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Genetics

Distribution of initial and persistent 2-acetylaminofluorene-induced DNA adducts within DNA loops

(**^{32}P-labeling assay/hepatocarcinogenesis**)

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ABSTRACT **The intranuclear distribution of initial and persistent DNA adducts induced *in vivo* after four weekly injections of the hepatocarcinogen 2-acetylaminofluorene was examined in rat liver by using a protocol that fractionates chromatin from various regions of each of the multiple nuclear DNA loops. Ten hours after the initial dose, two acetylated [*N*-acetyl-*N*-(deoxyguanosin-8-yl)-2-aminofluorene and 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene] and one deacetylated [*N*-(deoxyguanosin-8-yl)-2-aminofluorene] adduct were detected by a ^{32}P-labeling assay and were found to have a random genomic distribution, as evident by their relative concentrations in various chromatin fractions. These data suggest that all regions of the DNA loops are equally susceptible to adduct formation. A nonrandom persistence of the deacetylated adduct in the regions where the DNA loops are constrained by the nuclear matrix was evident by 6 days after the last dose and was markedly apparent by 60 days. In contrast, all chromatin fractions had equally inefficient removal of the N^2-acetylated adduct by 6 days as well as 60 days but had complete removal of the C8-acetylated adduct. These findings suggest that pronounced regional differences in adduct repair along the DNA loops may play a role in chemically induced hepatocarcinogenesis.**

Aromatic amines such as 2-acetylaminofluorene (AcNHFln) have been shown to be potent tumorigenic agents in several tissue types (1–3). *In vivo* modification of AcNHFln to electrophilic metabolites and subsequent formation of covalent DNA adducts are believed to be essential steps in hepatocarcinogenesis (1, 2). AcNHFln or its *N*-hydroxyl derivative forms three guanine adducts (3–7) and one tentatively identified adenine adduct (7) in rat liver DNA *in vivo*—namely, *N*-acetyl-*N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AcNHFln), 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AcNHFln), *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-NHFln), and *N*-acetyl-*N*-(deoxyadenosin-8-yl)-2-aminofluorene. Formation of the predominant liver DNA adduct dG-C8-NHFln has been shown recently to be directly related to *in vivo* mutagenesis (8, 9).

In light of recent evidence that AcNHFln adducts can lead to base-pair substitution during DNA replication (10) and that single base-pair substitution can result in activation of oncogenes (11–13), differential genomal localization of DNA adducts may be one important event in chemical carcinogenesis.

It is presently unknown whether dG-C8-NHFln lesions occur randomly throughout the genome or whether particular regions (demarked either by gene sequences or chromatin structure) are differentially involved. Based on exogenous nuclease digestion studies, nuclear euchromatic regions have been proposed to be preferentially susceptible to adduct formation (14–18). Nonrandom formation and removal of DNA adducts within rat repetitive genomic DNA sequences has also been observed recently (19).

The eukaryotic genome has at least three levels of organization—the nucleosome (20), the solenoid of 6–10 nucleosomes (21), and the supercoiled DNA loops (22). An emerging model for DNA loop organization of transcribed genes proposes that flanking DNA sequences containing regulatory regions of structural genes or gene clusters are positioned close to the point(s) where individual loops are constrained by a protein scaffolding structure or nuclear matrix (23–26). The transcribed portion of the gene therefore lies within the DNA loop, portions of which may also be associated with matrix by virtue of transcriptional complexes (26).

Taking advantage of a procedure for isolating chromatin fractions from discrete regions along DNA loops and evaluating adduct levels by a sensitive ^{32}P assay, we observed that AcNHFln-induced DNA adducts are formed in all regions of the loops. However, different DNA adducts exhibited different extents of persistence (or removal), and removal of the adduct dG-C8-NHFln was markedly inefficient from regions of DNA loops in the vicinity of their association with matrix.

