Volume 6 Number 1 January 1979 Nucleic Acids Research

The influence of chromatin structure on the distribution of DNA repair synthesis studied by nuclease digestion

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Received 8 September 1978

ABSTRACT

The influence of chromatin structure on the distribution of DNA repair synthesis was studied by enzymatic digestion of "repair labeled" nuclei of mouse mammary cells: "repair labeled" nuclei were isolated from pregnancy mammary tissue fragments, treated *in vitro* with methylmethanesulfonate (MMS) or methylnitrosourea (MNU), and pulse-labeled with ^{3}H-thymidine in the presence of hydroxyurea in the culture medium. Micrococcal nuclease digestion of "repair labeled" nuclei indicates that at early hours after treatment with the alkylating agents 70-80% of the total repair synthesis is located in the linker portion of the nucleosome. However, 6-12 hours after treatment DNA repair synthesis is more evenly distributed throughout the core and linker portion of the nucleosome. "Repair labeled" mammary cell nuclei were also digested with DNase I under conditions selective for transcriptionally active chromatin. A two-fold higher level of repair synthesis was found in the transcriptionally active chromatin of "repair labeled" nuclei isolated from MMS or MNU treated mammary fragments, pulse-labeled at different times after treatment. The results indicate that structural constitution of the chromatin may influence the distribution of DNA repair synthesis both at the nucleosome level, and at higher levels of chromatin organization. This may be due to 1) nonrandom base alkylation in chromatin or 2) areas in chromatin with increased accessibility for the repair enzymes to the alkylated bases.

INTRODUCTION

Cellular chromatin is a nucleoprotein complex composed of DNA, RNA, acidic and basic chromosomal proteins. It has been shown that chromatin is organized into a repeating structural unit termed nucleosome. Nucleosomes consist of a core particle portion which has 140 base pairs of DNA complexed with two each of the histones H2A, H2B, H3 and H4, and a linker portion which has 25-65 base pairs of DNA complexed with one H1 molecule giving a nucleosome repeat length of 165-205 base pairs[3,4]. These nucleosomes can also form higher-ordered superhelical structures resulting in a denser packaging of the chromatin[5]. Since each cell contains more genetic information than is expressed at one time, a majority of the information remains in a repressed state. Thus with regard to its functional properties chromatin may be

distinguished into transcriptionally active and inactive fractions. The nucleosome structure occurs in both transcriptionally active and inactive chromatin, but in the former the structure of the nucleosome may be modified by acidic chromosomal proteins[6-9].

Protein-nucleic acid interactions have been shown to play an important role in the regulation of both DNA transcription[10,11] and replication[12,13], but until recently a possible influence of protein-nucleic acid interactions on DNA repair has not been studied. In a preliminary communication we have reported that micrococcal nuclease digestion of "repair labeled" nuclei isolated from MMS[2]-treated mammary tissue fragments in culture showed a non-uniform distribution of DNA repair synthesis. The chromatin fraction, digested during the first 5 minutes of incubation was the primary site for DNA repair synthesis[14]. Cleaver[15] and Smerdon *et al.*[46], while studying the distribution of UV-induced DNA repair synthesis in the chromatin of human fibroblasts, have also reported similar results. A reduced DNA repair activity observed in mouse satellite DNA compared to main band DNA after treatment with MMS or MNU was interpreted as due to the location of satellite DNA in the condensed heterochromatic portion of the cell nucleus[16]. Autoradiographic analysis of WI-38 cells after treatment with X-rays, UV or alkylating agents also has shown a preferential location of DNA repair synthesis in the euchromatic portion of the cell nucleus[17].

Previously we have reported that treatment of mammary fragments with the alkylating agents MMS or MNU in the presence of 5 mM HU results in a 5- to 7-fold higher incorporation of ^{3}H-Tdr into the mammary cell DNA than treatment with HU alone[16,18]. The results of CsCl density gradient analysis and autoradiography have shown that this alkylating agent-induced, hydroxyurea-resistant incorporation of ^{3}H-Tdr into DNA reflects DNA repair synthesis in the mammary epithelial cell nuclei[18]. In the present studies we have investigated the distribution of DNA repair synthesis in chromatin after digestion of "repair labeled" nuclei with micrococcal nuclease or DNase I. DNase I and micrococcal nuclease are different in their specificites for chromatin digestion, the former preferentially digests transcriptionally active chromatin[6-8], while the latter does not[9]. Micrococcal nuclease digests the linker portion of the nucleosome until it reaches the core particle portion[19-21]. Thus the characteristic specificities for chromatin digestion by micrococcal nuclease and DNase I provide the conditions to study the influence of chromatin structure on the distribution of DNA repair synthesis, and this report presents the results of the study on the distribution of MMS or MNU

induced DNA repair synthesis in mammary cell chromatin.

MATERIALS AND METHODS

Culture and Treatment Procedures: Fragments of mammary glands from late pregnant (18-19 day) BALB/c mice were treated with MMS in a hormone-supplemented culture medium as previously described[14]. MNU treatment of the mammary tissue fragments in culture was done as follows: MNU, dissolved in sterile .01 M phosphate buffer (pH 6.0) was added to the culture medium to give a final concentration of 3 mM. After 1 hr treatment with MNU, the medium was replaced by fresh medium. The tissue fragments were then pulse-labeled with ^{3}H-Tdr (10 μCi/ml, 50 Ci/mM - New England Nuclear Corp.) for 2 hr either immediately, 6 hr or 12 hr after treatment with MNU. Three hours prior to labeling with ^{3}H-Tdr, HU (K and K Chemicals) was added to the culture medium to a final concentration of 5 mM. HU was also present in the medium during the 2 hr pulse-labeling period. After pulse-labeling, the tissue fragments were collected, quick-frozen, and stored at -80°C.

