restriction Endonuclease Fragmentation of DNA from Rats Treated 5 Days with N-2-Acetylaminofluorene

DNA Fragment[b]	NI		NII	
	Treated[c]	Control[d]	Treated	Control
Eco RI				
A	4.8[e]			
A	4.5	4.5	4.5	4.5
B	3.8	3.8	3.9	3.9
C	3.0	3.0	Not detected	Not detected
D	2.35	2.35	---	---
E	Not detected		1.95	1.95
Kpn I				
	23.5	23.5	23.5	23.5
A	18.5	18.5	18.5	18.5
B	Not detected	9.7	Not detected	9.3
C	4.4	4.4	Not detected	4.4
D	Not detected	Not detected	Not detected	3.7
E	Not detected	Not detected	Not detected	3.0
F	Not detected	2.2	Not detected	2.25

[a]Purified DNA from rats was digested with Eco RI (0.15 units/ 1 μg DNA) or Kpn I (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 5 days prior to sacrifice.

[d]Rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) for 5 days prior to sacrifice.

[e]Data expressed in terms of Kilobases.

With up to 7 days of AAF treatment, no effect was observed on the ability of Eco Rl to recognize and/or cleave at its specific sites (Figure 18, Table 18). Thus, carcinogen modification of DNA following progressive AAF treatment resulted in an altered ability of Kpn 1, but not of Eco Rl, to recognize and/or cleave at its specific sites.

When rats were maintained 7 days without treatment following the end of 1, 3, 5 or 7 days of AAF treatment, rats appeared to recover as indicated by lower liver/body weight ratios (Table 19). Eco Rl restriction fragments of NI and NII DNA containing albumin gene sequences were not altered by treatment with AAF for up to 7 days. Likewise, these patterns of digestion were not altered following 7 days without treatment (Figures 19, 20, 21, 22). However, the altered patterns of labelled restriction fragments produced by Kpn 1 in DNA of NI and NII nuclei following AAF treatment did not return to the digestion pattern exhibited by controls (Figures 19-22, Tables 20-23). Thus, carcinogen modification of DNA during treatment with AAF resulted in changes in DNA detected by Kpn 1 digestion that could not be repaired in 7 days following cessation of treatment.

Figure 18. Eco R1 digestion of liver DNA from rats treated 7 days with N-2-acetylaminofluorene (AAF) or with vehicle. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) or with vehicle (0.75 ml/100 g corn oil: DMSO) for 7 days. Nuclei of hepatic parenchymal cells (NI) and nonparenchymal cells (NII) were isolated and DNA purified (see Methods). DNA (40 μg) was digested by Eco R1 and electrophoresed, then transferred and hybridized (see legend to Figure 13). Digestion of DNA from NI and NII nuclei of treated rats by Eco R1 is represented in Lanes a, b and d, e. Eco R1 digested DNA from control rats is represented in Lanes c and f. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed 3 days at -90°C.

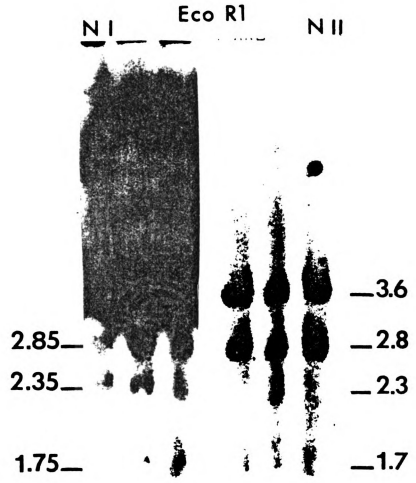


Figure 18

TABLE 18

Eco RI and Kpn I Restriction Endonuclease Fragmentation of DNA from Rats Treated 7 Days with N-2-Acetylaminofluorene

DNA Fragment[b]	NI		NII	
	Treated[c]	Control[d]	Treated	Control
Eco RI				
A	Not detected	Not detected	Not detected	Not detected
B	Not detected	Not detected	3.6	3.6
C	2.85[e]	2.85	2.8	2.8
D	2.35	2.35	2.3	2.3
E	1.75	1.75	1.7	1.7

[a]Purified DNA from rats was digested with Eco RI (0.15 units/ 1 μg DNA) or Kpn I (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 7 days prior to sacrifice.

[d]Rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) for 7 days prior to sacrifice.

[e]Data expressed in terms of Kilobases.

TABLE 19

Effect of Daily N-2-Acetylaminofluorene Treatment Followed by 7 Days Without Treatment on Liver/Body Weight Ratios[a]

Day	Treated[b]	Control[c]
1	0.050±0.002	
3	0.050±0.003	
5	0.051±0.005	
7	0.045±0.007	0.045±0.001

[a]Determined as liver weight/body weight on day of sacrifice.

[b]Male, Sprague-Dawley rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene in corn oil: DMSO (6:1, v/v) for 1, 3, 5 or 7 days, followed by 7 days without treatment.

[c]Male, Sprague-Dawley rats were injected i.p. with 0.75 ml/100 g corn oil:DMSO (6:1, v/v) for 7 days followed by 7 days without injection.

Figure 19. Eco R1 and Kpn 1 digestion of liver DNA from rats treated 1 day with N-2-acetyl-aminofluorene (AAF) followed by 7 days without treatment. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g), then maintained without treatment for 7 days. NI and NII nuclei (derived from parenchymal and nonparenchymal cells, respectively) were isolated, and DNA was purified (see Methods). DNA (40 μg) was digested by Eco R1 or Kpn 1 and electrophoresed, then transferred and hybridized (see legends to Figures 13 and 14). Digestion of DNA from NI and NII nuclei from AAF-treated rats is indicated in Lanes a-d after Eco R1 digestion, and Lanes a-d after Kpn 1 digestion. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed for 3 days at -90°C.

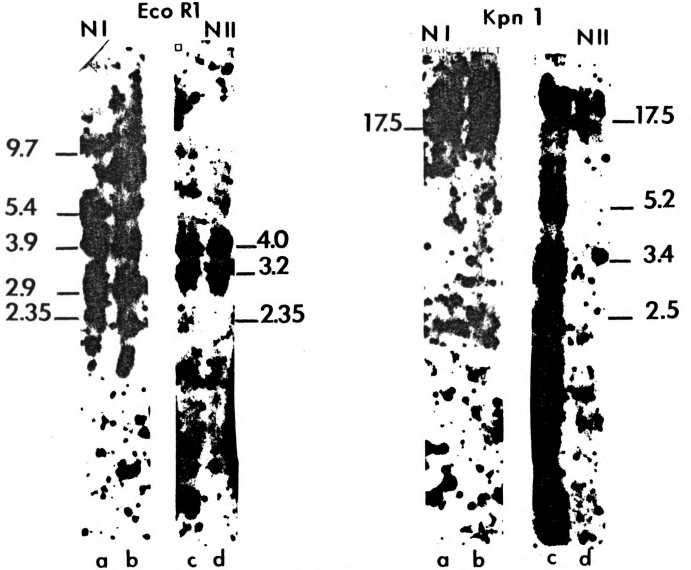

Eco R1
N I
N II
9.7
5.4
3.9
2.9
2.35
4.0
3.2
2.35
a b
c d
Kpn 1
N I
N II
17.5
17.5
5.2
3.4
2.5
a b
c d

Figure 19

Figure 20. Eco R1 and Kpn 1 digestion of liver DNA from rats treated 3 days with N-2-acetylaminofluorene (AAF) followed by 7 days without treatment. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g), then maintained without treatment for 7 days. NI and NII nuclei (derived from parenchymal and nonparenchymal cells, respectively) were isolated, and DNA was purified (see Methods). DNA (40 μg) was digested by Eco R1 or Kpn 1 and electrophoresed, then transferred and hybridized as indicated (see legends to Figures 13 and 14). Digested DNA from NI and NII nuclei of AAF-treated rats is indicated in Lanes a-f following Eco R1 treatment, and Lanes a-e following Kpn treatment. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed for 3 days at -90°C.

