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The distribution of benzo(a)pyrene DNA adducts in mammalian chromatin

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ABSTRACT

This paper describes the distribution of DNA-lesions generated by the potent carcinogen benzo(a)pyrene (BP) or its ultimate metabolic derivative 7α,8β,di-hydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) within mammalian chromatin using the enzymic probe micrococcal nuclease. We have shown that the progress of the nuclease on naked DNA is unaffected by the presence of the hydrocarbon lesion at moderate extents of digestion. Digestion of nuclei isolated from murine erythroleukaemic cells immediately following BPDE treatment, and analysis of micrococcal nuclease resistant DNA by TCA precipitation, hydroxyapatite chromatography and gelelectrophoresis demonstrates a non-random distribution of lesions. Approximately three times more binding occurs on the linker DNA regions between nucleosome cores than on the nucleosome core DNA itself. A similar result was obtained with BPDE treated primary mouse embryo cells; however nuclei isolated from these cells after prolonged treatment with BP (to allow metabolic activation) showed no such preferential binding. Post-treatment incubation of BPDE-treated cells shows that this difference can be accounted for by the loss of preferential localisation with time.

INTRODUCTION

The initial work of Brookes and Lawley (1) demonstrated a close correlation between the carcinogenicity of a series of chemical carcinogens and their binding to DNA, but not to RNA or protein. Further work has demonstrated a good correlation between the DNA binding, mutagenicity and carcinogenicity of a series of polycyclic aromatic hydrocarbons (2,3). Since eukaryotic DNA is condensed with histones into a highly organised structure displaying alternating regions of resistance (nucleosome core DNA) and sensitivity (nucleosome linker DNA) to micrococcal nuclease (4), it seemed likely that the interaction of carcinogens with DNA might be non-random. This may be important in the light of recent work indicating a non-random arrangement

of nucleosomes upon certain DNA sequences. Varshavsky et al have demonstrated that the replication origin in SV40 mini-chromosomes is particularly sensitive to restriction enzymes (5), suggesting that this functionally important region is deficient in nucleosome core particles. Subsequent work has shown a non-random arrangement of nucleosomes with respect to sequences of rat satellite I DNA (6), the genes coding for the major heat shock protein of Drosophila (7) and the constant region of the kappa light chain immunoglobulin gene (8) when in the non-expressed state. The alignment of nucleosomes on the oocyte-5S RNA genes in Xenopus has also been explored by micrococcal nuclease and restriction enzyme digestion (9). At least four possible modes of organisation were considered, and in each case regions of importance in the control of transcription (start, termination or middle control regions) occurred within the linker region between nucleosome cores. Also, 5-methylcytosine, considered to be important in gene regulation (10), is distributed non-randomly in relation to nucleosome structures, occurring to a greater extent in regions resistant to micrococcal nuclease digestion (11).

Non-random binding of carcinogens to chromosomal DNA might therefore have profound effects on gene expression. Indeed there is evidence for a non-random modification of chromatin by agents such as BMBA (12), BP plus microsomes (13) and AAAF (14). However these are all direct-acting agents or systems capable of generating such agents and the results obtained may not be relevant to the distribution of lesions generated from the pro-carcinogen in vivo. The present paper reports the distribution of adducts generated by treatment with either a direct-acting agent (BPDE) or its parent hydrocarbon (BP) within the chromatin of mammalian cells.

MATERIALS AND METHODS

Radiochemicals

Methyl[^{14}C]-thymidine (56 mCi/mmol) and [^{3}H]BP (35 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks, England. [^{3}H]-BPDE (466 mCi/mmole) was supplied by Midwest Research Institute, Missouri, U.S.A., via the N.C.I., Bethesda,

U.S. Contract Programme.

Cell Culture and Treatment

Murine erythroleukaemic (MEL) cells were obtained from Dr. Harrison, Beatson Institute for Cancer Research, Glasgow, Scotland and were routinely maintained in suspension culture by twice weekly dilution to 10^5 cells/ml in Dulbecco's modified MEM supplemented with 10% foetal calf serum (Gibco-Biocult Ltd.). MEL cells (10^5/ml) were pre-labelled with 0.1 µCi/ml [^{14}C]-thymidine, grown to 10^6 cells/ml, resuspended at $5x10^6$ cells/ml in medium minus serum and treated with 1.0 µg/ml [^{3}H]-BPDE for 30-60 min in the dark. Treatment with this relatively high dose of agent gave an extent of reaction of approximately 180 µmole BP/mole DNA-P, equivalent to one lesion per 14 nucleosomes. Cells were harvested immediately following treatment and nuclei isolated as described later.