MATERIALS AND METHODS

Chemicals. AcNHFln was purchased from Sigma. Materials required for the ^{32}P assay were the same as described (6).

Animal Treatment. Adult male Sprague–Dawley rats (450–500 g; three rats per group) were administered four weekly i.p. injections of 60 mg of AcNHFln per kg of body weight in 0.3 ml of dimethyl sulfoxide. Animals were sacrificed by decapitation at indicated times, livers were excised, minced, and pooled within each group, and nuclei were immediately isolated as described (27, 28).

Preparation of Chromatin Subfractions and Isolation of DNA. Details for chromatin and nuclear matrix isolation have been published (28, 29). In brief, nuclei digested endogenously at 37°C for 45 min were separated by low-ionic strength extractions [10 mM Tris·HCl, pH 7.4/0.2 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$); LS buffer] into a soluble bulk-chromatin fraction (75–80% of total nuclear DNA) and a remaining low-salt nuclear matrix (20–25% of total nuclear DNA). Low-salt matrix structures were then extracted in high-ionic strength buffer (10 mM Tris·HCl, pH 7.4/2 M NaCl/0.2 mM $MgCl_2$/1 mM $PhMeSO_2F$; HS buffer) to release a high-salt-soluble chromatin fraction (18–23% of total nuclear DNA). High-salt-resistant nuclear matrix contained 1–3% of the total nuclear DNA and was subsequently treated with 0.4% Triton X-100 in LS buffer to remove nuclear envelope components.

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Abbreviations: AcNHFln, 2-acetylaminofluorene; dG-C8-AcNHFln, *N*-acetyl-*N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG-N^2-AcNHFln, 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene; dG-C8-NHFln, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; LS chromatin, low-salt chromatin; HS chromatin, high-salt chromatin.

DNA was isolated from nuclei and nuclear subfractions by incubation with proteinase K followed by rapid extraction with phenol and Sevag (chloroform/isopropanol, 24:1) as described (19), except that prior to incubation with proteinase K (19), the high-salt-soluble chromatin fraction was dialyzed against deionized water at 4°C and the nuclear matrix fraction was digested with a mixture of RNases A and T1 (100 μg/ml and 50 units/ml, respectively) to obtain more complete removal of RNA.

Gel Electrophoresis. Four micrograms of DNA from each fraction was resolved on 1% agarose gels with 90 mM Tris/90 mM boric acid/3 mM EDTA, pH 8.3/0.1 μg of ethidium bromide per ml as gel and running buffer.

^{32}P-Labeling Assay. DNA was digested with a mixture of micrococcal nuclease and spleen phosphodiesterase to deoxyribonucleoside 3′-monophosphates, which were then converted to 5′-^{32}P-labeled deoxyribonucleoside 3′,5′-bisphosphates by T4 polynucleotide kinase-catalyzed transfer of [^{32}P]phosphate from [γ-^{32}P]ATP (6). The ^{32}P-labeled adducts were analyzed by a four-directional TLC procedure. To calculate adduct levels, total nucleotides were analyzed by one-dimensional PEI-cellulose TLC after appropriate dilution of the labeled digest (7). Adduct levels were determined as described (6, 7). Calculations were done according to relative adduct labeling (RAL). RAL = [cpm in adduct nucleotide(s)/cpm in total nucleotides] × (1/dilution factor). Considering 100% recovery of adducts (the actual values may be somewhat lower, see ref. 6), the RAL values were then translated into fmol of adduct(s) per μg of DNA (7, 19).