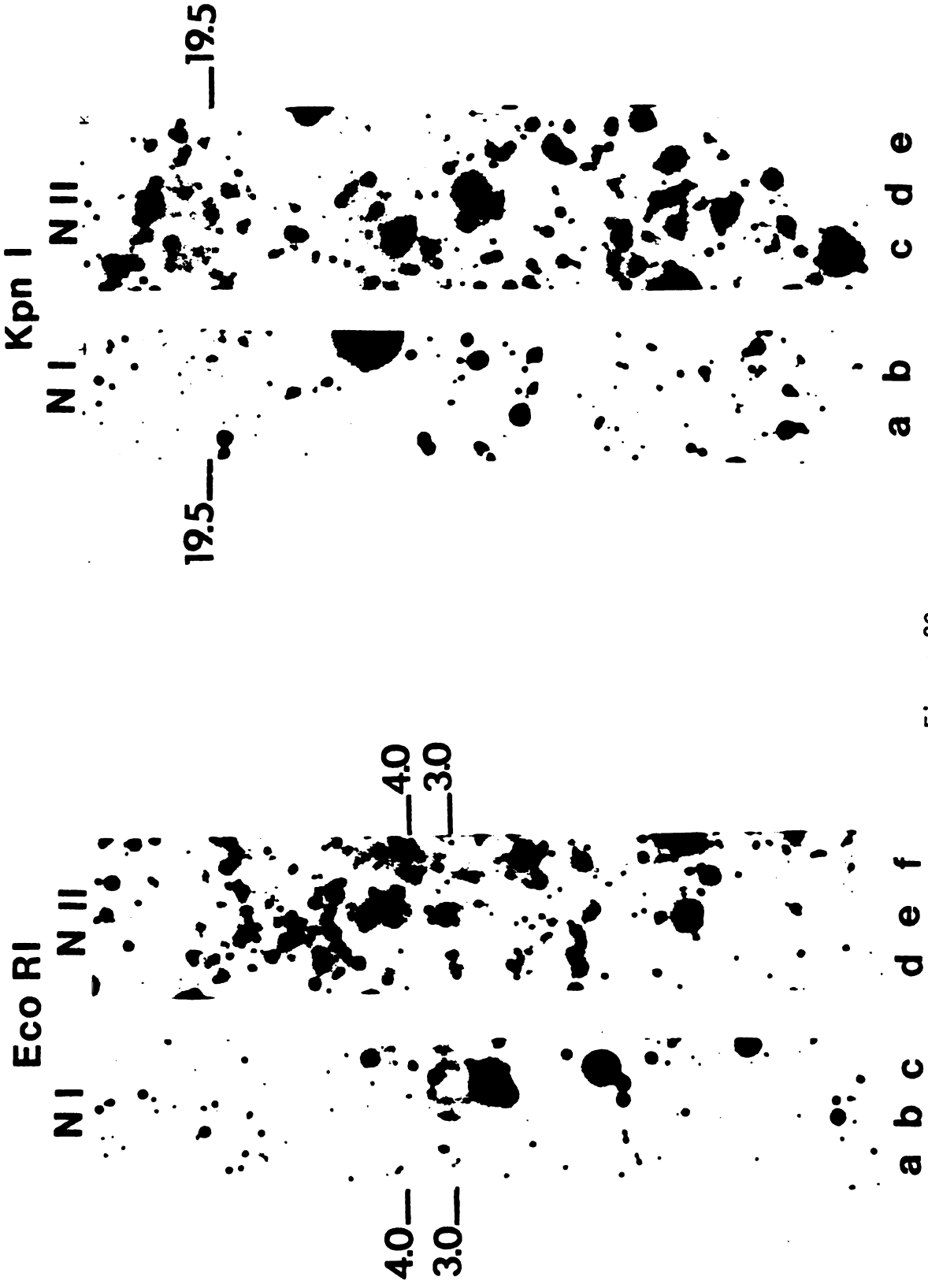


Figure 20

Figure 21. Eco R1 and Kpn 1 digestion of liver DNA from rats treated 5 days with N-2-acetyl-aminofluorene (AAF) followed by 7 days without treatment. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) then maintained without treatment for 7 days. NI and NII nuclei (derived from parenchymal and nonparenchymal cells, respectively) were isolated, and DNA was purified (see Methods). DNA (40 μg) was digested by Eco R1 or Kpn 1 and electrophoresed, then transferred and hybridized as indicated (see legends to Figures 13 and 14). DNA from NI and NII nuclei of rats treated with AAF and digested by Eco R1 or Kpn 1 is represented in Lanes a-f. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed for 3 days at -90°C.

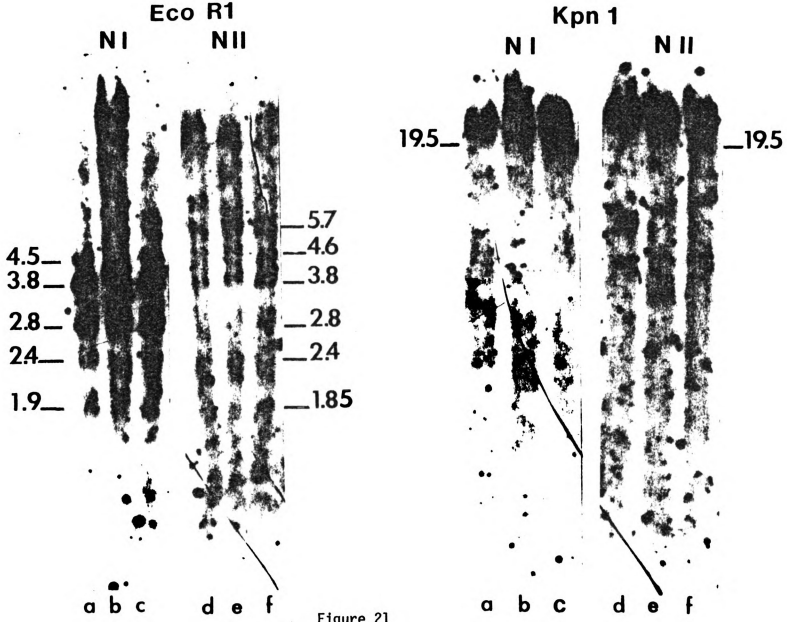

Eco R1
N I
N II
4.5
3.8
2.8
2.4
1.9
5.7
4.6
3.8
2.8
2.4
1.85
a b c
d e f
Kpn 1
N I
N II
19.5
19.5
a b c
d e f

Figure 21

Figure 22. Eco Rl and Kpn 1 digestion of liver DNA from rats treated 7 days with N-2-acetyl-aminofluorene (AAF) or vehicle followed by 7 days without treatment. Male Sprague-Dawley rats were injected i.p. with AAF (15 mg/100 g) then maintained without treatment for 7 days. NI and NII nuclei (derived from parenchymal and nonparenchymal cells, respectively) were isolated, and DNA was purified (see Methods). DNA (40 μg) was digested by Eco Rl or Kpn 1 and electrophoresed, then transferred and hybridized as indicated (see legends to Figures 13 and 14). DNA from NI and NII nuclei of AAF-treated rats following Eco Rl or Kpn 1 digestion is shown in Lanes a, b, a and b. Eco Rl and Kpn 1 digested DNA from control rats is shown in Lanes c, c and d. Sizes indicated are in kilobases (Kb). Hybridized blots were autoradiographed for 3 days at -90°C.