Primary monolayer cultures of mouse embryo cells were initiated as previously described (15) and grown in 175 cm^2 flasks containing 50 ml of the above medium. Twenty four hours after plating, the medium was changed and the cells pre-labelled by the addition of 0.3 nCi/ml [^{14}C]-thymidine and grown to confluence (three days) prior to treatment. Cells were treated with 10 µCi/ml [^{3}H]-BP (0.09 µg/ml), incubated for a further three days to allow metabolic activation, and harvested. The extent of reaction obtained was approximately 1.3 µmoleBP/mole DNA-P. For BPDE treatment of mouse embryo cells the medium was replaced with 20 ml medium minus serum, and the cells treated with 0.7 µg/ml [^{3}H]-BPDE for 30-60 min. The extent of reaction obtained was about 50 µmol BP/mole DNA-P. Cells were then either harvested with trypsin, or the original medium replaced and incubation continued for a further 3 days. (24 µmol BP/mol DNA-P)

Isolation of Nuclei

All procedures were carried out at 4°C. MEL cells were pelleted, washed once in PBS and suspended in 20 volumes of 0.25 M sucrose, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.3% NP40, 10 mM Tris, pH 7.4. The cells were homogenised with a motor-driven teflon-glass homogeniser (5 strokes), centrifuged at 800 g for 10 min, resuspended and the homogenisation repeated. The crude nuclear preparation was underlaid with a half-volume of 0.88 M sucrose

and centrifuged at 1,000 g for 10 min. The pellet of nuclei was resuspended in digestion buffer (0.35 M sucrose, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM KCl, 10 mM Tris, pH 7.4 - 16) and aliquots frozen in liquid nitrogen.

Mouse embryo cells were trypsinised, washed in PBS, suspended in 20 volumes of reticulocyte standard buffer (1.5 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris, pH 7.4), centrifuged at 800 g for 5 min, resuspended in the same buffer, allowed to swell for 15 min and homogenised in a hand-held Wheaton Dounce homogeniser (20 strokes, pestle A). The homogenate was centrifuged at 800 g for 10 min, the homogenisation step repeated and the nuclei resuspended in digestion buffer.

Micrococcal Nuclease Digestion and Assay

Nuclei were suspended in digestion buffer (see above) at a DNA concentration of 500-1,000 µg/ml, micrococcal nuclease (Sigma) added to 10 µg/ml and digestion performed at 37°C. Aliquots (10-20 µl) were removed at various times and the reaction stopped by the addition of an equal volume of 20 mM Tris, 20 mM NaCl, 10 mM EDTA, pH 8.0, followed by Sarkosyl NL (Ciba-Geigy, Manchester) to 0.5%. Pancreatic RNase (Sigma, pre-incubated at 2 mg/ml in 0.15 M NaCl at 80°C for 30 min) was added to 20 µg/ml and the lysate incubated at 37°C for 10 min. Proteinase K (BDH) was then added to 100 µg/ml and the incubation continued at 45°C for 60 min. An equal volume of phenol reagent (500 parts phenol: 50 parts m-cresol: 0.5 parts 8-hydroxyquinoline: 55 parts water, by weight) was added and the mixture vortexed. Following centrifugation (3,000 g - 5 min), the aqueous phase was removed and the phenol phase extracted twice with a half-volume of 20 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 8.0. The combined aqueous phases were again extracted with phenol as above and the final aqueous phase made to 100 µl with water and mixed with 100 µl of 5 mg/ml Bovine serum albumin (Sigma), and 300 µl 8% ($^{w}/v$) TCA. Following precipitation at 4°C for 30 min the solution was centrifuged at 16,000 g for 3 min and the TCA supernatant removed. The TCA precipitate was dissolved by the addition of 25 µl of 5 M KOH at 80°C for 30 min, followed by the addition of 25 µl of 4.5 M HCl and 900 µl of 0.05 M Tris, pH 8.0 to neutralise the solution. The phenol phases, TCA supernatant

and dissolved TCA precipitate were assayed for radioactivity by scintillation counting in ES299 scintillant (Packard, Caversham, Berks) in a Packard 3255 liquid scintillation spectrometer using appropriate double-label settings.

Calculation of the Adduct Concentration in Linker DNA

Assuming that the nucleosome repeat size is 200 base-pairs (figure 6) and that the core size is 146 base-pairs (4) the fraction of the BP-DNA lesions within micrococcal nuclease sensitive and resistant regions may be determined as follows. If X equals the concentration of lesions on undigested DNA (determined from the $[^{3}H]/[^{14}C]$ ratio of purified DNA), Y equals the concentration of lesions on the DNA of core particles i.e. at terminal (27%) digestion and Z equals the concentration of lesions on the DNA of micrococcal nuclease sensitive regions, then:

$$200X = 146Y + 54Z$$

The concentration of lesions in micrococcal nuclease sensitive regions (linker) relative to micrococcal nuclease resistant regions (core) is simply:

$$Z/Y = [X-(0.73Y)]/0.27Y$$

Hydroxyapatite Chromatography

Hydroxyapatite chromatography was performed as previously described (17).

