DISTRIBUTION OF COVALENTLY BOUND BENZO(a)PYRENE IN CHROMATIN

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Received April 5,1977

Summary

The distribution of the covalently bound polycyclic aromatic hydrocarbon carcinogen, benzo(a)pyrene, in nuclease digestible and undigestible regions of chromatin was studied. Binding of [^{3}H]benzo(a)pyrene was effected by incubating the carcinogen with calf thymus nuclei in the presence of NADPH and rat liver microsomes. The time-course of digestion of carcinogen-modified nuclei by Staphylococcal nuclease and DNase I was determined. Both enzymes result in preferential digestion of [^{3}H]benzo(a)-pyrene modified DNA, demonstrating that the carcinogen binds more extensively to nuclease accessible regions of DNA in chromatin. Comparison of the kinetics of digestion by the two enzymes suggests that the carcinogen primarily binds to the outermost "spacer" regions of DNA in the nucleosome.

Introduction

Covalent attachment of carcinogens to macromolecules has been well documented and has been implicated as a key initiating step in carcinogenesis (1). The carcinogen,benzo(a)pyrene, has been shown to bind to DNA following metabolism by microsomal enzymes (2,3). Although the metabolic intermediate of BP[2] which binds to DNA and the structure of the BP-DNA adduct have been elucidated (4,5), little is known concerning the intra-chromatin distribution of the carcinogen. Considering the role of organized chromatin structure in the regulation of gene activity, analysis of the distribution of the carcinogen within this structure may be of considerable importance in defining the molecular mechanism(s) of carcinogenesis by PAHs.

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[2]Abbreviations: BP, benzo(a)pyrene; PAH, polycyclic aromatic hydrocarbon; AAF, acetylaminofluorene.

ISSN 0006-291X

Several reports have described *in vitro* binding of PAHs to chromatin components accompanying incubation of carcinogen with nuclei, and have demonstrated the requirement for NADPH-dependent, mixed-function oxidases localized in either the nuclear envelope or endoplasmic reticulum (6-10). We have developed a similar system employing calf thymus nuclei, NADPH, and rat liver microsomes to effect activation and binding of BP. Nuclease digestion of chromatin has been used to distinguish the distribution of [^{3}H]BP in regions of DNA which differ in susceptibility to digestion because of their location in nucleosomes.

Materials and Methods

[^{3}H]BP (8.3 or 26 Ci/mmole) was obtained from Amersham-Searle. DNase I (EC 3.1.4.5.) at 2900 U/mg and Staphylococcal nuclease (EC 3.1.4.7.) at 22,000 U/mg were purchased from Worthington Biochemicals. Liver microsomes from 3-methylcholanthrene-induced rats were prepared as described by Pietrapaolo and Weinstein (11).

Nuclei were isolated from frozen calf thymus by modification of the procedure of Johnson *et al.* (12). Homogenization in a Virtis Model 60 homogenizer was carried out for 3 min at 1200 RPM in 0.32 M sucrose-3 mM $MgCl_2$-0.5 mM $CaCl_2$. Nuclei were washed twice in 0.25 M sucrose-3 mM $MgCl_2$-0.5 mM $CaCl_2$.

Nuclei (1-2 x 10^8/ml) were incubated with 0.2 mCi/ml of [^{3}H]BP (8-24 μM), 1 mM NADPH, and 1 mg/ml of microsomal protein in the 0.25 M sucrose solution at 37°C for 30 min. Binding of 10-100 pmoles/mg of DNA was routinely obtained in our system. Following incubation, the nuclei were washed twice in the 0.25 M sucrose solution containing 0.5% Triton X-100, washed once with the 0.25 M sucrose solution, and resuspended in the digestion buffer consisting of 3% sucrose-1 mM Tris-HCl-0.1 mM $CaCl_2$, pH 7.9 (13).

Staphylococcal nuclease or DNase I was added to a concentration of 5-20 μg/ml and digestion was allowed to proceed at 37°C. Aliquots were periodically removed and after addition of EDTA to 5 mM were: 1) precipitated by addition of 1 M $HClO_4$-1 M NaCl, 2) layered on 5-20% sucrose gradients (in 5 mM EDTA) and sedimented 16 hr at 25,000 RPM in a Beckman SW41 rotor; 3) extracted twice with phenol, twice with chloroform-isoamyl alcohol, and precipitated with ethanol. Extracted DNA was analyzed on 3% acrylamide-0.5% agarose gels in 0.04 M Tris-0.02 M sodium acetate-2 mM EDTA, pH 7.0. Gels were stained with ethidium bromide (1 μg/ml) and sliced and counted in Biofluor after solubilization in Protosol (New England Nuclear).