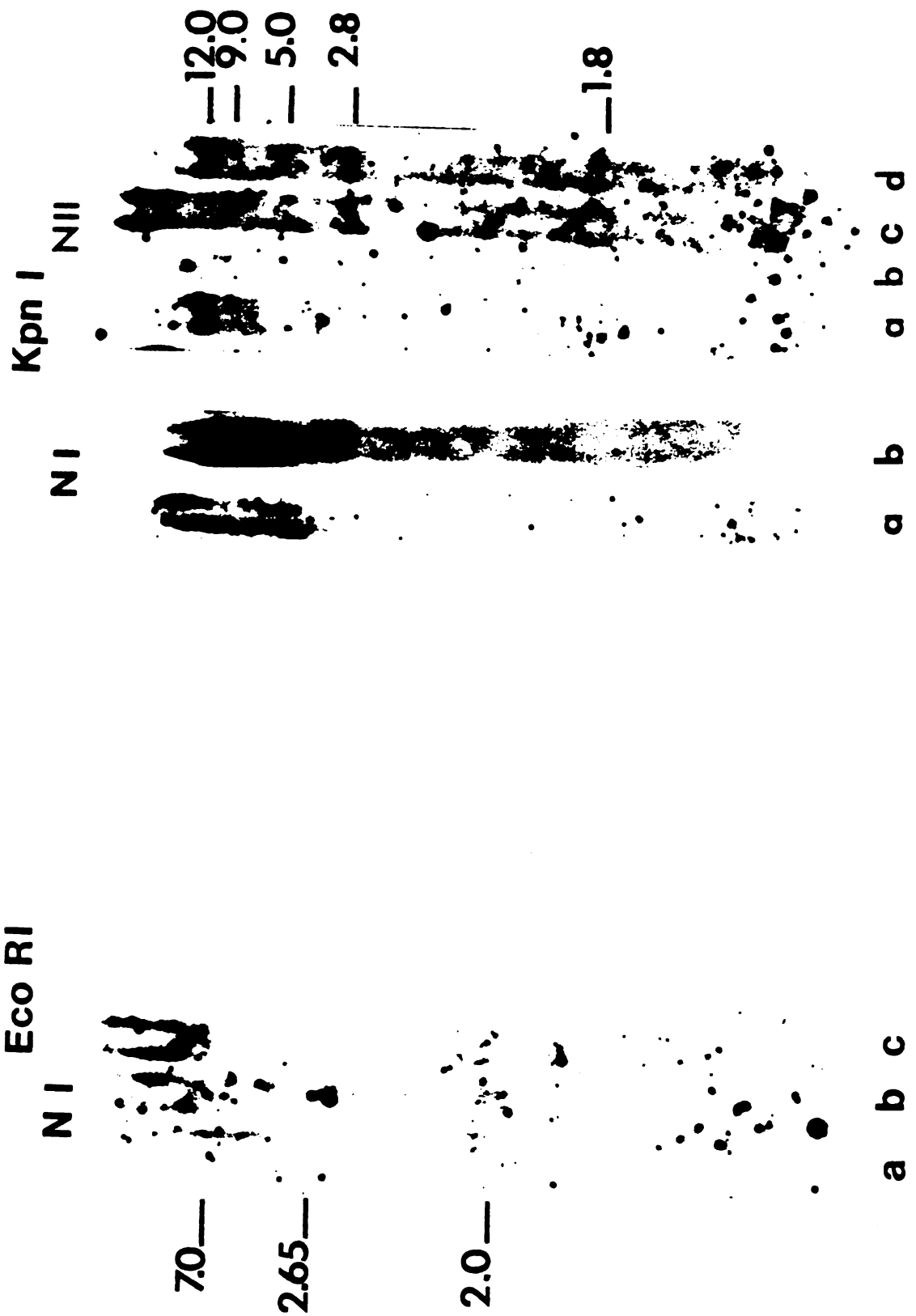


Figure 22

TABLE 20

Eco R1 and Kpn 1 Restriction Endonuclease Fragmentation of DNA from Rats Treated 1 Day with N-2-Acetylaminofluorene Followed by 7 Days Without Treatment[a]

DNA Fragment[b]	NI Treated[c]	NII Treated
Eco R1		
	9.7[d]	
A	5.4	Not detected
B	3.90	4.0
C	2.9	3.2
D	2.35	2.35
E	Not detected	Not detected
Kpn 1		
	17.5	17.5
A	Not detected	Not detected
B	Not detected	Not detected
C	Not detected	5.2
D	Not detected	3.4
E	Not detected	2.5
F	Not detected	Not detected

[a]Purified DNA from rats was digested with Eco R1 (0.15 units/1 μg DNA) or Kpn 1 (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 1 day followed by 7 days without treatment.

[d]Data expressed as kilobases.

TABLE 21

Eco R1 and Kpn 1 Restriction Endonuclease Fragmentation of DNA from Rats Treated 3 Days with N-2-Acetylaminofluorene Followed by 7 Days Without Treatment[a]

DNA Fragment[b]	NI Treated[c]	NII Treated
Eco R1		
A	Not detected	Not detected
B	4.0[d]	4.0
C	3.0	3.0
D	Not detected	Not detected
E	1.9	1.9
Kpn 1		
	19.5	19.5
A	Not detected	Not detected
B	Not detected	Not detected
C	Not detected	Not detected
D	Not detected	Not detected
E	Not detected	Not detected
F	Not detected	Not detected

[a]Purified DNA from rats was digested with Eco R1 (0.15 units/1 μg DNA) or Kpn 1 (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 3 days followed by 7 days without treatment.

[d]Data expressed as kilobases.

TABLE 22

Eco R1 and Kpn 1 Restriction Endonuclease Fragmentation of DNA from Rats Treated 5 Days with N-2-Acetylaminofluorene Followed by 7 Days Without Treatment[a]

DNA Fragment[b]	NI Treated[c]	NII Treated
Eco R1		
A	4.5[d]	5.7
B	3.8	3.8
C	2.8	Not detected
D	2.4	2.4
E	1.9	1.85
Kpn 1		
	19.5	21.0
A	Not detected	Not detected
B	Not detected	Not detected
C	Not detected	Not detected
D	Not detected	Not detected
E	Not detected	Not detected
F	Not detected	Not detected

[a]Purified DNA from rats was digested with Eco R1 (0.15 units/1 μg DNA) or Kpn 1 (8 units/1 μg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 5 days followed by 7 days without treatment.

[d]Data expressed as kilobases.

TABLE 23

Eco Rl and Kpn 1 Endonuclease Fragmentation of DNA from Rats Treated 7 Days with N-2-Acetylaminofluorene or Vehicle Followed by 7 Days Without Treatment

DNA Fragment[b]	NI		NII	
	Treated[c]	Control[d]	Treated	Control
Eco RI				
	17.5[e]	17.5		
A	7.0	7.0		
B	Not detected	Not detected		
C	2.65	2.65		
D	Not detected	Not detected		
E	2.0	2.0		
Kpn 1				
			17.5	
A	12.0	12.0	Not detected	12.0
B	Not detected	9.0	Not detected	9.0
C	Not detected	5.0	Not detected	5.8
D	Not detected	Not detected	Not detected	Not detected
E	Not detected	Not detected	Not detected	2.8
F	Not detected	Not detected	Not detected	1.8

[a]Purified DNA from rats was digested with Eco RI (0.15 units/ 1 µg DNA) or Kpn I (8 units/1 µg DNA) for 2 hours at 37°C.

[b]Molecular weights as labelled in Figures 14 and 15.

[c]Rats were injected i.p. with 15 mg/100 g N-2-acetylaminofluorene for 7 days followed by 7 days without treatment.

[d]Rats were injected i.p. with 0.75 ml corn oil:DMSO (6:1, v/v) per 100 mg for 7 days followed by 7 days without treatment.

[e]Data expressed in terms of Kilobases.

