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NUCLEASE-SENSITIVITY OF METHYLATED DNA AS A PROBE FOR CHROMATIN RECONSTITUTION BY GENOTOXICANTS*

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DNA in Chinese hamster ovary cells was labeled with [^{14}C]thymidine and [methyl- ^{3}H]-l-methionine in culture, and their nuclei were digested with micrococcal nuclease. Not until 10 percent of bulk DNA was digested did methylated DNA appear in the acid-soluble fraction. When these cells were exposed to UV-radiation, alkylating agents and intercalating agents in culture, the resistance of methylated DNA to digestion by the nuclease was largely or completely eliminated. The change in the sensitivity of methylated DNA to the nuclease indicates a conformational change in chromatin induced by the genotoxicants.

INTRODUCTION

The bulk of DNA in eukaryotes is known to be packed into approximately 200-base pair units, the nucleosome. A nucleosome core consists of about 146 base pairs of DNA winding around an octamer of two molecules each of histones H2a, H2b, H3 and H4, and a nucleosome linker region consisting of about 60 base pairs of DNA (1,2). The arrangement of the nucleosome relative to the DNA sequence ("phasing") has received much interest because the specific location of a nucleosome in a gene may be a means of regulating gene expression and replication. There is consensus that a non-random distribution of nucleosomes in genes is generally obtained (3). Nucleosome structure can also significantly influence the distribution of DNA damage induced by physical/chemical agents and repair processes. Nuclease-digestion studies have been invaluable probes; however, the results of such studies are subject to contradictory interpretations because of the possible reorganization of nucleosome structure

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by damage and repair processes (4,5). Further development of the methods for the detection of conformational changes in chromatin with respect to DNA sequences are required.

In mammals, 5-methylcytosine (MeCyt) is the only minor base in DNA and accounts for 2 to 7 percent of total cytosine residues (6). Non-random distribution of MeCyt has been reported with respect to DNA sequences and nucleosome structure (7); the abundance of MeCyt in highly repetitive sequences of DNA is several times higher than in moderately repetitive or unique sequences (8, 9), and up to 75 percent of total MeCyt is located in nucleosome core portions (10, 11). In this study, we investigated the possibility of using a differential nuclease-sensitivity of methylated and bulk DNA to detect changes in nucleosome reconstitution induced by genotoxicants.

MATERIALS AND METHODS

Chemicals: Doxorubicin was supplied by The Drug Synthesis and Chemical Branch, National Cancer Institute (Bethesda, MD). Dichlorvos was supplied by Analytical Chemistry, U.S. Environmental Protection Agency (Research Triangle Park, NC). Ethyl methanesulfonate (EMS) was obtained from Sigma (St. Louis, MO). [methyl- ^{3}H]-l-methionine (specific activity: 12 Ci/mmole) was obtained from New England Nuclear (Boston, MS). [2-^{14}C] thymidine (54 mCi/mmole) and [methyl- ^{3}H]thymidine (48 Ci/mmole) were from Schwarz/Mann (Orangeburg, NY). Micrococcal nuclease was obtained from Worthington (Freehold, NJ).

Cell culture: Chinese hamster ovary (CHO) cells were grown as a monolayer in RPMI 1640 medium supplemented with 10 percent fetal calf serum, an additional 2 gm/L glucose, 10 ng/ml insulin and penicillin/streptomycin at 37 °C in a water-jacketed CO_2 incubator. Radio-labeling of DNA was done overnight by adding [methyl-^{3}H]methionine, [^{14}C]thymidine or [^{3}H]thymidine at concentrations of 5, 0.05 and 1 μCi/ml, respectively. For the treatment with genotoxicants the medium containing radioactivity was discarded, and cells were washed once with phosphate buffered saline (PBS), chased with fresh medium for 2 hr, and then exposed to chemicals for 3 hr or UV-light (predominantly 254 nm) in PBS.