Gel Electrophoresis

DNA samples, purified as described above with the omission of the TCA precipitation step, were analysed by electrophoresis in 2% agarose slab gels. Prior to electrophoresis, 4 µl of 30% Ficoll and 2 µl of 0.5% bromophenol blue, 2% sodium dodecyl sulphate, 0.1 M EDTA were added to the samples to give a final volume of 20-25 µl. Agarose gels were run in 0.04 M Tris, 0.005 M sodium acetate, 0.001 M EDTA, pH 7.5. ØX174 DNA digested with Hae III (18) was used for gel calibration. Following electrophoresis, gels were stained for 20 min with 1 µg/ml of ethidium bromide and illuminated using a long-wave transilluminator (C-62, ultraviolet products, Winchester, Hants). Photographs were taken with a Polaroid CU5 camera using a Kodak Wratten 23A filter and 665 film.

RESULTS

Prior to examining the distribution of BP-DNA adducts in regions of chromatin accessible to micrococcal nuclease it was necessary to show that the presence of the hydrocarbon lesion on the DNA did not affect the course of the digestion. This was determined by digesting naked DNA (either isolated from [^{14}C]-thymidine pre-labelled, [^{3}H]-BPDE treated cells, or DNA modified *in vitro* with [^{3}H]-BPDE), with micrococcal nuclease and following the fraction of the two labels remaining TCA precipitable i.e. remaining undigested. Figure 1 shows a plot of the percentage [^{3}H] remaining TCA precipitable as a function of the fraction of the DNA remaining TCA precipitable from eight independent micrococcal nuclease time-courses. If the presence of the lesion had no effect on the progress of the digestion, then a plot of TCA precipitable [^{3}H]-label versus TCA precipitable [^{14}C]-label would give a straight line with a slope of one. If the lesion reduced the rate of digestion, then the experimental points would fall below this line. Figure 1 shows that up to about 60% digestion there was little effect on the progress of the digestion, how-

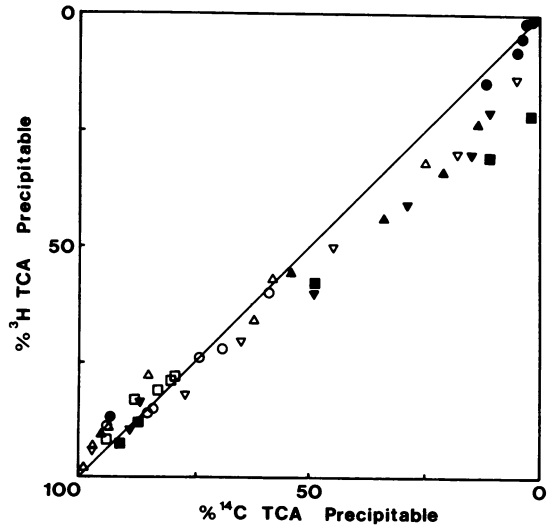

Figure 1 The fraction of [^{3}H]-BP lesions on DNA remaining undigested by micrococcal nuclease as a function of the total DNA [^{14}C]-label) remaining undigested.

ever beyond this point there was some deviation. Thus at moderate extents of digestion, the bound hydrocarbon appeared to have little effect on the rate at which the DNA becomes acid-soluble.

A second point to be considered was that, in the case of BPDE modification of MEL cells, only 15-30% of the [^{3}H]-BPDE found in nuclei after a 30 minute treatment was covalently bound to DNA, the remainder being bound to other macromolecules or being unreacted BP-tetrol. It was therefore necessary to remove hydrocarbon bound to protein and RNA, as well as non-covalently bound material (19), prior to analysis. This was achieved by removing aliquots of nuclei at various times from the micrococcal nuclease digest, simultaneously stopping the digestion and lysing the nuclei with EDTA and sarkosyl, incubating with pancreatic RNase followed by proteinase K, and phenol extracting twice to remove protein digestion products. Undegraded DNA was then precipitated from the aqueous phase by the addition of TCA.

It was important to determine the fate of the [^{3}H]-BP-DNA digestion products throughout this protocol and this was examined in the following experiment. DNA, isolated from cells pre-labelled with [^{14}C]-thymidine and treated with [^{3}H]-BPDE, was digested with micrococcal nuclease. At various times aliquots were removed and DNA isolated as outlined above. Figure 2 (top) shows the distribution of the two labels in each of the extraction phases. The [^{14}C]-label indicates that as the digestion proceeds, the fraction of thymidine nucleotides recovered in the TCA precipitate falls whilst the fraction in the TCA supernatant rises by a corresponding amount and little is found in the combined phenol phases. However Figure 2 (bottom) shows that as the digestion proceeds a significant fraction of the [^{3}H]-label appeared in the phenol phase demonstrating the greater phenol solubility of BP-DNA digestion products in comparison with unmodified digestion products. At even greater extents of digestion (data not shown) most of the [^{3}H]-label was found in the phenol phases and little remained in the TCA supernatant.