DISCUSSION

Covalent binding of chemical carcinogens to cellular macromolecules, especially DNA, appears to be a critical early event in chemically-induced carcinogenesis (50,85,86). Polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and aflatoxins are known to bind to tissue macromolecules (50,85,86), yet they vary widely in chemical structure. It is now recognized that most often the non-reactive parent compounds must be metabolically activated to a highly electrophilic form, which is then capable of binding to nucleophilic sites within cells such as RNA, DNA and protein (85,86).

Treatment of rats *in vivo* with AAF results in several carcinogen-DNA reaction products (64,65,69): N-(deoxyguanosin-8-yl)-AAF (C8-gua-AAF), N-(deoxyguanosin-8-yl)-AF (C8-gua-AF), and 3-(deoxyguanosin-N^2-yl)-AAF (N^2-gua-AAF). Kriek (66) found that the ratio of adducts produced *in vitro* was dependent upon the pH of the reaction mixture, i.e., a higher proportion of C-8 adducts were produced at lower pH, while N^2 adduct formation was favored at more alkaline pH. Following formation of adducts, the C8-gua-AF adduct has been shown to be unstable in an alkaline environment (64), as well as the N-acetyl group on C8-gua-AAF adducts (64,125). The C8-gua-AAF adduct is degraded to C8-gua-AF in alkali, which further degrades into 2 alkaline-stable, structurally unidentified products (125). Kriek has recently

found that C8-gua-AAF might degrade *in vivo* in a manner similar to exposure of the C8-gua-AF adduct to 0.1 N NaOH at 37°C, i.e., formation of a stable product in which the imidazole ring has opened (54). This aromatic pyrimidine derivative might then offer the possibility for mispairing with a thymine or adenine base (54).

In view of the numerous contributing factors to instability of carcinogen adducts, it is desirable to employ a procedure for isolation of DNA from carcinogen-treated rats that minimizes degradation of adducts. Beland *et al.* (9) presented a hydroxyapatite column chromatographic procedure which was relatively rapid, and minimized degradation of adducts. RNA and protein were eluted from the hydroxyapatite column at a low phosphate concentration, then DNA could be eluted at a higher phosphate concentration. This DNA isolation procedure could be performed at room temperature and neutral pH. We have modified this procedure by employing centrifugal force rather than a peristaltic pump to elute the column. Thus, numerous columns can be run simultaneously in shorter periods of time.

The yield of DNA from the hydroxyapatite column did not change by employing centrifugal force to elute the columns, i.e., a quantitative recovery of known amounts of DNA applied was achieved (Table 1). As shown in Table 2, RNA and protein contamination of the DNA-containing 0.48 M sodium phosphate buffer fraction was less than 1% of the total macromolecules. Beland *et al.* (9) found the $A_{260/280}$ ratio of the 0.48 M phosphate fraction was 1.88, indicating highly purified DNA in this fraction. In addition, only carcinogen adduct peaks corresponding to those of DNA standards were observed upon HPLC analysis of the

0.48 M phosphate buffer fraction (9). Thus, employing centrifugal force to elute multiple columns simultaneously did not appear to alter the yield or purity of eluted DNA.

Carcinogen-modified DNA from rats treated *in vivo* with [ring-^{3}H]-N-OH-AAF (1.8 μmoles/100 μCi/100 g) was isolated from hydroxyapatite columns to assess the recovery of adducts by this method. Chromatin isolated from carcinogen-treated rats was digested by DNase II, followed by selective $MgCl_2$ precipitation (44) resulting in a nuclease resistant pellet, P1, a $MgCl_2$-insoluble pellet, P2, containing putative transcriptionally inactive regions of chromatin, and $MgCl_2$-soluble supernatant, S2, containing putative transcriptionally active regions of chromatin (Figure 2). The greater carcinogen modification of the S2, transcriptionally active, fraction compared to the P2, transcriptionally inactive, fraction (Figure 3) is in agreement with previous reports in which DNA from S2 and P2 was isolated free of protein by hydrolysis in acid following alkaline hydrolysis of RNA (114). Thus, the modified hydroxyapatite DNA isolation procedure does not alter the extent of carcinogen modification determined in DNA from various chromatin fractions. Further verification is illustrated in Table 3. When known amounts of carcinogen-modified DNA were added to hydroxyapatite columns, 97% of the radioactivity applied (which corresponds to μmoles carcinogen adduct) was recovered. Beland *et al.* (9) found that even at levels as high as one carcinogen adduct per 100 nucleotides, a quantitative recovery of DNA was obtained, suggesting that this DNA retained enough of its double-stranded character to elute as such from a hydroxyapatite column.

It is well known that highly electrophilic forms of chemical carcinogens can bind to any of a number of nucleophilic macromolecules in cells (57,78,85,86,128). The extremely low percent of the injected carcinogen noted in Table 4 which binds to DNA (0.004%) indicates that the activated carcinogen must encounter many potential binding sites before reaching DNA in target organs. It has been suggested that the degree of nucleophilic sites that a carcinogen encounters before reaching the target macromolecule might be one important factor in determining susceptibility of a cell type to carcinogenesis by a particular chemical (79). While 1.5% of the injected dose binds to total hepatic macromolecules, only 0.08% of the injected dose binds to acid precipitable material of liver cell nuclei, suggesting that many binding sites for metabolically activated N-OH-AAF exist in the cytoplasm. Irving and Veazey (54) have found that the $t_{1/2}$ of carcinogen adducts in RNA is only 3 days, while the $t_{1/2}$ of adducts in DNA is approximately 7-10 days following a single dose of AAF. When there are no detectable adducts remaining in RNA, 10% of the initially bound DNA adducts remain (54). Thus, factors such as rate of repair of adducts from various macromolecules, and rates of turnover of various macromolecules affect accumulation of carcinogen damage. Results from Tables 4 and 5 indicate that, following a single carcinogen treatment, carcinogen adducts are lost more rapidly from total hepatic macromolecules compared to DNA, indicating that repair and/or macromolecular turnover occur to a greater extent with macromolecules other than DNA in rat liver.

Data in Table 5 illustrate 2.5- to 3.0-fold more carcinogen binding following treatment with N-OH-AAF compared to an equimolar dose of AAF. N-OH-AAF is the first metabolite along the presumed pathway to the ultimate carcinogenic form (50,69,85-87). Thus, when rats are given AAF or the N-hydroxymetabolite in an equimolar dose, the liver cells are able to metabolize rapidly the N-hydroxy metabolite to a reactive species (Table 5). When AAF or N-OH-AAF was administered to rats in the diet, tumors developed in the forestomach of rats given N-OH-AAF, but not following AAF treatment (88). Additionally, when AAF or N-OH-AAF was injected i.p., peritoneal tumors developed in rats injected with N-OH-AAF, but not with AAF (88). Guinea pigs are resistant to carcinogenesis following AAF treatment, but developed tumors following N-OH-AAF administration (87). Thus, N-hydroxylation appears to be a critical step in the carcinogenicity of AAF. Once N-hydroxylation of AAF occurs, subsequent metabolism of the ultimate carcinogenic form can occur rapidly in many cell types.

The mammalian liver is composed of a variety of cell subpopulations. Though hepatocytes (parenchymal cells, PC) constitute 90% of the liver weight, they represent only 65% of the total number of cells present (912). The remaining 35% of nonparenchymal cells (NPC) include fat-storing cells, endothelial cells, pit cells, bile duct cells and Kupffer cells which constitute 40% of nonparenchymal cells (91).