RESULTS

Following endogenous nuclease digestion (37°C for 45 min), purified rat liver nuclei were exhaustively and sequentially extracted with LS buffer and HS buffer. This procedure resulted in liberation of 70–75% of the total nuclear DNA in LS buffer (LS chromatin) and 22–23% of the total nuclear DNA in the HS buffer (HS chromatin) and left 1.8–2.5% of the total nuclear DNA attached to nuclear matrix. The DNA attached to nuclear matrix corresponded to DNA fragments that contained the region(s) where DNA loops were constrained. The weighed average size of DNA in all nuclear subfractions was the same, 320 base pairs, and showed a typical nucleosomal repeat pattern (Fig. 1). Recovery of DNA in each nuclear subfraction and digestion pattern were both similar to those reported by others (22, 30, 31). In those studies, the progression of replication forks along DNA loops was used to show that DNA in HS chromatin is contiguous with DNA at the points where DNA loops are constrained by nuclear matrix (Fig. 2). DNA in LS chromatin is contiguous with that in HS chromatin and includes the distal regions of the loop. Using this nuclear fractionation protocol we examined the general distribution of DNA adducts within DNA loops following a multiple-dose regimen of AcNHFln.

The concentration of individual DNA adducts in unfractionated nuclei, LS chromatin, HS chromatin, and matrix DNAs was measured by PEI-cellulose TLC analysis of ^{32}P-labeled adduct nucleotides and total nucleotides, followed by determination of Cerenkov radiation (6, 7). As reported (7), ^{32}P mapping of *in vivo* AcNHFln-damaged DNA showed two acetylated (dG-C8-AcNHFln and dG-N^2-AcNHFln) and one deacetylated (dG-C8-NHFln) adducts. The dG-C8-AcNHFln, obtained under our digestion conditions as a mixture of mono- and dinucleotides (19), was detected only 10 hr after the initial dose (not shown) but was no longer detected at 6 and 60 days after the last dose (i.e., 34 and 88 days after the initial dose, respectively). Typical "fingerprints" obtained from unfractionated nuclei and various nuclear subfractions 60 days after the last dose are shown in Fig. 3. In addition to the presence of two known

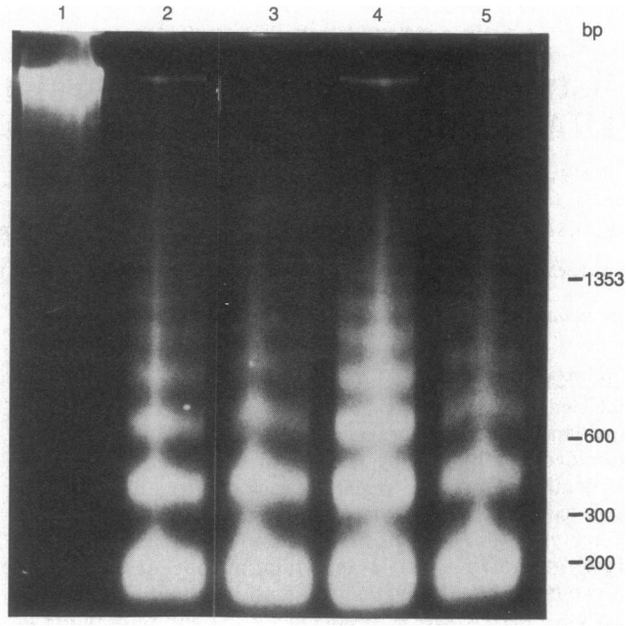

FIG. 1. Agarose gel electrophoretic analysis of the DNA in nuclear subfractions. DNA was resolved on 1% agarose gels in the presence of ethidium bromide. Lanes 1–5 corresponded to DNA from undigested nuclei, postdigestion nuclei, LS chromatin, HS chromatin, and nuclear matrix, respectively. Assignment of molecular weight was based on the migration of *Hae* III restriction fragments of PM-2 phage DNA. bp, Base pairs.

adducts, dG-C8-NHFln and dG-N^2-AcNHFln (7), two unknown minor adducts (spots 10 and 17, Fig. 3) were also detected at the two later time points; these unknown adducts were undetectable initially. Qualitatively, no differences were observed between DNA-bound metabolites in unfractionated nuclei, LS chromatin, HS chromatin, and nuclear