A similar analysis was performed on micrococcal nuclease digested, BPDE-modified nuclei, and the distribution of [^{3}H] in each extraction phase is shown in figure 3. At zero-time digestion, the bulk of the hydrocarbon (77%) was phenol soluble

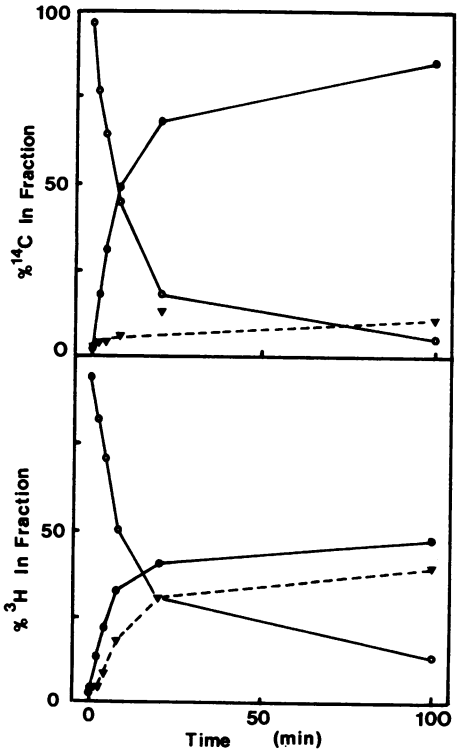

Figure 2 The fraction of total DNA ([^{14}C]-label, top figure) or BP-DNA lesions ([^{3}H]-label, bottom figure) appearing in either the phenol phase (▼), TCA supernatant (●) or TCA precipitate (○) during a DNA isolation procedure on material at different times of digestion of DNA by micrococcal nuclease.

indicating either covalent linkage to protein or possibly lipid-soluble unreacted BP-tetrol. Furthermore at zero-time digestion, a small but significant fraction of the [^{3}H] (6%) was present in the TCA supernatant and this is likely to represent part of the BP-RNA binding (though some may be soluble in the phenol extraction phase due to the pancreatic RNase digestion).

Since the [^{3}H] appearing in the TCA supernatant at any one time will be a combination of non-phenol soluble BP-RNA digestion products and non-phenol soluble BP-DNA digestion products (the extent of phenol solubility varying with the extent of digestion, figure 2), we feel that little information can be gained about

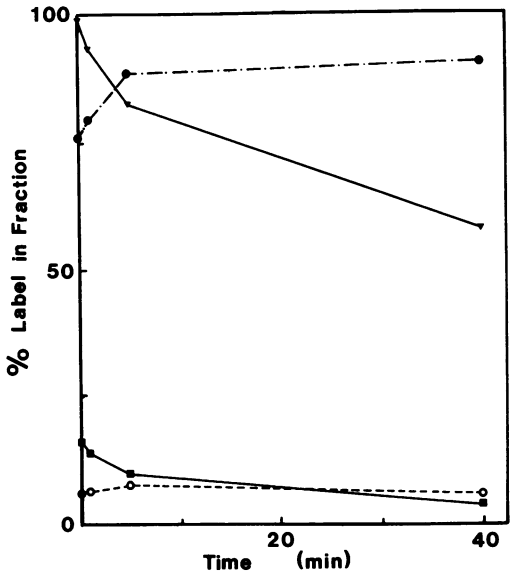

Figure 3 The fraction of the total nuclear [^{3}H]-hydrocarbon present in the phenol phase (●), TCA supernatant phase (○) and TCA precipitate (■) during a DNA isolation procedure on material at different times of digestion of nuclei by micrococcal nuclease. Also shown is the fraction of nuclear DNA (▼) remaining TCA precipitable (undigested) at these times of digestion.

the rate of digestion of BP-DNA adducts from an analysis of TCA soluble material. (This may not necessarily be the case for agents other than BPDE which give high extents of DNA reaction relative to RNA, protein and hydrolysis products). However determination of the [^{3}H] present in the TCA precipitate as a function of DNA digested, can be used albeit indirectly, to estimate the distribution of BP-DNA adducts in regions of chromatin accessible to micrococcal nuclease. This is shown in figure 4 where the relative adduct concentration ([^{3}H] /[^{14}C] ratio of TCA precipitable material at various times of digestion divided by the [^{3}H]/[^{14}C] ratio of TCA precipitable material prior to digestion) is plotted as a function of the fraction of DNA digested during micrococcal nuclease digestion of nuclei from three independent BPDE treated MEL cell preparations. This result indicates that BPDE treatment of MEL cells leads to