A variety of techniques exist for separation of various subpopulations of liver cells (62,94,151). Following perfusion of the liver with collagenase, hepatocytes may be obtained from the mixed cell suspension by centrifugation at 50 x g (94,151). Alternatively,

addition of trypsin or pronase results in lysis of hepatocytes, allowing for isolation of NPC (151). However, this method yields a population of NPC contaminated with hepatocytes. Kupffer cells can be isolated by selective adherence to glass, or use of a magnetic field to attract Kupffer cells which have ingested colloidal iron (151). Isolation of cells by these methods might result in loss of other liver cell populations during a particular procedure (94), relatively poor yield of the desired cell population (94,151) and/or contamination of the desired cell population by cells of an undesired cell population (94,151). The technique of centrifugal elutriation has been employed for separation and isolation of PC and NPC (62,74,151) as well as for subpopulations of PC (139). This method allows for separation of cells from a mixed liver cell suspension following collagenase perfusion on the basis of cell size and density, resulting in a high yield of purified fractions of PC and NPC which are viable (62,72,151). Using this method for liver cell isolation from rats treated with AAF or N-OH-AAF, the binding of carcinogen to DNA of target (PC) and nontarget (NPC) cells could be assessed. The DNA of elutriated PC was found to contain a higher concentration of carcinogen adducts than NPC following treatment with the procarcinogen, AAF, but not following treatment with the N-hydroxy metabolite (Figures 4 and 5) at the time of peak binding (18 hours and 2 hours, respectively) as well as 3 days later.

The results of these studies suggest the possibility that PC may be more efficient at carrying out the initial N-hydroxylation reaction than nonparenchymal cells. However, once AAF is N-hydroxylated,

subsequent metabolism to the ultimate binding form occurs to a similar extent in both target and nontarget liver cell populations. As cited above, it is well-established that N-hydroxylation of AAF is a critical step in AAF-induced carcinogenesis (24,50,69,85-88). Following N-hydroxylation, it is generally accepted that the N-hydroxy group is esterified, then degrades to a reactive electrophile, but the ester formed may vary in different species and tissues (28,58,82,85,86,103). From data presented in Figures 4 and 5 it appears that N-hydroxylation is a limiting step in the ability of PC and NPC to metabolically activate AAF to a form capable of covalently binding to DNA. However, once carcinogen binds to DNA of both PC and NPC, both cell populations appear to be able to repair carcinogen-DNA adducts to a similar extent. It is unlikely that loss of carcinogen from hepatic DNA over 3 days following a single injection was due to cell death because feeding of rats a 0.028% (w/w) AAF-containing diet for 5 weeks does not cause cellular necrosis (148).

Based on total DNA attributable to PC and NPC liver cell populations, there is approximately 20 times more carcinogen bound to PC DNA than to NPC DNA following AAF treatment, and approximately 8 times more carcinogen in PC DNA compared to NPC DNA following N-OH-AAF treatment (Table 6). When data are expressed in these terms, it is clear that binding of N-OH-AAF to DNA would still favor the induction of hepatocellular tumors.

An alternative procedure has been employed for isolation of nuclei derived from different liver cell populations based on nuclear density in a discontinuous sucrose gradient (19). When this procedure

was used for separation of nuclei into nuclei derived from PC (class NI nuclei) and those derived from NPC (class NII nuclei), Bushnell et al. (19) reported a preferential incorporation of ^{3}H-orotic acid and ^{3}H-cytidine into RNA of NI nuclei, concluding that NI nuclei were derived mainly from hepatocytes, and NII nuclei were derived from nonparenchymal cells. These results were confirmed by similar studies of Albrecht (2). Following treatment of rats with the hepatocarcinogen 3'-methyl-4-dimethylaminoazobenzene for 42 days, Sneider et al. (124) found a marked decrease in DNA associated with NI nuclei and an increase in DNA of NII nuclei which correlates with a persistence of ^{3}H-thymidine labelling of NI nuclei and a rapid turnover of ^{3}H-thymidine labelled DNA from NII nuclei. Samples of livers of these same animals were used by Rabes et al. (104) for histological and autoradiographic analyses. Histologic evidence demonstrated that the changes in the numbers of hepatocytes and nonhepatocytes as a percentage of total cell number corresponded to the change in percentage of total DNA distributed in NI and NII nuclei during carcinogen treatment (104). Autoradiographic analysis revealed the changes in ^{3}H-thymidine label in histologically-identified hepatocytes and nonhepatocytes corresponded to the change in ^{3}H-thymidine labelling of DNA from NI and NII nuclei populations (104). Therefore, discontinuous sucrose gradient centrifugation of rat liver homogenate results in separation of nuclei into a population derived from PC and a population derived from NPC which can be used to study carcinogen binding to DNA of target (NI) and nontarget (NII) nuclei populations following AAF treatment in a manner similar to that described above employing centrifugal elutriation for cell isolation.

As illustrated in Figure 6, there is no significant difference in the amount of carcinogen bound to DNA of NI or NII nuclei. This may be due, in part, to the fact that NII nuclei are derived from the entire population of nonparenchymal cells, i.e., bile duct cells, oval cells, fat storing cells, Kupffer cells, endothelial cells and pit cells (19,91), while the elutriated NPC were composed only of Kupffer and endothelial cells. It is possible that cells other than Kupffer and endothelial cells may bind carcinogen to DNA to a greater extent, thus accounting for the greater overall binding of carcinogen to DNA derived from nuclei of all liver nonparenchymal cells. As reported in the studies involving elutriated cell populations, following a single injection of N-OH-AAF, there was no difference in carcinogen concentration in the DNA of NI or NII nuclei (Figure 7). These results provide additional evidence that once AAF is N-hydroxylated, both cell types are able to carry out further steps in the metabolic activation to a DNA-binding form to a similar extent. Furthermore, approximately 50-60% of the initially bound carcinogen is lost by 3 days following treatment, providing support that both cell types are able to carry out repair to a similar extent.

The fact that approximately 50% of the initially bound adducts are lost within 3 days of treatment of rats with AAF or its N-hydroxy metabolite does not rule out the persistence of damage, such as single strand breaks or depurination, or that damage is not repaired in an error-free fashion, which might result in a mutation. Bolognesi *et al.* (16) has shown that several aromatic amines produce single-strand breaks in DNA of mouse liver following a single injection, and that

this damage persists at least up to 12 hours following injection. In addition, it has been shown that replication can proceed past carcinogen-induced lesions in DNA (152).

Tulp et al. (132,133) have separated various ploidy classes of hepatocyte nuclei as well as stromal (nonparenchymal) nuclei by velocity sedimentation at unit gravity. Binding of carcinogen to DNA at these classes of nuclei was assessed following treatment of rats with N-OH-AAF (approximately 4 μmoles carcinogen/100 g). At 24 hours following i.p. injection, 2.6-3 times more carcinogen was bound to DNA of parenchymal cell nuclei (diploid) compared to stromal cell nuclei (132,133). DNA of tetraploid parenchymal cell nuclei bound approximately twice as much carcinogen as their diploid counterpart (132, 133). The reasons for differing results of Tulp et al. concerning binding to DNA following N-OH-AAF administration may be several. In the studies of Tulp et al. (132,133), rats were sacrificed 24 hours following injection as the earliest time point. In our binding studies, rats were sacrificed 2 hours following N-OH-AAF injection because peak binding of carcinogen to DNA occurs at this time (90). The differences due to a dissimilar nuclei separation technique and DNA isolation method cannot be evaluated. However, the fact that these investigators found preferential binding to DNA of target cell nuclei is not inconsistent with our results taking into consideration total DNA of PC at risk for carcinogen attack (Table 6), thus favoring hepatic parenchymal as targets for eventual development of hepatocellular carcinomas.

A great deal of evidence has supported a nonrandom interaction of carcinogens with DNA (4,35,60,70,75,84,90,102,106,114,115,131). Therefore, it was of interest to determine whether or not there is a nonrandom interaction of carcinogen with DNA of target (NI) or non-target (NII) rat liver nuclei populations following treatment of rats with AAF or its N-hydroxy metabolite.

DNase I is a well-characterized probe for specifically digesting transcriptionally active regions of chromatin (27,42,43,56,121,138, 140). It has been shown that when only 10% of the total DNA from chick erythrocytes was digested by DNase I, approximately 75% of the active globin gene sequences are no longer present (140), indicating that DNase I is highly specific for actively transcribing genes.