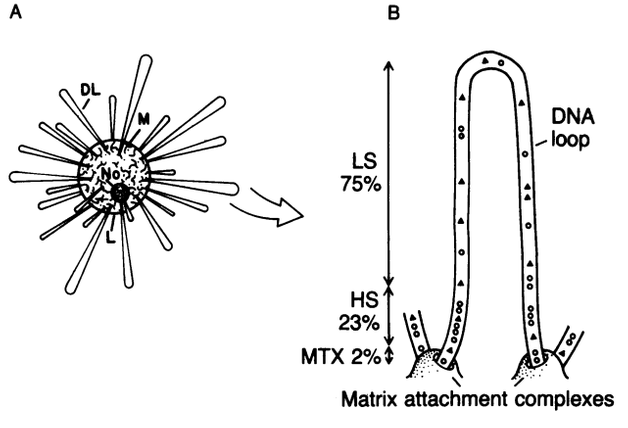

FIG. 2. (*A*) Hypothetical structure of DNA loops. Following removal of histones and the majority of nonhistone proteins, DNA remains attached to the scaffolding structure of the nucleus—i.e., the nuclear matrix—in the form of multiple DNA loops of varying lengths of 5–200 kilobase pairs (22). Only a few of the DNA loops are shown for diagramatic purposes. L, fibrous pore-complex lamina; M, internal nuclear matrix protein fibers; DL, DNA loops; No, nucleolar region. (*B*) Cartoon of an enlarged region of a DNA loop shows its attachment to regions of the nuclear matrix. The qualitative and quantitative (relative percent DNA recovery) distribution of the loop DNA in chromatin fractions, based on the topological progression of replication forks along the DNA loop (22), is shown to the left. LS, LS chromatin; HS, HS chromatin; MTX, high-salt-insoluble matrix DNA fragments. The relative distribution of dG-C8-NHFln (○) and dG-N^2-AcNHFln (△) along the DNA loops is based on the data obtained at 60 days after the last dose of AcNHFLn (see Fig. 4).

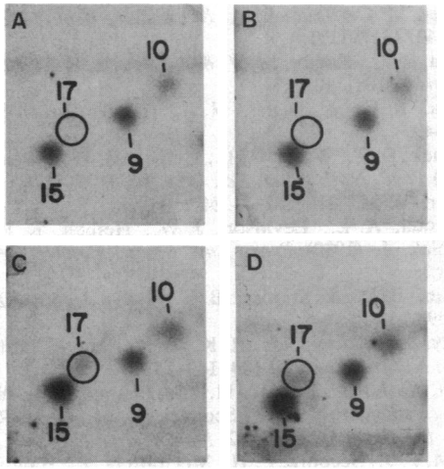

FIG. 3. ^{32}P fingerprints of DNA from unfractionated nuclei (*A*), LS chromatin (*B*), HS chromatin (*C*), and nuclear matrix (*D*) from rat liver 60 days after administration of AcNHFln (60 mg/kg) once a week for 4 weeks. The various subfractions were prepared by endogenous digestion of the nuclei, followed by differential extraction of chromatin with low- and high-ionic strength buffers. DNA was digested and subjected to ^{32}P-labeling and mapping by four-directional PEI-cellulose TLC (6, 7). About 300 μCi (1 Ci = 37 GBq) of labeled digest was chromatographed. Screen-enhanced autoradiography was at −80°C for 15 hr. Spots 9 and 15 are 3′,5′-bisphosphates of dG-N^2-AcNHFln and dG-C8-NHFln, respectively. Spots 10 and 17 have not been identified. For consistency, adduct numbering is the same as described (7).