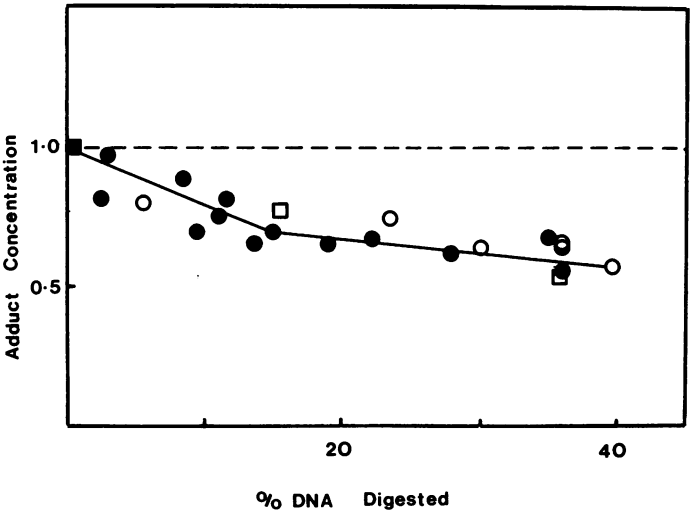

Figure 4 The relative adduct concentration in the micrococcal nuclease resistant DNA fraction as a function of the percentage of total DNA made TCA soluble during the digestion of nuclei from [^{3}H]-BPDE treated MEL cells.

preferential binding of hydrocarbon to that fraction of the chromosomal DNA which is accessible to micrococcal nuclease. Using values of 146 base-pairs for the core size and 54 base-pairs for the linker size (see below), at 27% digestion (the fraction of DNA in linker), the relative adduct concentration falls to 65% of that of undigested DNA. Since the relative adduct concentration of the core particle is 65% of the total, then the digested linker must have an adduct concentration of $[1-(0.65 \times 0.73)]/0.27 = 1.95$ times that of total DNA and hence 3.0 times that of the core DNA (see Materials and Methods for details of calculation).

To confirm that the fall in specific activity of the TCA precipitable material genuinely reflected a lower adduct concentration in the micrococcal nuclease resistant DNA, the material was chromatographed on hydroxyapatite. Nuclei isolated from [^{14}C]-thymidine pre-labelled, [^{3}H]-BPDE-treated MEL cells were digested with micrococcal nuclease to 34% [^{14}C]TCA solubility. The DNA was purified as described above, with the omission of the TCA precipitation step, and applied to a column of hydro-

xyapatite pre-equilibrated at 60°C in 0.05 M sodium phosphate, pH 6.8. The column was successively washed with 0.05 M sodium phosphate (to elute digestion products), 0.12 M sodium phosphate (to elute any single-stranded material) and finally 0.4 M sodium phosphate (to elute double-stranded material) as described previously (17). A second column was run using DNA isolated from undigested nuclei. Figure 5 shows that with undigested DNA, a small fraction of the [^{14}C]-label elutes in the 0.05 M (8%) and 0.12 M (4%) washes, but that the bulk was eluted as expected with 0.4 M sodium phosphate. The [^{3}H] profile was similar except that a higher proportion (24%) was eluted in the 0.05 M fraction, presumably due to BP-RNA digestion products. The digested material shows a quite different elution profile, with 38% of the [^{14}C] being eluted in the 0.05 M fraction. This corresponded well with the value of 34% digestion as assayed by TCA solubility. The fraction of [^{3}H] eluted by 0.05 M sodium phosphate had also increased to 43% due to that fraction of BP-DNA digestion products which were not extracted by phenol. This experiment provided an independent method of separating duplex

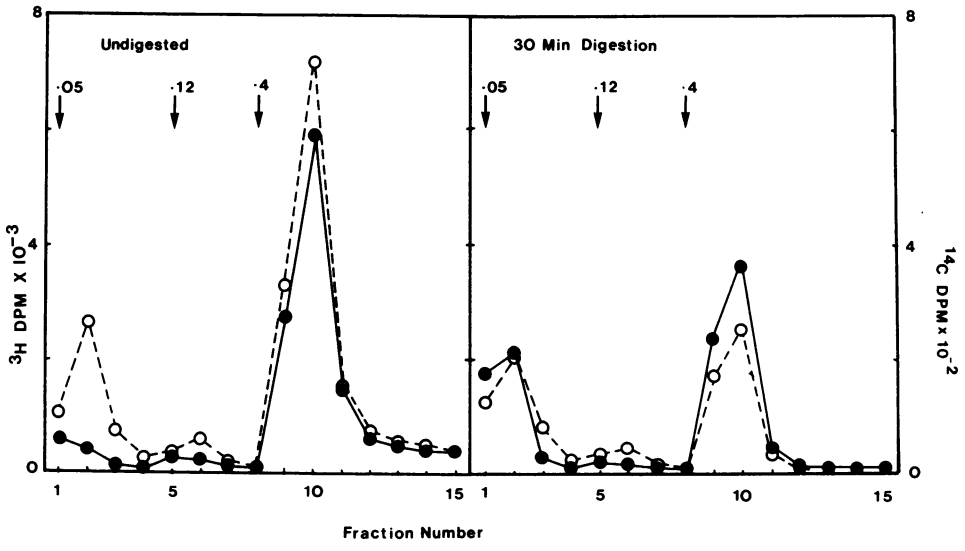

Figure 5 Hydroxyapatite chromatography of DNA from [^{14}C]-thymidine pre-labelled, [^{3}H]-BPDE treated MEL cell nuclei before and after micrococcal nuclease digestion. [^{3}H]-label (○), [^{14}C]-label (●).