DNA extracted from [^{3}H]BP modified nuclei was digested in the same buffer with 1 μg/ml of either enzyme and 0.5 mg/ml of DNA. The reaction was stopped by adding EDTA to 5 mM, and the samples were precipitated with $HClO_4$ or extracted as described above.

Results and Discussion

The time-course of digestion of [^{3}H]BP modified nuclei by Staphylococcal nuclease and DNase I is presented in Figures 1 and 2.

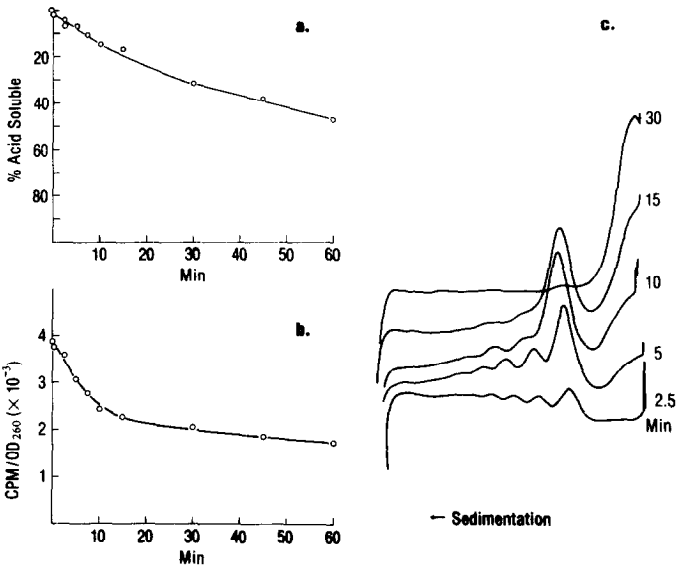

Figure 1. Staphylococcal nuclease digestion of [^{3}H]BP modified nuclei.
(a) % Acid-soluble products versus time of digestion determined from A_{260} in $HClO_4$ supernatant.
(b) Specific activity versus time of extracted, undigested DNA (cpm and A_{260} determined on same sample).
(c) Recorded optical density profiles (A_{254}) of sucrose gradients of digested chromatin at times indicated.

Digestion with Staphylococcal nuclease proceeds to 50% acid-soluble products (Figure 1a) and results in buildup of monomer-sized particles (Figure 1c), followed by conversion of the monomer to smaller material, while digestion with DNase I proceeds to 80% (or greater) acid-soluble products (Figure 2a) and results in direct conversion to a size smaller than monomer (Figure 2c).

As can be seen in Figures 1b and 2b, treatment of nuclei with either enzyme decreases the specific activity of the undigested DNA. In comparison, when DNA is extracted from the modified nuclei and digested with either enzyme, the undigested DNA has the same specific activity at all time points (not illustrated). Thus, the carcinogen appears to be preferentially located in digestible regions of DNA in chromatin. With Staphylococcal nuclease (Figure 1b) the [^{3}H]BP DNA is preferentially

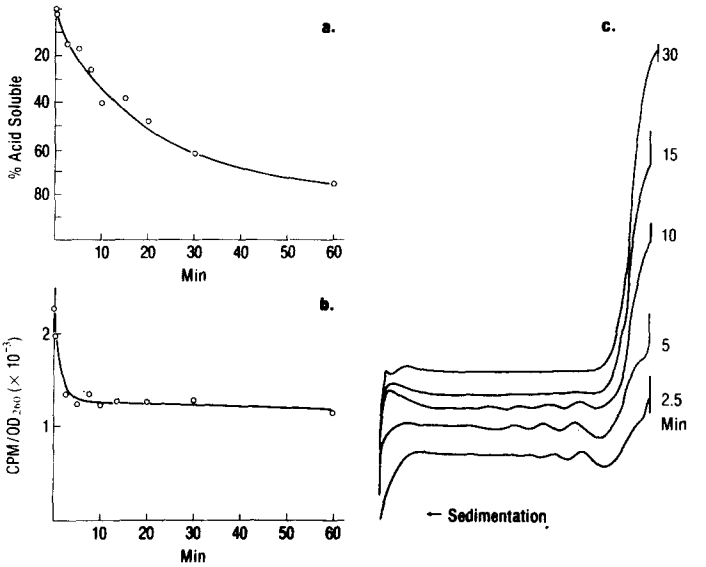

Figure 2. DNase I digestion of [^{3}H]BP modified nuclei. (a), (b), (c), as indicated for Figure 1.