matrix DNAs but significant quantitative differences were observed (Fig. 4). Initially (10 hr after the first dose) all adducts were distributed randomly (Fig. 4*A*). However, 6 days after the last dose, the concentration of the dG-C8-NHFln in matrix-associated DNAs was found to be ≈1.5 times higher than in unfractionated nuclei, LS chromatin, or HS chromatin, indicating a partial selectivity in the persistence of this adduct from loop regions (Fig. 4*B*). Sixty days after the last dose, this DNA lesion continued to persist in the proximity of the DNA loops attached to matrix, as DNA fragments recovered from both matrix and HS chromatin contained 2 times higher concentration of the lesion compared to unfractionated nuclei or LS chromatin (Fig. 4, see also Fig. 2). The other known (dG-N^2-AcNHFln) and unknown (spots 10 and 17, Fig. 3) lesions were also persistent but were present at comparable levels in the various fractions.

We also determined normal nucleotide compositions of DNAs recovered from the various chromatin fractions to ascertain whether the adduct distribution observed was influenced by guanine content. The results indicated this not to be the case since guanine was distributed similarly in LS chromatin (20.9%), HS chromatin (20.7%), and matrix (20.4%) DNAs.

DISCUSSION

We have examined the intranuclear distribution of DNA adducts in rat liver following a multiple-dose regimen of AcNHFln by biochemically fractionating DNA loops into three regions corresponding to: DNA at the base of the loop containing the sites for nuclear matrix attachment (nuclear matrix-attached DNA fragments), DNA proximal to the loop attachment sites (high-salt-soluble chromatin DNA fragments), and DNA distal to the loop attachment sites constituting the bulk of DNA loops (low-salt-soluble chromatin DNA fragments) (see Fig. 2 and refs. 22 and 31). Our data suggest that 10 hr after the initial dose, DNA adducts

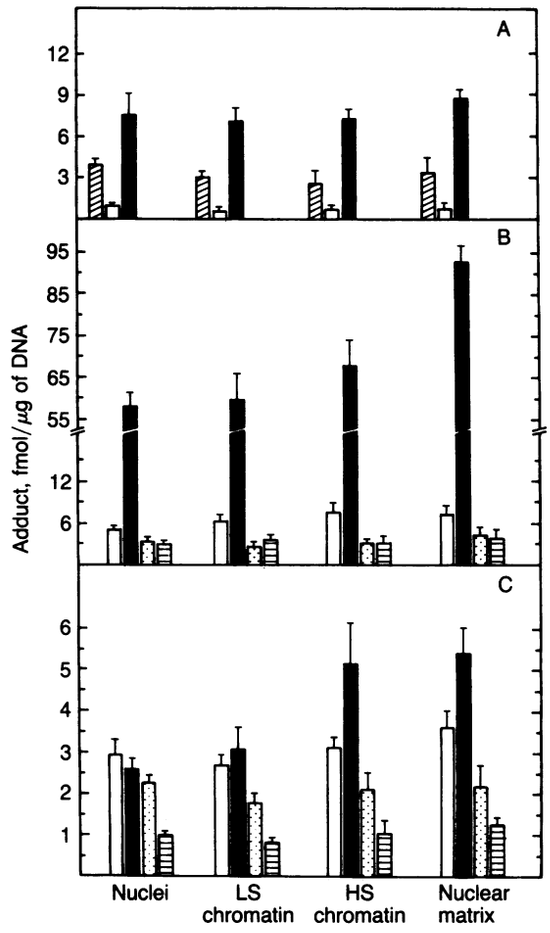

FIG. 4. Distribution of DNA adducts in soluble chromatin and nuclear matrix fractions from rats following administration of four weekly i.p. injections of 60 mg of AcNHFln per kg of body weight. Adduct concentrations were evaluated as follows: (*A*) 10 hr after the first dose; (*B*) 6 days after the fourth dose; (*C*) 60 days after the fourth dose. ▨, dG-C8-AcNHFln; □, dG-N^2-AcNHFln; ■, dG-C8-NHFln; ⊞ and ▤, unknowns.

dG-C8-AcNHFln, dG-N^2-AcNHFln, and dG-C8-NHFln have formed rapidly and apparently randomly in chromatin fractions from all regions of the DNA loops. At the level of resolution performed in this study, there appears to be no general restriction for formation of DNA adducts in any region of DNA loops.