Digestion by nuclease: Preparation of CHO cell nuclei followed the method reported by Muramatsu et al (12). Nuclei were suspended in a buffer consisting of 0.25 M sucrose, 0.1 mM $CaCl_2$, 1 mM Tris-HCl (pH 7.8) at a concentration of 10^{8} nuclei/ml. Micrococcal nuclease was added at a concentration of 50 U/10^{8} nuclei. Digestion was continued in a 37 °C water bath and terminated by adding an equal volume of 10 percent perchloric acid (PCA) or 10 mM EDTA and chilling in ice. Digestion of bulk DNA was determined by measuring the radioactivity of [^{14}C]thymidine in the cold PCA-soluble and -insoluble fractions of nuclease-treated nuclei, after solubilization of DNA with 5 percent PCA by heating at 90 °C for 20 min.

Extraction of DNA: The EDTA-added nuclei were treated with RNase A (0.5 mg/ml), pronase (0.5 mg/ml) and sodium dodecyl sulfate (1 percent) in a buffer

containing 0.2 M NaCl, 5 mM EDTA and 10 mM Tris-HCl (pH 7.8) at 37 °C for 1, 4 and 2 hr, respectively. DNA was extracted twice with phenol: chloroform: isoamyl alcohol (24: 24: 1) and precipitated overnight with 3 volumes of ethanol with 5 µg calf thymus DNA as carrier at -20 °C. For measurement of radioactivity, DNA was solubilized in 5 percent PCA by heating at 90 °C for 20 min and added to a xylene-based scintillation counting fluid. Radioactivity of [methyl-^{3}H] methionine in the ethanol-precipitated DNA was rendered completely acid-soluble by DNase I, but not by RNase A or pronase. The possibility that radioactivity resides in the methyl group of thymine and purine rings can not be excluded (11, 13, 14), since the radioactivity among the DNA bases was not determined.

RESULTS

Digestion kinetics of methylated DNA (Met-DNA) and bulk DNA by micrococcal nuclease are shown in Fig 1 A. Not until 10 percent of bulk DNA was rendered acid soluble did the digestion of Met-DNA occur. We obtained similar results, using mouse L cells (unpublished observations), and this nuclease- resistance of Met-DNA is in accord with the reports of others (10,11). In Fig. 1 B, the digested Met-DNA is compared with digested bulk DNA. Digestion of Met-DNA, once initiated, proceeded in parallel manner with bulk DNA. When digestion of bulk DNA reached a plateau (40 percent of total DNA remained undigested), 50 - 60 percent of Met-DNA remained undigested.

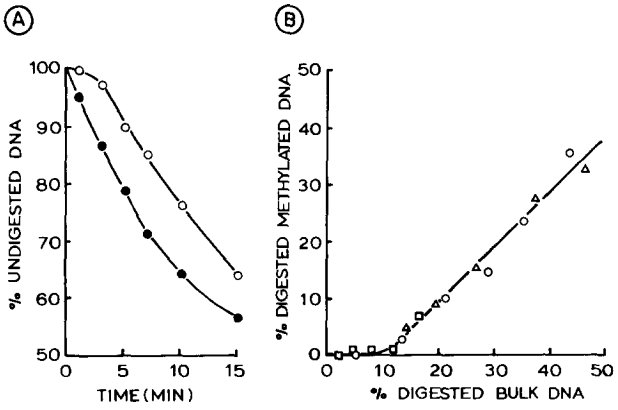

Fig. 1. Digestion of methylated DNA and bulk DNA in nuclei by micrococcal nuclease. A: kinetics of digestion. CHO cells were labeled overnight with [methyl-^{3}H]-l-methionine and [^{14}C]thymidine, 5 and 0.05 µCi/ml, respectively. Isolated nuclei were digested with micrococcal nuclease (50 U enzyme/10^8 nuclei) at 37 °C for the indicated periods. Undigested DNA was purified, and the radioactivity was determined by scintillation counting. o: methylated DNA ([^{3}H]). •: bulk DNA ([^{14}C]). B: Digestion of methylated DNA as a function of bulk DNA. Digestion of methylated DNA was plotted against bulk DNA. o, △, □ represent three different experiments.