removed throughout the digestion, but with differing kinetics after the first 15% digestion (10 min). As shown in Figure 2b, DNase I results in an initial drop in specific activity up to 15% digestion (2.5 min), after which the carcinogen-modified DNA is removed at the same rate as unmodified DNA. Increasing the amount of [^{3}H]BP bound (initial specific activity) results in slower removal of the carcinogen-modified DNA by DNase I, suggesting that increasing the extent of modification results in increased binding to DNase I susceptible sites.

The sucrose gradient profiles and acid solubility curves demonstrate that typical digestion patterns are achieved after modification of nuclei with the carcinogen. The digestion kinetics and products as characterized by these techniques agree with previous studies which have demonstrated that Staphylococcal nuclease digests DNA between nucleosomes, whereas DNase I digests DNA within as well as between nucleosomes (14-16). Analysis of the undigested DNA fragments by polyacrylamide gel electro-

phoresis has confirmed these results and has further shown that the presence of the carcinogen has no effect on the cleavage pattern of the enzymes, as the profiles of radioactivity at various time points parallel the DNA banding patterns obtained by staining with ethidium bromide.

The difference in kinetics of [^{3}H]BP DNA removal by the two enzymes indicates that preferential binding occurs primarily to the Staphylococcal nuclease sensitive sites. Other studies have shown that of the DNA digested by Staphylococcal nuclease, at least 30% of the total 50% digestion results in conversion of the approximately 200 base pairs/nucleosome to the minimal 140 base pair monomer (15,17,18); thus, the majority of the [^{3}H]BP must be bound to this interbead or spacer sequence. The very early drop in specific activity seen with DNase I could also be accounted for by digestion between particles as the sucrose gradient profiles at the early times of DNase I digestion show discrete multimers of particles. However, because DNase I continues to digest at more sites within the nucleosome, it has an equal chance of attacking modified and unmodified sites, as indicated by the leveling off of the specific activity.

Other studies of the digestion of chromatin modified by N,N-dimethylnitrosamine and AAF have indicated an uneven distribution of these carcinogens in DNA resistant or sensitive to digestion (19-21). These reports have primarily used *in vivo* modified DNA and limit digests of chromatin, making their results difficult to compare with ours. Metzger *et al.* (22) have characterized the distribution of N-acetoxy-AAF after *in vitro* modification of duck reticulocyte chromatin and have found a twofold higher binding of AAF in Staphylococcal nuclease digestible regions of DNA. In contrast to our results, they demonstrated parallel kinetics of release of carcinogen and acid-soluble products throughout the digestion. This difference may be related to the approximately 1000 times higher level of modification with AAF than with BP, since most of the sites accessible to carcinogen must be bound.

It is apparent from these studies that the accessibility of DNA in chromatin can determine the distribution of binding of a carcinogen. The consequences of binding of BP in regions within versus between nucleosomes are difficult to access. Sequences which are actively transcribed are known to be equally distributed between these regions of chromatin, although some evidence exists for increased susceptibility of transcribed sequences to DNase I (23). One consideration is that the amount of carcinogen which a single cell actually binds and which could initiate carcinogenesis is much smaller than the amount studied in models such as this. If a hierarchy of accessible sites was defined, it seems possible that the DNA most accessible to carcinogen might be in regions of chromatin which are genetically most active. Partial support for this has come from a report by Moses et al. (24), who fractionated chromatin from cells incubated with several PAH carcinogens by shearing and sedimentation on sucrose gradients, and demonstrated that the carcinogen binding occurred in the fractions most active in transcription.

Acknowledgments

This work was supported by NCI Grants CA 17085 and CA 08748. C.L.J. is a predoctoral trainee supported by USPHS Grant GM 01918.

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