Binding of benzo[a]pyrene to different chromatin domains following activation at the nuclear membrane

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When isolated liver nuclei from methylcholanthrene-treated rats are incubated with benzopyrene, covalent adducts are formed between DNA and the ultimate carcinogen, benzopyrene diol epoxide. Brief digestion with DNasel, or micrococcal nuclease has been used to demonstrate that benzopyrene metabolites bind more readily to DNA in chromatin regions with a more open, active conformation than to inactive chromatin.

Chemical carcinogens, or their metabolites, are thought to initiate cell transformation by reacting covalently with the DNA of target tissues, thus causing mutations (1). The ubiquitous pollutant benzo[a]pyrene (BP) is metabolically activated by microsomal or nuclear forms of aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase (EH) (2,3) to anti-BP diol epoxide (BPDE). BPDE is highly mutagenic and carcinogenic, and is the major BP metabolite which binds to DNA, mainly to guanine (4). However, little is known of the specificity of binding of this carcinogen with respect to DNA sequence.

The packaging of nuclear DNA by chromatin proteins clearly restricts the binding of BPDE, and other carcinogens, since internucleosomal linker DNA is at least two to three times more accessible to carcinogen attack than nucleosomal core DNA (5-8). Since most of the genome is packaged into nucleosome-like particles (9), the functional significance of these observations is not clear. Transcribed DNA sequences exist in chromatin in an altered, more open conformation than bulk DNA, making them very sensitive to nucleases (10-12). For example, sites hypersensitive to DNasel are present near the 5' end of many genes, in tissues where they are expressed (13). If these regions were readily accessible targets for carcinogenic damage, the resulting lesions might have profound effects on the cell, including activation of cellular oncogenes (14).

We have begun to investigate this problem by determining the distribution of BP binding to DNA in different domains of rat liver

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chromatin, defined by their nuclease sensitivity, following metabolic activation of the carcinogen at the nuclear membrane.

Materials and Methods

G-[\textsuperscript{3}H]BP (40 Ci/mmol) was from Amersham International, and G-[\textsuperscript{3}H]BPDE ((\(\pm\))trans-7,8-dihydrobenzo[a]pyrene-7,8-diol-9,10-epoxide (anti), 479 mCi/mmol) from Midwest Research Institute, Mo., U.S.A., through the National Cancer Institute.

Incubation of nuclei with [\textsuperscript{3}H]BP

Wistar rats were induced for AHH activity by injecting 3-methylcholanthrene (MC) at 40 mg/kg, 44-48 h before sacrifice. Liver nuclei were prepared as in reference 15 except that MgCl\textsubscript{2} was replaced by 50 mM Tris/HCl, pH 7.5, 25 mM KCl, 5 mM MgCl\textsubscript{2} (TKM). Rat liver microsomes were isolated (16) with 0.32 M sucrose, 1.5 mM MnCl\textsubscript{2} as homogenizing medium. Isolated nuclei were incubated with [\textsuperscript{3}H]BP (0.37 Ci/mmol, 20 \(\mu\)Ci/ml, 54 \(\mu\)M) at 37°C for 60 min in 0.32 M sucrose, 1.5 mM MnCl\textsubscript{2}, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM NADPH. The cationic composition of this buffer is sufficient to completely inhibit endogenous rat liver endonucleases (17).

Nuclei were diluted tenfold with ice-cold 0.25 M sucrose in TKM, centrifuged at 1500 g, 10 min, and washed twice with diluting buffer. In some experiments 5 mM sodium butyrate was included in all solutions for nuclear isolation, [\textsuperscript{3}H]BP incubation, and nuclease digestion.

Analysis of [\textsuperscript{3}H]BP binding to nuclear macromolecules

[\textsuperscript{3}H]BP-labelled samples were resuspended in 8 M urea, 1% SDS 0.001 M EDTA, 0.15 M sodium phosphate, pH 6.8, and stirred for 30 min at room temperature. The nuclear lysate was extracted twice with chloroform-iso-amyl alcohol-phenol (CIP) (24:1:25). Proteins were recovered from the combined organic phases (18), with inclusion of two ethyl acetate washes, for quantitation (19) and scintillation counting. Combined aqueous phases from the CIP extraction were extracted exhaustively with ethyl acetate and diethyl ether. Covalently bound [\textsuperscript{3}H]BP in the aqueous phase was then either (a) assayed directly for radioactivity and DNA content (20), following dialysis and precipitation of nucleic acids with 0.5 M HClO\textsubscript{4}; or (b) after separation of RNA and DNA by hydroxyapatite (HAP) chromatography. The aqueous phase was applied to a 1.5-x-2-cm HAP column, equilibrated in 8 M urea, 0.15 M sodium phosphate, pH 6.8, and sequentially washed with urea-phosphate buffer to elute residual protein and some RNA, 0.15 M phosphate, pH 6.8, and 0.18 M phosphate, pH 6.8, which elute remaining RNA, and finally 0.48 M phosphate, which elutes DNA. Fractions containing DNA were dialysed against water, and DNA recovered by lyophilization or precipitation (0.5 M HClO\textsubscript{4}). HAP-purified DNA contained less than 2% RNA, and less than 1.6% of nuclear protein. The specific activity of [\textsuperscript{3}H]BP-modified, HAP-purified DNA is usually slightly lower than [\textsuperscript{3}H]BP-DNA obtained by CIP extraction and RNase/protease digestion. HAP chromatography is claimed to be the superior method for isolating carcinogen-modified DNA (21). To analyse BP-nucleoside adducts,
BENZO[A]PYRENE BINDING TO CHROMATIN DOMAINS