Differences were observed in both the relative level of persistence and localization of individual persistent DNA adducts. Removal of dG-C8-AcNHFln was complete by 6 days of the last dose in all chromatin fractions. In contrast, a significant amount of dG-N^2-AcNHFln remained in all chromatin fractions even 60 days after the last dose. These data suggest that removal of the acetylated adducts, dG-C8-AcNHFln and dG-N^2-AcNHFln, from DNA may depend largely on repair enzyme specificity rather than localization along DNA loops. However, as suggested by Leadon and Hanawalt (32), further studies will be necessary before the effect of chromatin structure on the differential rate of removal of these two lesions can be fully evaluated.

Supporting the possibility that the localization of adducts within chromatin may influence removal rates are the data demonstrating nonrandom distribution of the deacetylated adduct, dG-C8-NHFln 6 days after the last dose. This phenomenon became more apparent by 60 days. Though dG-C8-NHFln adducts were formed with equal efficiency over the entire DNA loop, those lesions close to the loop's

attachment to matrix (HS chromatin and matrix DNA fragments, see Fig. 2) were markedly more inefficiently removed. We believe this reflects a true regional selectivity for loss or repair of dG-C8-NHFln adducts because all DNA loop regions showed a similar G·C content, equally efficient removal of dG-C8-AcNHFln adducts, and equally inefficient removal of dG-N^2-AcNHFln adducts. At present it is unclear whether dG-C8-NHFln adducts accumulate in HS chromatin and matrix DNAs randomly or as clusters within a subpopulation of DNA sequences. If adducts are indeed clustered, then certain regions of DNA loops would presumably show an even higher enrichment for persistent adducts than what we observed using total HS chromatin and matrix DNAs.

Though our studies report on the distribution of persistent DNA adducts relative to the DNA loop, benzo[*a*]pyrene adducts have been shown to form preferentially with DNA in the region of DNA loop's association with matrix (33–35). We found no evidence for selective formation of AcNHFln–DNA adducts throughout the DNA loop. One major reason for this discrepancy may lie in the fact that in this work we measured adduct levels, whereas in the previous studies only radiolabel associated with DNA was measured (33–35). It is not clear from the previous studies how much of the label may have been associated with DNA alone as opposed to protein components of the tight protein–DNA complexes, which have been shown to be a contaminant of even the purest DNA preparations (36, 37). However, we cannot rule out the possibility that the differences in the initial adduct formation observed with AcNHFln and benzo[*a*]pyrene may be due to their chemical nature.

Our findings coupled with the observation that dG-C8-NHFln is directly correlated with mutagenicity (8, 9) suggest intriguing possibilities for mechanisms involved in hepatocarcinogenesis. Since LS chromatin constitutes the majority of DNA loops (Fig. 2 and refs. 22, 31) and contains structural portions of transcribed genes (23–26), a significant portion of the dG-C8-NHFln adducts are in this region. These lesions are therefore in a position where they may give rise to mutations that may be important to conversion of protooncogenes to oncogenes (11–13). Alternatively, aberrant gene expression leading to hepatocarcinogenesis may be brought about by accumulation of dG-C8-NHFln adducts within regulatory gene sequences that have been shown to be enriched in DNA in the proximity of DNA loop's attachment sites (23–26). Also of potential significance might be persistence of dG-N^2-AcNHFln adducts throughout the DNA loops. In this regard, Hanawalt and coworkers (38) recently have reported deficient repair of pyrimidine dimers in the 5′ region of the dihydrofolate reductase gene in Chinese hamster ovary cells. Further studies on distribution of DNA adducts within other defined gene sequences will be necessary before these possibilities can be fully evaluated.

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