DNA from digestion products, and thus an independent measure of the relative adduct concentration in nucleosome cores. The data of figure 5 allows a value of 62% to be calculated for this parameter (a 2.8 fold preferential modification of linker DNA over core DNA) compared with the value of 61% (2.8 fold preferential modification) obtained by independent TCA precipitation.

Additionally, aliquots of DNA isolated from a micrococcal nuclease digestion time-course of [^{14}C]-thymidine pre-labelled, [^{3}H]-BPDE modified MEL cell-nuclei, were subject to electrophoresis through a 2% neutral agarose gel as shown in figure 6. Comparison of distance migrated with the known sizes of the bacteriophage ØX174-Hae III DNA fragments (18) allows an estimate to be made of the DNA size of each band in the ladder. The unit nucleosome size, determined by averaging the difference between the first and the sixth bands (20), is 200 base-pairs. Assuming a core size of 146 base-pairs (4) this gives a linker size of 54 base-pairs. The relative adduct concentrations in various regions of a similar gel were determined and are shown in Table 1. It can be seen that the relative adduct concentration in the

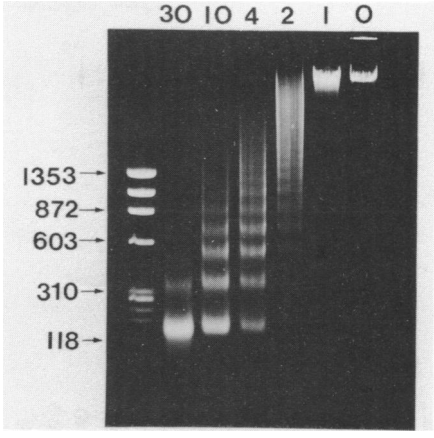

Figure 6 Agarose gel electrophoresis of DNA isolated from a micrococcal nuclease digestion time course of MEL cell nuclei. Aliquots were removed from the digestion mixture at 0,1,2,4,10 and 30 minutes, the DNA isolated and applied to the gel. Also shown is a marker HaeIII digest of ØX174 DNA. Similar gel profiles were obtained from mouse embryo cell nuclei.

Table 1. Relative DNA-adduct concentration of nucleosomes at different times of micrococcal nuclease digestion.

	Relative Adduct Concentration (% of DNA in fraction)			
Digestion Time (min)	0	1	5	30
> Tetramer	1.00(100%)	0.96(47%)	0.93(7%)	-
Dimer-Tetramer	-	0.95(34%)	0.88(31%)	-
Monomer	-	1.33(19%)	0.76(62%)	0.54(100%)
Total Relative Adduct Concentration On Gel	1.00	1.03	0.81	0.54

monomer peak at late digestion times (28% [^{14}C] TCA soluble) falls to 54% of that of total DNA, in close agreement with the value of 58% determined by TCA precipitation. These values correspond to a 4.0 and 3.6 fold increased modification of linker over core DNA. It is interesting to note that early in the digestion the monomer fraction (which accounts for only 19% of the DNA in that lane) appeared to have a slightly higher specific activity than that of total DNA.

Although the 7,8-diol-9,10-epoxide is the ultimate carcinogenic metabolite of BP, yielding DNA products identical to those obtained in BP-treated cells, it is clearly important to investigate the distribution of hydrocarbon-DNA adducts generated from BP metabolised in vivo. The previous experiments were all performed using murine erythroleukaemic cells. These cells were chosen because of the high yield of BP-DNA adducts obtained per unit BPDE used, presumably as a consequence of the treatment of cells growing in suspension rather than as monolayers. However these cells cannot metabolise BP to DNA-binding products. Primary mouse embryo cells capable of the metabolic activation of BP were therefore chosen for subsequent experimentation. An added advantage of using a primary mixed cell population is that it is more closely related to in vivo systems than is a suspension cell-line grown in culture over a number of years.