Weintraub and Groudine (140) have shown that digestion of chromatin from chick erythrocyte nuclei by DNase I begins to level off at 30 minutes of digestion, a time at which approximately 40% of the total DNA has been rendered acid-soluble by the enzyme. Data represented in Figure 8 show that when total nuclei isolated from rat liver homogenate are digested with DNase I, digestion levels off at 50-60 minutes, a time at which approximately 20% of the total DNA has been rendered acid-soluble by the enzyme.

When chromatin of nuclei populations derived from different cell types within the liver are digested by DNase I, differential digestion occurs (Tables 9 and 10). Digestion of NI nuclei for 10 minutes by DNase I results in acid-solubilization of approximately 60% of total NI DNA (Table 9) in nuclei from control rats as well as from rats treated with AAF or its N-hydroxy metabolite. However, at 10 minutes

of digestion of NII nuclei by DNase I, only 20% of the total DNA is digested in NII nuclei from control or treated rats (Table 10). Thus, digestion patterns of nuclei obtained from total liver may not represent DNase I sensitivity of chromatin in target (NI) and nontarget (NII) cell nuclei for AAF carcinogenicity. It is possible that the high percentage of nuclease-accessible DNA from NI nuclei may reflect a high degree of transcriptional activity (Table 9). Several investigators have found preferential incorporation of RNA precursors, orotic acid and cytidine, into NI nuclei (2,80) as opposed to NII nuclei, suggesting that chromatin of NI nuclei is more transcriptionally active than that of NII nuclei. The high degree of DNase I sensitivity of chromatin from NI nuclei, and the low sensitivity of chromatin from NII nuclei illustrated in Tables 9 and 10 are further exemplified in Figures 11 and 12. Following selective nicking of NI and NII chromatin in transcriptionally active regions with DNase I in a sufficiently low concentration, gaps were filled in with ^{32}P-labelled deoxyribonucleotide triphosphates by E. coli DNA polymerase I. This procedure has been shown to preferentially incorporate radioactivity labelled nucleotides in DNase I-sensitive regions (presumed to be transcriptionally active (43). When equal amounts of labelled DNA from NI and NII nuclei were applied to agarose gels, it is clear that DNA of NI nuclei is more heavily labelled than an equal amount of DNA from NII nuclei following autoradiography for 4 and 24 hours (Figures 11 and 12). It is reasonable to conclude, therefore, that a greater degree of transcriptional activity occurs in liver parenchymal cell DNA compared to nonparenchymal cell DNA. This is to

be expected in view of the increased amount of RNA synthesis noted in nuclei of parenchymal compared to nonparenchymal cells (2,124) indicating increased transcriptional activity of these cells. Furthermore, the parenchymal cells are polyploid (diploid, tetraploid, and some octaploid cell nuclei), and are involved in synthesis of export proteins such as albumin (48), whereas the nonparenchymal cells are not observed to have such functions.

From Tables 9 and 10, it appears that the presence of carcinogen in DNA of NI or NII nuclei following treatment of rats with AAF or its N-hydroxy metabolite does not affect the ability of DNase I to recognize and/or cleave DNA either at the time of peak binding for the carcinogens, or 3 days later.

Following treatment of rats with AAF, there is significantly less carcinogen bound to DNase I-sensitive DNA of NI nuclei (target nuclei) compared to undigested DNA (similar results were obtained following N-OH-AAF treatment, but not statistically significant). However, there is a great deal more carcinogen bound to DNase I-sensitive DNA of the nontarget cell nuclei (NII) at the time of peak binding for either carcinogen (Figure 10). The fact that carcinogen is rapidly lost from these regions 3 days following peak binding might, in part, explain why nonparenchymal cells are not the targets for AAF-induced hepatocarcinogenesis.

Data on carcinogen binding to nuclease-inaccessible regions of NI and NII nuclei following treatment of rats with AAF or its N-hydroxy metabolite are compiled in Tables 10 and 11. Following treatment with either carcinogen, adduct concentration is higher in DNase I-inaccessible regions (transcriptionally repressed) of target cell nuclei (NI)

at the time of peak binding and 3 days later. While carcinogen adducts are lost rapidly from nuclease accessible regions of nontarget cell nuclei (NII) (Figure 10), adducts persist in nuclease-resistant regions of NII chromatin (Tables 10 and 11). These results might aid in explaining AAF-induced carcinogenesis targeted to hepatic PC in that adducts form and persist in transcriptionally active and inactive DNA of target cells, yet adducts only persist in transcriptionally inactive regions of nontarget cells. As long as these regions of chromatin remain transcriptionally repressed, damage will not become manifest. As mentioned above, however, loss of carcinogen adducts does not rule out the possibility of persisting DNA damage (16), and mere persistence of carcinogen adducts can occur in target as well as nontarget tissue (10).

Two groups of investigators have reported on carcinogen binding to DNase I-accessible and inaccessible regions of chromatin from total rat liver following treatment in vivo with AAF (84) or with N-OH-AAF (106). In both studies, carcinogen was found to preferentially bind to DNase I-inaccessible regions of chromatin from total rat liver, and adducts persisted for up to one week in these regions (84,106). These results are not inconsistent with those described above in that approximately 60-65% of the total liver cell number is hepatic parenchymal cells. Data from DNase I digestion of chromatin from NI nuclei (Figures 9 and 10) are consistent with studies on DNase I digestion of nuclei from total liver (84,106).

Treatment of rats with AAF daily for up to 5 days results in decreased amounts of carcinogen bound to total hepatic macromolecules

(Table 12), probably due to a decreased ability of liver cells to metabolically activate AAF (114,123). Other investigators have noted that pretreatment with AAF increased C8-adduct formation in regions of the genome containing C8-adducts compared to N^2 adducts (114). In addition, pretreatment of rats with AAF for 3 feeding cycles (0.06% w/w AAF-containing diet) for up to 12 weeks resulted in enhanced removal of O^6-methylguanine from rat liver DNA, but not kidney DNA when a pulse of DMN was given at the end of AAF pretreatment (18, 23). However, pretreatment of rats with dialkylnitrosamines decreased the removal (increased the persistance) of O^6-methylguanine in rat liver DNA following injection of DMN, suggesting the possibility that pretreatment with these dialkylnitrosamines produces similar products which interfere with removal of O^6-methylguanine from rat liver DNA (95). Following treatment of rats with AAF (15 mg/100 g) for 1, 3 and 5 days, there was no difference in binding of a tracer dose of [ring-^{3}H]-AAF to DNase I sensitive DNA of PC or NPC compared to vehicle-control-injected rats (Table 13). These results suggest that pretreatment of rats with AAF for up to 5 days does not affect subsequent binding of carcinogen to DNase I-sensitive DNA of target (NI) or nontarget (NII) DNA. Even though pretreatment with carcinogen decreases the ability of the liver to metabolically activate subsequently administered carcinogen (Table 12), the amount of carcinogen which binds to DNA is not affected by pretreatment as evidenced by similar amounts of carcinogen adducts in DNase I-sensitive DNA of AAF- and vehicle-pretreated rats (Table 13).

Schwartz et al. (61) reported a 16-fold specificity of AAF for transcriptionally active areas of rat liver chromatin detained in DNase II-digested, $MgCl_2$-soluble fractions. These studies investigated binding of carcinogen to specific regions of the genome from a total rat liver, i.e., DNA isolated from parenchymal as well as nonparenchymal cells. Results from studies depicted in Figures 9 and 10 emphasize the importance of assessing carcinogen binding to DNA of cell populations within the target organ. DNase I-accessible DNA from NI contains less carcinogen adducts than undigested NI DNA, while DNase I-accessible DNA from NII contains a great deal more carcinogen adducts than undigested NII DNA. These results are not inconsistent with those of Schwartz et al. if it is considered that hepatocytes comprise 60% of the total liver cell number, while nonparenchymal cells comprise the remaining 40% (91). Thus, DNA from transcriptionally active chromatin regions exhibiting high concentrations of carcinogen adducts from the studies of Schwartz et al. (114) may be regions analogous to the DNase I-sensitive regions of nonparenchymal cells which contained large amounts of carcinogen adducts.