[\(^3\text{H}\)]BP-modified DNA, purified by HAP chromatography, was hydrolysed to nucleosides as described (22). Nucleosides and nucleoside adducts were separated by Sephadex LH20 chromatography, using a 40-to-100% methanol gradient (23). Salmon-sperm DNA (1.8 mg/ml) was incubated for 27 h at 37°C in 10 mM Tris/HC1, pH 7.4, 10 mM MgCl\(_2\) with [\(^3\text{H}\)]BPDE (15.8 µCi/ml), digested, and analysed in the same way.

Digestion of nuclei with nucleases

[\(^3\text{H}\)]BP-modified nuclei were washed three times in 10 mM Tris/HC1, pH 7.4, 10 mM NaCl, 3 mM MgCl\(_2\), resuspended in the same buffer at 250 to 500 µg DNA/ml and digested with 1-2 µg/ml (2.6-5.2 units/ml) DNaseI at 37°C. At various time intervals, duplicate samples were removed and EDTA added to 10 mM. Undigested DNA was recovered by low-speed centrifugation after adjusting to 0.7 M HClO\(_4\). Acid-soluble nucleotides in the supernatant were quantitated (\(A_{260}\)) to estimate the extent of digestion. Undigested DNA was extracted with CIP and ethyl acetate before exhaustive dialysis and lyophilization. DNA was redissolved in 1 mM EDTA, 20 mM sodium phosphate, pH 6.8, and digested with heat-treated RNase (100 µg/ml) for 2 h. SDS was added to 0.1%, and the sample digested for 2 h with proteinase K (200 µg/ml). DNA was recovered and its concentration and [\(^3\text{H}\)]BP content measured (24).

[\(^3\text{H}\)]BP-modified nuclei (0.25 mg of DNA/ml) were washed in 0.25 M sucrose, 1.5 mM MgCl\(_2\), containing firstly 0.2% and then 0.1% Triton X-100, resuspended in medium B (25) at 2.5 mg DNA/ml and digested with 0.2 units/ml micrococcal nuclease at 29°C for 1.5 min. The reaction was stopped by adding EGTA to 2 mM and the mixture fractionated, exactly as described (12,25). Fraction S was precipitated with 0.1 vol. 3 M sodium acetate, 0.1 M magnesium acetate, and 3 vol. ethanol. Chromatin fractions were analysed for [\(^3\text{H}\)]BP binding to DNA by CIP extraction and HAP chromatography.

Results

We have investigated the binding of BNP to DNA in different chromatin domains, using isolated liver nuclei in which metabolic activation to form the ultimate carcinogen, BPDE, is dependent on AHH and EH activities associated with the nuclear membrane (2,3).

When liver nuclei are incubated with [\(^3\text{H}\)]BP at 37°C, in a buffer mimicking the unbound cation concentrations of the intact cell (26,27), carcinogen binds covalently to nucleic acid and proteins (Table 1). Binding to both macromolecules is greatly stimulated when liver AHH activity is induced in vivo by pretreatment with 3-methylcholanthrene (MC), and is almost completely dependent on exogenous NADPH (Table 1). Addition of liver microsomes, from MC-treated rats, slightly decreased the extent of binding to both nucleic acids and proteins. This may reflect contamination of the washed nuclear pellet with poorly labelled microsomal RNA and protein; a potential complication in nuclear incubation systems which use microsomes for carcinogen activation (5). BP binding to nuclear macromolecules in MC-induced nuclei does not increase significantly during longer
**Table 1. Binding of [3H]BP to macromolecules of isolated rat liver nuclei**

Nuclei from normal or MC-induced rats were incubated for 1 h at 37°C (see Materials and Methods) with 54 μM [3H]BP and other additions as indicated. Microsomes were added at a final concentration of 1 mg protein/ml. Results are averages of two duplicates.

<table>
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<td>Proteins (pmol/mg protein)</td>
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* 27 μM BP, not 54.

incubations (not shown) or when the [3H]BP concentration is increased from 27 to 54 μM (K_m for nuclear AHH is reported as 15 μM BP (2)).