Primary mouse embryo cells were pre-labelled with [^{14}C]-thymidine and treated with either 0.09 µg/ml [^{3}H]-BP for 3 days or 0.63 µg/ml [^{3}H]-BPDE for one hour, and harvested either immediately or after incubation for a further three days. Isolated nuclei were digested with micrococcal nuclease, aliquots removed at various times and the DNA purified and TCA precipitated as described for figure 4. Figure 7 shows that in the case of the one hour BPDE-treated cells, harvested without post-treatment incubation, there was a clear drop in the relative adduct concentration with time of enzyme incubation, as was found with the MEL cells. This drop in specific activity corresponds to a 2.6 fold increase in the modification of linker over core compared with the 2.8 to 4.0 fold increase found for MEL cells. This result shows that the preferential modification of linker over core by BPDE is not confined to MEL cell chromatin, but also occurs in chromatin from a primary mixed cell population. However, as figure 7 shows, DNA from cells treated with [^{3}H]-BP for 72 hours shows no drop in specific activity during micrococ-

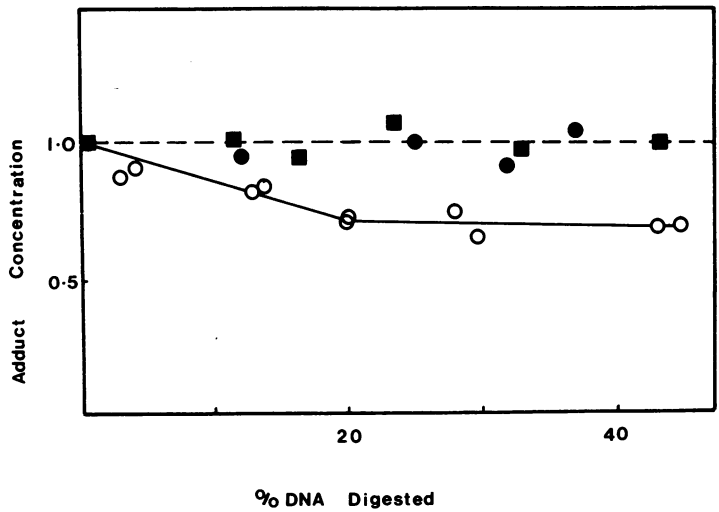

Figure 7 The relative adduct concentration in the micrococcal nuclease resistant DNA fraction as a function of the extent of total DNA digestion in nuclei from BPDE treated mouse embryo cells harvested one hour after treatment (○) or three days after treatment (●), or from BP treated cells (■).

cal nuclease digestion, indicating a random location of BP-DNA adducts with respect to nucleosome structure. It seemed possible therefore that the diol epoxide derivative of BP generated *in vivo* reacted differently to BPDE added directly to cells. An alternative explanation involved the time-course of metabolic activation. Since the mouse embryo cells were incubated in the presence of the parent hydrocarbon for a considerable length of time it was possible that the initial modification did indeed show nucleosome linker specificity but that this was lost during subsequent incubation. Evidence in favour of this view was obtained by digestion of nuclei from BPDE-treated cells following 3-day post-treatment incubation, which as shown in figure 7, also demonstrated no preferential linker binding.

DISCUSSION

Recent work investigating the arrangement of nucleosomes on DNA indicates a non-random arrangement of nucleosome cores on certain genes, as described more fully in the introduction. Such nucleosome phasing may be important in gene expression as a consequence of regulatory DNA sequences being exposed in the linker regions between nucleosome cores. If this is correct then it is possible that potent carcinogens such as the polycyclic aromatic hydrocarbons (which require doses as low as 5 µg in two-stage mouse-skin painting experiments to generate tumours) may mediate their effect through preferential binding to nucleosome linker and hence regulatory DNA sequences.

There have been a number of studies performed to determine the distribution of DNA-adducts within mammalian cell chromatin using micrococcal nuclease. AAAF modification of isolated chromatin has been shown to produce a 1.8 fold increased modification of nucleosome linker over core DNA (21). Reaction with chromatin of either the syn- or anti-isomers of BPDE leads to a 3-4 fold preferential modification of linker DNA (22,23) and BP plus a microsome activating system also leads to preferential linker binding in treated calf thymus nuclei (13). However these *in vitro* systems may not reflect the distribution of adducts occurring *in vivo*. Also in the case of the microsome-mediated binding study it is likely that the spectrum of DNA-adducts

produced is not the same as that generated *in vivo* (24,25). There have however been studies in which cells in culture or whole animals have been treated with carcinogens. Dimethylnitrosamine treatment of rats has been reported to lead to preferential binding of adducts to micrococcal nuclease sensitive regions of DNA (26), however a subsequent study was unable to confirm this (27). AAF treatment of rats (28) led to a nuclear preparation in which there was a higher proportion of carcinogen released than DNA digested, implying a preferential linker localisation. BMBA treatment of normal human fibroblasts also led to a non-random interaction of carcinogen with micrococcal nuclease sensitive regions of chromatin (12). Methylnitrosourea has been shown to be preferentially released during micrococcal nuclease digestion of treated HeLa cell nuclei but in the same study 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was released with quite different kinetics (29).