The resistance of Met-DNA to nuclease digestion was abolished when cells were treated with doxorubicin (20 μM), dichlorvos (10 mM) and EMS (20 mM) for 3 hr. Fig. 2 A shows the digestion kinetics of Met-DNA and bulk DNA in dichlorvos-treated nuclei; both DNAs were equally digested by the nuclease. Similar results were obtained with doxorubicin and EMS. Fig. 2 B shows digestion of Met-DNA as a function of bulk DNA in nuclei treated with the genotoxicants; the initial resistance of Met-DNA to digestion disappeared, and the digestion proceeded in a parallel fashion in both bulk and Met-DNA. Doxorubicin is a well known intercalating anticancer agent (15). EMS is a strong mutagen and alkylates DNA and protein (16). Dichlorvos, an anti-cholinesterase organophosphate pesticide, preferentially methylates and phosphorylates protein rather than DNA (17). The three chemical genotoxicants showed a similar effect on the nuclease sensitivity of chromatin; although UV-radiation (100 J/m^2) did not abolish the resistance of Met-DNA completely, a dose response curve in the abolition of this resistance was observed (Fig. 3).

Effect of the chemicals on digestion kinetics of bulk DNA was also investigated; [^{3}H]thymidine-labeled cells were treated with the drugs in

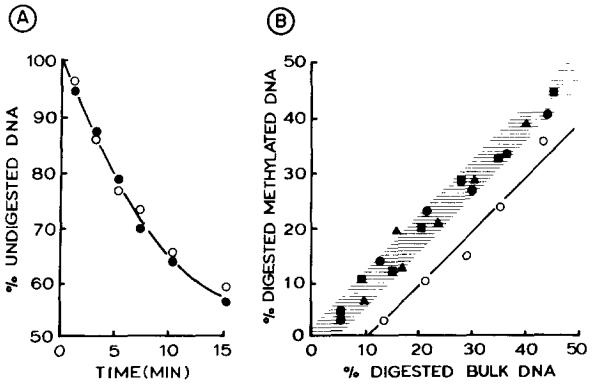

Fig. 2. A: Digestion kinetics of methylated and bulk DNA in nuclei treated with dichlorvos. The radiolabeled cells were treated for 3 hr with 10 mM dichlorvos in culture, and isolated nuclei were subjected to digestion by micrococcal nuclease under similar conditions to Fig. 1. o: methylated DNA. •: bulk DNA. B. Effects of dichlorvos, EMS and doxorubicin on the nuclease sensitivity of methylated DNA. This figure is represented in a similar way to Fig. 1 B. o: control. •, ▲, ■ : cells were treated with 10 mM dichlorvos (•), 20 mM EMS (▲) and 20 uM doxorubicin (■) for 3 hr in culture.

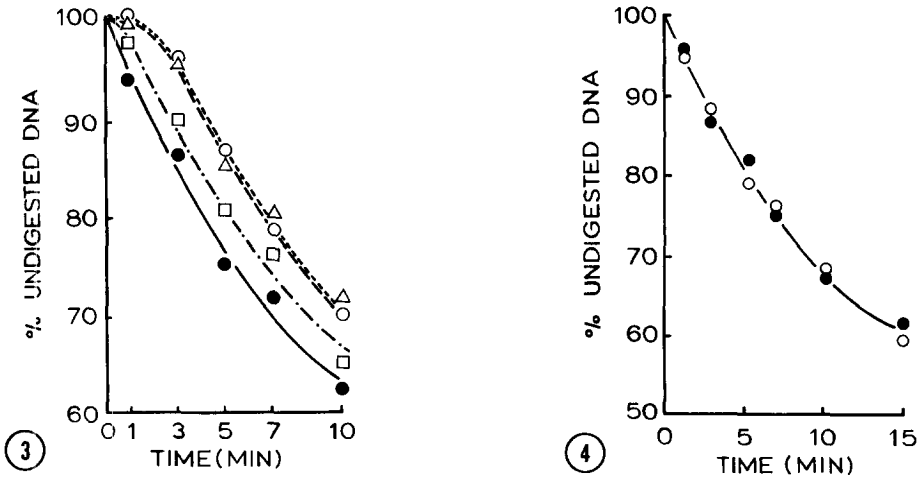

Fig. 3. Effects of UV-radiation on the digestion kinetics of methylated DNA. Cells were exposed to UV-radiation at 8, 20 and 100 J/m² and cultured for an additional 3 hr. The condition for digestion was similar to Fig. 1. ●: bulk DNA. o, △, □: methylated DNA, irradiated 8 (△), 20 (o) and 100 J/m² (□).