In subsequent experiments liver nuclei from MC-induced rats were incubated for 60 min with 1 mM NADPH, but without microsomes. When the DNA from such an incubation is purified by HAP chromatography 15 to 25 pmol of BP are routinely bound per mg of DNA. The [3H]BP-modified DNA recovered from HAP columns has been exhaustively extracted with ethyl acetate to remove non-covalently bound hydrocarbon. The radioactivity still associated with DNA is completely DNaseI-sensitive, but not RNase- or protease-sensitive, indicating minimal contamination with BP-modified RNA or protein.

To prove the covalent nature of the binding of [3H]BP to nuclear DNA [3H]BP-nucleoside adducts were isolated by exhaustive enzymic digestion and Sephadex LH20 chromatography (23). When the digest of [3H]BP-modified nuclear DNA is analysed (Fig. 1a) the first radioactive peak (A) elutes with unmodified nucleosides. This material has been claimed to include nuclease-resistant modified oligodeoxyribonucleotides, or phosphotriesters, and contaminating modified proteins (28). In the present work the contribution of protein contaminants should be very low because DNA has been purified by CIP extraction and HAP chromatography (21). The major radioactive peak (B) elutes in a similar position to the major nucleoside adduct
Fig. 1. Sephadex LH20 chromatography of nucleoside adducts derived from $[^3H]$BP-modified nuclear DNA. 
(a) Liver nuclei were incubated with $[^3H]$BP, and DNA was isolated by CIP extraction and HAP chromatography, enzymatically hydrolysed, and fractionated on Sephadex LH20. 
(b) Salmon testis DNA was modified by reaction with $[^3H]$BPDE, hydrolysed, and fractionated as in a. Peaks were identified by comparison with (29), as nucleoside adducts substituted at following positions; B, N2-deoxyguanosine; D, O6-deoxyguanosine; E, N7-deoxyguanosine and N6-deoxyadenosine. ($\bullet$), $A_{260}$; (-), d.p.m. $[^3H] \times 10^{-3}$.

formed when the ultimate carcinogen BPDE reacts with purified DNA (Fig. 1b) and is presumed to represent the adduct formed between BPDE and the 2-amino group of deoxyguanosine (29). Minor adducts produced in the nuclear incubation system coincide with other components of BPDE-modified DNA, and are tentatively identified as O6- and N7-substituted deoxyguanosine and N6-substituted deoxyadenosine (29). Negligible amounts of late-eluting adducts (peak C) corresponding to 9-hydroxy BP derivatives (3,28) were observed (Fig. 1a). This contrasts with other reports on incubation of BP with isolated nuclei, and may indicate high levels of EH activity in our nuclei (3).

BP-induced DNA damage includes covalent nucleoside adducts, phosphotriesters, and strand breaks (3,29,30). Others have conclusively demonstrated that BP-modified DNA (over the range 10 to 150 pmol BP/mg DNA), purified from nuclei or cells incubated with BP, shows identical digestion kinetics to unmodified DNA, upon digestion with DNaseI or micrococcal nuclease (5,7). Our results with DNA modified to the extent of 15-20 pmol/mg DNA confirm these observations (not
Fig. 2. Changes in specific radioactivity of [3H]BP-modified nuclear DNA during digestion with DNase I. DNA was purified from [3H]BP-modified nuclei after various periods of digestion with DNase I, and its specific radioactivity determined (see Materials and Methods). In one experiment sodium butyrate (5 mM) was present during nuclear isolation, incubation with BP, washing, and DNase I digestion. Results are plotted as the mean ± S.E.M., (●) without butyrate (n = 6); (○), with butyrate (n = 2).

shown). Thus, BP-induced lesions do not appear to affect the reaction of these nucleases with DNA per se.

Actively transcribed genes are packaged in an open conformation that allows their digestion to non-hybridizable fragments by brief treatment of nuclei with DNase I, whereas non-transcribed DNA is relatively resistant to digestion (10). Binding of [3H]BP to transcribed regions of nuclear DNA may therefore be estimated indirectly by comparing the specific radioactivity of DNase I-resistant (non-transcribed) DNA with that of total nuclear DNA measured before DNase I digestion has commenced. When [3H]BP-modified rat liver nuclei are digested with DNase I, there is a rapid drop in specific radioactivity of DNA (Fig. 2) at an early stage of digestion (less than 5%), implying that hydrolysed fragments of transcribed DNA bound much more BP than resistant, non-transcribed DNA.