Some of the differences reported in these *in vitro* and *in vivo* studies may be accountable by the differing nature of the chemicals used. For example, the bulky adducts may be subject to more steric constraints and hence react non-randomly, whilst the smaller molecules, particularly those reacting in a predominantly S_N1 manner (e.g. methylnitrosourea or dimethylnitrosamine), may be expected to react more randomly. Another consideration is that in the main these studies have been confined to following the distribution of adducts by the release of acid-soluble label during micrococcal nuclease digestion. However, as Pegg and Hui have pointed out (27) unless it is clearly shown that the label made acid-soluble originates from DNA modification and not either RNA or other macromolecular binding, or even reincorporation of breakdown products then the results may be misleading.

A better method of assay involves purification of the nuclease-resistant DNA prior to examination of the adduct distribution. Such DNA purification must be carried out rigorously since, as Jahn and Litman have shown, contradictory results may be obtained depending on the extent to which the DNA is purified (30,13). Using this type of approach in conjunction with gel and sucrose gradient fractionation it has recently been shown

that aflatoxin B_1-DNA adducts are preferentially located in linker regions of nucleosomes following treatment of rainbow trout (31).

Another point of interest is the persistance of any preferential carcinogen distribution since this will have important biological consequences. The preferential linker localisation of BMBA-generated lesions has been reported to be unaffected by post-treatment incubation of cells (12), however it has recently been shown that AAAF treatment of similar cells leads to a preferential localisation which is lost during post-treatment incubation (14).

With these considerations in mind we have carried out an examination of the distribution of adducts generated by the direct-acting agent BPDE in the chromatin of an established mammalian cell line and of a primary mixed cell population using three independent methods of DNA fractionation, and comparying this distribution at different times with that obtained using the parent hydrocarbon, BP.

It was shown that the digestion of DNA by micrococcal nuclease was not affected by the presence of the BP-DNA lesion up to at least 50% DNA digestion and that digestion of BP-DNA adducts over this period was random (Figure 1). However micrococcal nuclease digestion of nuclei from BPDE modified MEL cells resulted in a preferential release of DNA-bound adducts early in the digestion (Figure 4) indicating preferential modification of the linker regions between nucleosome cores. This result was confirmed by agarose gel electrophoresis of DNA isolated from nuclei at various stages of micrococcal nuclease digestion (Figure 6 and Table 1) where it can be seen that nucleosome core DNA at limit digestion has a lower concentration of adducts than total DNA. Early in the digestion a fraction of mononucleosomes are released which have a slightly higher DNA-adduct concentration. These particles may correspond to actively transcribing genes which are known to be preferentially released early in micrococcal nuclease digestion (16).

Knowledge of the nucleosome unit size, core size and hence linker size allows an estimate to be made of the extent of preferential modification of linker DNA over core DNA. From the

specific activity of total DNA and core DNA, as determined by three independent methods (TCA precipitation, hydroxyapatite chromatography and gel electrophoresis), it is estimated that there is about a 3-fold increased modification of linker over core DNA during a 30-60 minute treatment of MEL cells with BPDE. Experiments were performed with a primary mixed cell population of mouse embryo cells to test the generality of this preferential modification and as figure 7 shows BPDE again produces an approximately 3-fold increased modification of linker over core DNA at short treatment times. These results are similar to those obtained when isolated chromatin was treated with BPDE (22,23) indicating that the constraints in the reactivity of linker and core DNA persist during chromatin isolation.

However, treatment of mouse embryo cells with the parent hydrocarbon BP, which requires much longer cell treatment to allow metabolism to DNA-binding intermediates, does not lead to preferential linker modification (figure 7). Similar differences in the binding pattern of BP and BPDE to linker and core DNA have been reported by Feldman et al (32,33). We show here that this apparent difference in the behaviour of the two agents can be explained by a time-dependent process which randomises the initial preferential modification since post-treatment incubation of BPDE-treated cells leads to a loss of preferential binding (Figure 7).

There are at least three possible mechanisms which would result in the loss of preferential binding to linker DNA. Firstly, the nucleosome histone core may be free to move along the DNA backbone. Alternatively if nucleosome cores are unable to slide along the DNA molecule, it is possible that during DNA replication, the histone core and DNA become separated and subsequently realign in a different orientation. Finally, just as linker DNA is preferentially modified by BPDE, presumably as a consequence of steric constraint, then the DNA repair process may also be directed towards linker DNA. Further work is in progress to distinguish between these three possibilities.

If the preferential binding of adducts to linker regions of chromatin is relatively short-lived, then it might be thought that this phenomenon is not important in carcinogenesis.

However, it is possible that the time-dependent loss may be due to preferential excision of DNA-adducts from linker regions. If such excision is error-prone then this would lead to a preferential location of mutations within linker regions between nucleosome cores and hence within sequences of potential importance in gene expression.

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ABBREVIATIONS

MEL	:	Murine erythroleukaemic
BP	:	Benzo(a)pyrene
BPDE	:	7α,8β-di-hydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene
AAF	:	2-acetylaminofluorene
AAAF	:	N-acetoxy-N-2-acetylaminofluorene
BMBA	:	7-bromomethylbenz(a)anthracene

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