Fig. 4. Digestion kinetics of bulk DNA in nuclei treated with dichlorvos. CHO cells were labeled with [^{3}H]- or [^{14}C]thymidine overnight. [^{3}H]-labeled cells were treated with dichlorvos under similar conditions to Fig. 1. Isolated [^{3}H]- and [^{14}C]-labeled nuclei were mixed and subjected to digestion by micrococcal nuclease. o: dichlorvos-treated DNA. ●: control DNA.

culture, and the digestion was compared with that of [^{14}C]thymidine-labeled controls. Fig.4 shows that there are no differences in nuclease-sensitivity between dichlorvos-treated and control cells. Similar results were obtained with doxorubicin and EMS (data not shown).

DISCUSSION

An explanation for the micrococcal nuclease-resistance of Met-DNA in the early course of digestion (Fig. 1) is that Met-DNA is preferentially distributed in nucleosome cores (10, 11). There are, however, conflicting reports on the distribution of MeCyt in chromatin: it is randomly (18) or preferentially (19) distributed in the linker portion of nucleosome. Hence, whether or not nuclease-sensitivity of Met-DNA will be a useful "marker" for the nucleosome core requires further investigation.

Many studies are currently under investigation dealing with the relationship between methylated DNA and gene activity (20). There is agreement

that hypomethylation of a gene and neighboring sequences is correlated with active transcription (21-23). Transcriptionally "active" chromatin is topologically different from bulk chromatin (24); "active" chromatin is hypersensitive to pancreatic DNase I, and possibly to micrococcal nuclease (25). The possibility, thus, exists that the micrococcal nuclease-hypersensitive, hypomethylated DNA observed in our studies may correlate with "active" genes. Such genes may be associated with different nucleosome configurations as well as different higher order structures (compared with those occurring in bulk DNA), rendering their DNA more susceptible to acid hydrolysis.

Treatment with the genotoxicants, UV-radiation, an intercalating agent (doxorubicin), and alkylating agents (EMS and dichlorvos), either completely or almost abolished the nuclease-resistance of Met-DNA (Fig. 2 and 3). We considered two possibilities; the first one for these effects is that there is a conformational change between the nucleosome and DNA in such a manner that DNA sites, previously "covered" and, hence, opaque to nuclease action, are now susceptible. If this were the case, then the resistance of Met-DNA in the early course of digestion should be lost and, further, toxicant-treated Met-DNA curves would show a more abrupt and steeper slope. The fact that this steeper slope was not observed in drug-treated Met-DNA (Fig. 2 B), and that UV-radiation showed a dose response curve (instead of an all-or-none response, Fig. 3) lessens the feasibility of this first possibility.

A second possibility is that there is a toxicant-induced topological change in the higher order structure of transcriptionally "active" chromatin, normally hypomethylated and nuclease-hypersensitive, resulting in the loss of nuclease-hypersensitivity. Nuclease-hypersensitivity is somewhat dependent on histone acetylation and presence of high mobility group (HMG) of proteins (25, 26). Genotoxicant effects on these proteins is not presently known. We believe that conformational changes occurred between these proteins and DNA with the addition of genotoxicants and, as observed in our study, resulted in abolition of nuclease-hypersensitivity of the hypomethylated, "active" DNA. Further work is in progress.

Using radio-labeled Met-DNA as a probe, we showed that a change in the digestion kinetics of DNA by micrococcal nuclease was induced by several genotoxicants. Because nuclease-sensitivity of nuclear DNA is dependent on its association with chromatin proteins, the change probably indicates a structural rearrangement of chromatin by the toxicants. It is noteworthy that a comparative digestion of bulk DNA in drug-treated and control cells (Fig. 4) showed no differences; we interpreted this to mean that the putative conformational changes suggested by Met-DNA labeling are not large enough as to be detected by a gross increase in the nuclease-digestible fraction of whole chromatin DNA. We believe that the use of bulk DNA and Met-DNA assays will have general utility in the study of genotoxic agents.

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