In some cases, carcinogen binding to total DNA of a target organ might correlate with carcinogenicity of a particular chemical (144). However, many examples indicating that carcinogen accumulates in total DNA of nontarget organs as well as target organs (10,99) suggests that other factors are involved in carcinogenicity of a chemical. Therefore, it is reasonable to consider the nature of carcinogen specificity for particular regions of the genome in target vs. nontarget cells.

Restriction endonucleases are recognized as useful tools in studies concerning chromatin structure (53,96). Because these endonucleases are specific with regard to base sequence of DNA, digestion of DNA with these enzymes yields information not available with other nucleases such as DNase I or DNase II.

We proposed to employ restriction endonucleases as probes for determining whether or not carcinogen modification of DNA *in vivo* is specific for selected areas of the genome within target cells. Boehm and Drahovsky (15) reported that, following reaction of lambda bacteriophage DNA with various concentrations of MNU, the abilities of the restriction endonucleases Hae III, Hind III, Eco Rl, and Bam HI to recognize and cleave DNA at sites to which these enzymes are specific, was impaired in all cases. Thus, it was of interest to determine whether or not AAF treatment of rats *in vivo* resulted in carcinogen modification of DNA from target and nontarget cell nuclei such that restriction endonucleases no longer cleaved at their specific sites.

Treatment of rats for 1, 3, 5 and 7 days with AAF resulted in liver/body weight ratios significantly greater than control ($p<0.05$) (Table 14). Wilson *et al.* reported a dose-dependent decrease in growth weight with increasing concentration of AAF in the diet (146). The increased liver/body weight ratio is due to decreasing body weight simultaneous with increasing liver weight with progressive AAF treatment. Therefore, daily treatment of rats with 150 mg/kg AAF up to 7 days appears to be toxic to rats.

The restriction endonuclease Eco Rl recognizes the base sequence 5'-↓GAATTC-3'. The majority of carcinogen adducts in DNA following

treatment with AAF occurs at guanine (40,54,64,65,67,85,136), although some adducts occur to a limited extent at adenine (69,86,116). Therefore, if carcinogen modification occurs at a guanine within an Eco Rl-specific site, impairment of the ability of the enzyme to recognize or cleave at this sight might be expected.

Treatment of rats with AAF for 1, 3, 5 or 7 days did not alter the ability of Eco Rl to recognize and cleave at its specific sites (Figure 16, Table 15). In several cases (Figures 17, 18, Eco Rl panels), the intensity of equal molecular weight bands was less for NII DNA samples compared to NI, even though equivalent amounts of DNA were applied to the gel. Due to the ploidy differences of NI vs. NII nuclei (93), more albumin gene-containing sequences may be present in a determined amount of DNA from nuclei derived from polyploid hepatocytes than of diploid nonparenchymal cells. In Figure 18, lanes b and f of the Eco Rl panel represent 2 times the amount of DNA electrophoresed in lanes a and e, respectively. The intensity of the bands correlate with the quantity of DNA containing homologous sequences for the albumin gene probe. Because the carcinogen used was not radioactively labelled, the persistence of carcinogen adducts could not be determined. Several possible explanations would be considered for lack of an effect of AAF on Eco Rl digestion of DNA. Carcinogen adducts may not have occurred at the site for which Eco Rl is specific. Alternatively, if carcinogen adducts did occur at these sites, it is possible that adducts were rapidly repaired. Beland et al. (10) and Poirier (99) found that the C8-gua-AAF adduct is rapidly repaired, whereas the N^2-gua-AAF and, to a lesser extent, the C8-gua-AF, adducts

appear to persist in DNA of target as well as nontarget tissue. Thus, if C8-gua-AAF adducts formed predominantly at Eco Rl specific sites, repair of these adducts would be expected to occur rapidly.

Treatment of rats with AAF for 1, 3, 5 and 7 days did result in alterations in the ability of Kpn 1 to recognize and/or cleave at its specific sites in DNA of target (NI) and nontarget (NII) nuclei (Figures 17, 18 and Tables 16, 17). Digestion of DNA from rats treated for 3 days (Figure 17) and 5 days (Figure 18) resulted in loss of several labelled restriction fragments (16.0, 9.7, 8.0 and 2.3 Kb). Kpn I is specific for the base sequence GGTAC↓C. There is not a guanine base at the actual site of cleavage for Kpn I, yet Kpn I cannot act at some sites within carcinogen-modified DNA presumed to contain carcinogen-guanine adducts. Thus, it is possible that neighboring regions of chromatin contain sufficient concentrations of carcinogen-guanine adducts to inhibit the action of Kpn I. Furthermore, chromatin structure may be responsible for the presence of some guanines in carcinogen-accessible positions, while other guanines are inaccessible to carcinogen. When DNA is then purified and reacted with Kpn I, the restriction enzyme has access to regions previously protected from carcinogen attack.

Beard et al. (8) found nucleosome-like structure of the Simian virus 40 (SV-40) chromosome was an important determinant for preferential attack by N-acetoxy-AAF of a regulatory gene sequence, while the carcinogen randomly bound at guanine sites in the purified SV40 DNA. The nucleosomal structure of mammalian chromatin might allow for "hot spot" regions to which carcinogen binds. These hot spot regions might fall within the same areas that the restriction endonuclease,

Kpn I, recognizes. Thus, nucleosomal chromatin structure might be responsible for increased accessibility of certain regions of the rat liver genome (Kpn I-sensitive regions) for carcinogen modification.

Fuchs et al. (41) have reported that N-acetoxy-AAF can induce frameshift mutations in regions adjacent to the 6-nucleotide sequence GGCGCC of the plasmid pBR322. The fact that 3 mutations have been found near this nucleotide sequence indicates that hot spot regions within the genome can occur. Yoon et al. (150) have also reported on hot spot regions within the pBR322 plasmid following reaction with N-acetoxyacetylaminofluorene. Acetoxy-AAF induced GC base pair deletions in GC-rich regions. In addition, these investigators found the repeating sequence TCGATCGA to be a hot spot for mutations. Clustering of carcinogen adducts might be another factor involved in preferential binding of carcinogens to specific regions of the genome. Winkle and Krugh (147) found that, at equilibrium, N-acetoxy-AAF binds to ØX174RF in only four sites. Scatchard plot analysis of equilibrium binding revealed cooperativity of binding, i.e., binding of one molecule of AAF may facilitate binding of subsequent carcinogen molecules. Similarly, Schwartz et al. (114) found that following pretreatment of rats with AAF, 85% of the total bound carcinogen was clustered in less than 25% of the total genome. As carcinogen treatment progressed, cooperative binding was observed in that increasing binding to DNA correlated with increasing amounts of C-8 adducts which were concentrated within one-fourth of the total genome. It is conceivable that carcinogen adducts might cluster in regions of the genome sensitive to Kpn I, thus inhibiting enzyme activity.

Following treatment of rats with AAF for 1, 3, 5 and 7 days, rats were maintained for 7 days without treatment to allow for repair of carcinogen adducts. Sites recognized by Eco R1 within rat liver DNA from target (NI) and nontarget (NII) cell nuclei were not blocked as a result of carcinogen treatment for 1, 3, 5 and 7 days; therefore, allowing 7 days following carcinogen treatment to repair carcinogen-modified DNA had no effect on Eco R1 fragmentation patterns of DNA from NI and NII nuclei of carcinogen-treated rats (Figures 20-22, Tables 20-23). It has been shown that the presence of intercalating agents (actinomycin D, ethidium bromide, proflavin) and agents which bind to minor groove regions (distamycin A and netropsin) within pBR322 DNA can protect Eco R1 cleavage sites from the action of this restriction enzyme. However, it must be re-emphasized that conclusions from studies on carcinogen modified non-nucleosomal DNA structures might not be indicative of effects in DNA arranged in nucleosomes.