When sodium butyrate was included in all solutions during cell lysis, nuclear isolation, incubation, and digestion, to inhibit histone deacetylation (10,31), a significant drop in specific radioactivity of undigested DNA was again seen during DNase I digestion (Fig. 2) although the effect was less pronounced than with non-butyrate-treated nuclei. These experiments indicate that transcribed regions of chromatin DNA, as defined by DNase I sensitivity, bind 5 to 10 times more BP than
non-transcribed regions (e.g. in the absence of butyrate a 50% drop in radioactivity accompanies digestion of 5% of nuclear DNA).

Micrococcal nuclease preferentially attacks chromatin regions containing transcribed DNA (11,12,32), and in some cases its rate of attack is more sensitive to gene transcription rate than DNase I (33). Furthermore, unlike DNase I, it releases transcribed DNA sequences in macromolecular form, allowing a direct measure of BP binding to these sequences. We have fractionated BP-modified chromatin by a very mild micrococcal nuclease digestion procedure (23), which releases: a fraction (S) containing predominantly mono-nucleosomes; an oligonucleosome fraction (P2) enriched in actively transcribed DNA sequences (12) and engaged RNA polymerase II (25); and a bulk chromatin fraction (P1), depleted of transcribed sequences, and containing remnants of the nuclear structure (12,25). If similar fractions are prepared from rat liver nuclei, fractions P2 and S are moderately enriched in active genes (e.g. serum albumin) and depleted in inactive genes (e.g. seminal vesicle protein IV (34)), relative to fraction P1 (A. Ryan and M.B., in preparation).

When [3H]BP-modified liver nuclei were digested with micrococcal nuclease, and the DNA of each fraction purified by HAP chromatography, the degree of modification of DNA from P2 and S fractions varied considerably (Table 2). In the absence of butyrate, P2 DNA had a significantly higher level of BP binding (2- to 4-fold) than P1 DNA in only 2 out of 7 experiments. When butyrate was included, however, a more consistent pattern emerged: binding of BP to P2 DNA was greater (1.5- to 3-fold) than to P1 DNA in 3 out of 4 experiments. Carcinogen binding to DNA of fraction S was increased 1.5- to 2-fold over P1 DNA in 2 out of 7 experiments without butyrate, and 1 out of 4 experiments with butyrate. The increased binding of BP to S DNA partly correlated with increased amounts of DNA in the S fraction; and is consistent with the greater enrichment of active gene sequences in S with increasing digestion time (A. Ryan and M.B., in preparation).

Discussion

BP is not normally a hepatocarcinogen in the rat although it can induce liver tumours in certain circumstances (35,36). Efficient further metabolism of DNA-binding BP metabolites, and repair of hepatic DNA damage, have been advanced as possible reasons for the low incidence of BP-induced liver tumours (35,37). The isolated liver nuclear incubation system used here allows BP activation at the nuclear membrane and formation of the major BPDE-DNA adducts characteristic of target and non-target tissues (38), without interference from phase-II conjugating enzymes, and in the absence of DNA repair.

We provide evidence that BP metabolites bind more readily to regions of chromatin in an open, active conformation than to inactive chromatin, as defined by two different nucleases. Similar results with DNase I have been reported for cultured cell lines incubated with BP (24). Selective binding of BP could only be demonstrated consistently with micrococcal nuclease when butyrate was present during cell breakage and further manipulation. Although butyrate will preserve in
Table 2. Specific radioactivity of [3H]BP-modified DNA from chromatin fractions generated by micrococcal nuclease digestion

DNA was purified from each fraction by CIP extraction and HAP chromatography. Sodium butyrate (5 mM) was added as indicated to all solutions used in nuclear isolation, incubation with BP, washing, nuclease digestion, and chromatin fractionation. Rats were 150 g body wt. except experiments 7 and 11, where 90-g rats were used. Average % nuclear DNA in each fraction were: 92.4% P1; 4.5% P2; 3.0% S (without butyrate); and 97.2% P1; 1.2% P2; 1.6% S (with butyrate).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Butyrate</th>
<th>[3H]BP bound to DNA of chromatin fraction (pmol/mg DNA)</th>
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Vivo levels of acetylated histones, and these are claimed to be enriched in active chromatin, it has many other effects. Thus, it alters gene expression, DNA replication and repair, and phosphorylation of chromatin proteins, and can change the rate of chromatin digestion by nucleases (31,39). In our work, butyrate treatment doubled the rate of DNaseI digestion of nuclear DNA (not shown), yet halved the rate of micrococcal nuclease digestion (Table 2). This may explain the apparent failure of butyrate to augment selective BP binding to DNaseI-sensitive chromatin, in spite of its obvious effect in the micrococcal nuclease experiments.

The modest enrichment of BP-DNA adducts in active chromatin observed here has recently been confirmed (F.O., M.B., and A. Ryan, in preparation) using a different fraction procedure (11). Equivalent findings have been reported for the regulatory region of SV40 minichromosomes (6). The significance of this selectivity for oncogene expression and transformation remains to be determined.

Acknowledgements

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References