Immediately following treatment of rats with AAF for 3 or 5 days, several labelled fragments were not detectable compared to control (Figures 17 and 18). When rats were maintained 7 days without AAF treatment, it appeared that the carcinogen-damaged DNA was not repaired (Figures 21-22). However, liver/body weight ratios were returning to control levels, suggesting that rats were recovering from acute toxicity of AAF. Bands A-F presented in Figure 15 were not present following 7 days repair time after cessation of 3, 5 or 7 days AAF treatment. In all cases, a high molecular weight DNA fragment (17.5-19.5 Kb) was observed (Figures 21-22). If the presence of carcinogen damage at or near Kpn 1 sensitive sites remained after 7

days of repair, then one or several bands of higher molecular weight, i.e., incompletely fragmented DNA, might be expected. Thus, in Figures 21-22, the high molecular weight band may represent incompletely cleaved DNA due to an inhibition of Kpn 1 cleaving activity as a function of persistent carcinogen damage.

Carcinogen modification of DNA as a result of treatment of rats with AAF for 1, 3, 5 and 7 days does not inhibit the ability of Eco R1 to recognize specific sites within the genome. The fact that a band appears occasionally that is of a different molecular weight than bands A-E indicated in Figure 14 might indicate an occasional random inhibition of an Eco R1 site. However, these bands occurred in both treated vs. control DNA. Therefore, this is probably due to a more complete transfer of DNA from the gel to nitrocellulose in some cases.

Carcinogen modification of DNA from AAF-treated rats for 1, 3, 5 and 7 days does appear to inhibit the ability of Kpn I to act at sites for which it is specific (Figures 17 and 18). DNA damage detected immediately following carcinogen treatment is not repaired for up to 1 week following cessation of treatment (Figures 20-22). These results indicate that carcinogen modification of DNA following treatment in vivo is nonrandom with respect to the genome. The fact that guanine, a predominant target for AAF, is present within the base sequence for which both restriction enzymes are specific, yet only one restriction enzyme is inhibited from cleaving indicates the specificity of carcinogen for region of the genome is of a higher order. Thus, the neighboring base sequences (41,150) as well as chromatin structure (8,36) may play a more important part in determining specificity of a carcinogen for regions of the genome.

SUMMARY AND CONCLUSIONS

An analysis of early events resulting in AAF-induced carcinogenesis targeted a specific cell population and particular regions within the genome was investigated. When cell populations from the liver were isolated following treatment of rats with AAF or its N-hydroxymetabolite, AAF appeared to bind selectively to DNA of parenchymal cells, while there was no difference in the carcinogen binding to DNA of parenchymal and nonparenchymal cells following N-OH-AAF administration at the time of peak binding. Thus, it appeared that AAF may preferentially attack DNA of the target cells, parenchymal cells, due to a relative increased capacity of these cells to carry out the first step in metabolic activation of AAF to the ultimate reactive form, N-hydroxylation. Following carcinogen treatment, both cell types carry out repair to a similar extent. Similar studies were performed in which carcinogen binding to DNA of hepatic parenchymal cell nuclei (NI) and nonparenchymal cell nuclei (NII) was assessed following a single injection of carcinogen. At the time of peak binding, there was no difference in binding to DNA of NI and NII. This was probably due to the fact that NII nuclei were derived from the entire population of nonparenchymal cells of the liver, while elutriated nonparenchymal cells consisted of Kupffer and endothelial cells which are the predominant nonparenchymal cells in the liver.

These studies indicate that binding of carcinogen to DNA varies among cell populations within a target organ.

When nuclei from NI and NII populations following treatment of rats with AAF and its N-hydroxy metabolite were digested with DNase I the binding of carcinogen to transcriptionally active vs. inactive regions of chromatin in target and nontarget nuclei could be evaluated on the basis of the selectivity of DNase I for transcriptionally active DNA. Carcinogen bound preferentially to DNA of transcriptionally repressed regions of target cell nuclei (NI), but preferentially to transcriptionally active regions of nontarget cell nuclei (NII). Though damage following a single injection persisted for up to 3 days in transcriptionally repressed DNA of NI nuclei, damage was rapidly repaired from transcriptionally active DNA of NII nuclei. These studies stress the importance of investigating differences in carcinogen interaction with subpopulations of cells within a target organ. The persistence of carcinogen in the transcriptionally repressed regions of the quiescent hepatocyte population is important if these cells should be stimulated to proliferate, or if previously repressed regions of the genome should become transcriptionally active, which might happen as the result of an epigenetic event, i.e. promotion of carcinogenesis. For example, carcinogen damage might be expressed in hepatocytes, resulting in an altered population of cells, generally considered to be a preneoplastic event. Transcription from a faulty template might result in alterations of the target cell population as well. Hepatocytes appear to be more transcriptionally active than nonparenchymal cell as indicated in this investigation.

DNase I digests approximately 50-60% of the genome of NI nuclei while only 10-20% of the nonparenchymal cell DNA is susceptible to this enzyme which selectively attacks transcriptionally active DNA. In addition, nick-translation of nuclei by DNase I which results in radioactive labelling of transcriptionally active regions of DNA indicates a greater degree of transcriptional activity in target cell nuclei.

Enzyme-altered foci which develop as rats are maintained on an AAF-containing diet consists of small islands of cells with increased gamma-glutamyl transpeptidase, and decreased adenine triphosphatase and glucose-6-phosphatase (100). This indicates that alterations in gene expression might occur as a result of carcinogen exposure. It is known that portions of chromosomes can move from one chromatid to another (25). Transposable elements, movable regulatory DNA sequences, could alter phenotypic expression if these elements change in position, structure or orientation as a result of carcinogen exposure (31). It has been found that DMN and aflatoxin B_1 can alter gene expression in Drosophila as a result of effects on DNA insertions by these carcinogens (31). Mutations in regulatory DNA sequences could cause altered gene expression. Thus, regional specificity of carcinogen with regard to the genome of target cells might be important in alterations of gene expression resulting in preneoplastic changes in the target cell population.

Investigations on the specificity of AAF for particular base sequence regions in DNA of NI and NII nuclei were carried out using restriction endonucleases which act at specific base sequences within

the DNA. Progressive treatment of rats with AAF had no effect on Eco Rl recognition of DNA sequences, but partially inhibited Kpn 1 from acting at sites for which this restriction endonuclease is specific. When rats were maintained one week without treatment following carcinogen exposure, damage at some Kpn I-specific sites was not repaired. These results indicate that AAF can be very specific with regard to location of carcinogen attack within the genome. Selective damage of regulatory gene sequences (8) could result in alterations of gene expression and phenotypic changes of a target cell population. Furthermore, masking of specific regions of chromatin DNA by carcinogens might alter the ability of proteins specific to base sequences or structural arrangement of chromatin to recognize these regions, resulting in decreased transcription of particular genes, or repression of some genes and derepression of other genes.

Thus, further investigations into the target specificity of chemical carcinogens must take into account not only events resulting in carcinogenesis targeted to a particular tissue, but critical events occurring in subpopulations of cells within the target organ. Increased knowledge in the field of molecular biology had yielded useful tools with which to probe the specific nature of carcinogen interaction with chromatin of target cell populations. Further studies on specific events resulting in alterations of gene expression as the result of exposure to a variety of chemicals might yield valuable information on critical early events that eventually result in carcinogenesis targeted to particular cell types. The interaction of genetic consequences of DNA damage by carcinogens with epigenetic

events resulting in the expression at malignant tumors must be more fully investigated.

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BIBLIOGRAPHY

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