Chromatin Isolation: The nuclei were isolated as previously described[14]. The nuclear pellet was suspended in 10 ml of 0.01 M Tris-HCl, pH 8.0, homogenized (5 strokes) with a motor-driven glass teflon homogenizer (.005"-.006" clearance - Arthur Thomas Co.), and centrifuged at 3000 rpm for 10 min in a Sorvall SS-34 rotor. The pellet was suspended in 2 ml of 0.01 M Tris-HCL, pH 8.0, and sonicated in an ice bath at 60 watts for 30 sec with a Branson sonicator using the microtip attachment. This procedure was repeated 3 times with 1 min cooling periods between sonication bursts. The solution was then centrifuged at 10,000 rpm for 10 min, and the supernate was collected. This supernate was used for the digestion assays.

Nuclease Digestion: Isolated nuclei or chromatin (125 μg DNA/ml) were incubated in 5 mM Tris 0.1 mM $CaCl_2$, pH 7.9, at 37°C, with 2.5 - 5.0 μg micrococcal nuclease (29,000 units/mg - Worthington Biochemical Corp.). Nuclei were also digested with 5.0 μg (DNase I (2197 units/mg - Worthington Biochemical Corp.) in .01 M Tris-HCl, .01 M NaCl, 3 mM $MgCl_2$, pH 7.4. The enzymatic reaction was stopped by the addition of 1.1 volume 1 N $HClO_4$, 1.4 M NaCl. The mixture was chilled for 30 min in an ice bath, then centrifuged at 10,000 rpm for 15 min. The supernate containing the acid-soluble radioactivity and acid-soluble nucleotides was pipetted out and saved. The acid-soluble nucleotides were determined by diphenylamine procedure[22]. The acid-insoluble pellet was dissolved overnight in 2 ml of 1 N NaOH, 1 M NaCl.

Liquid Scintillation Counting: The acid-soluble supernate (0.5 ml) was added to 1 ml of water plus 10 ml of Aquasol (New England Nuclear), and counted in a Beckman LS-350 liquid scintillation counter with an efficiency of approximately 24%. 0.5 ml of the alkali solubilized pellet was added to a mixture of 0.5 ml water, 0.5 ml 1 N glacial acetic acid, plus 10 ml Aquasol; and counted with an efficiency of approximately 31%.

Polyacrylamide Gel Electrophoresis: Isolated nuclei were digested with micrococcal nuclease as described above. The digestion was stopped by the addition of 0.1 volume 50 mM EDTA, and chilling the reaction in an ice bath. The solution was treated with RNase A (250 µg/ml, Sigma) and proteinase K (100 µg/ml, E. M. Laboratories) sequentially each for 1 hr. The DNA was purified as previously described[16]. After overnight ethanol precipitation the DNA was pelleted by centrifugation at 10,000 rpm for 20 min, and dissolved in a small volume of 1/10 diluted electrophoresis buffer[23]. The DNA samples (30 µg/tube) were electrophoresed on 9.5 cm 6% polyacrylamide cylindrical gels at 50 volts for 3.5 hr at 25°C in a buffer system described by Peacock and Dingman[23]. The gels were stained overnight in .005% Stainsall (Eastman) in 50% formamide, destained with H_2O, and scanned at 550 nm using a Gilford 2410S recording spectrophotometer.

RESULTS

Figure 1 shows micrococcal nuclease (2.5 or 5.0 µg) digestion of "repair labeled" nuclei isolated from MMS-treated fragments. It can be seen that the same results are obtained with both nuclease concentrations. After 5 min of digestion with either concentration of the nuclease the total radioactivity due to DNA repair synthesis converted to an acid-soluble form is over 2-fold greater than the amount of nuclear DNA rendered acid-soluble. After 30 min of digestion with 5.0 µg micrococcal nuclease, nearly 80% of the total radioactivity and 40% of the total DNA is acid-soluble. In contrast, earlier[14] we have shown that after micrococcal nuclease digestion of chromatin uniformly labeled by DNA replication the level of acid-soluble radioactivity was closely parallel to that of the acid soluble nucleotide.

Figure 2 compares the results after digestion of "repair labeled" nuclei and "repair labeled" chromatin isolated from MMS-treated tissue. After 30 min of micrococcal nuclease digestion of "repair labeled" chromatin, 73% of the total radioactivity and 49% of the nuclear DNA becomes acid soluble; similar results are also obtained with "repair labeled" nuclei.

Figures 1 and 2 also show that after 15 min of nuclease digestion little

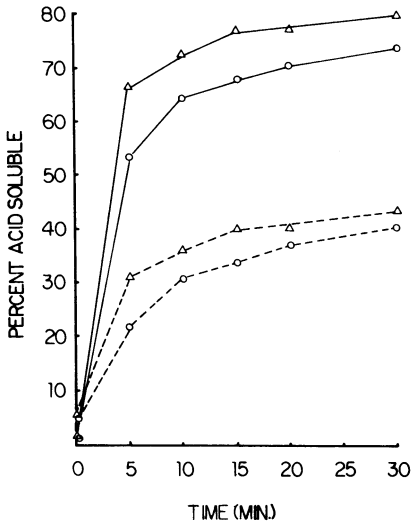

FIGURE 1. Micrococcal nuclease digestion of "repair labeled" nuclei from MMS-treated mammary tissue. Δ——Δ——Δ, A.S. radioactivity and Δ----Δ----Δ, A.S. nucleotides after digestion with 5.0 μg micrococcal nuclease; O——O——O, A.S. radioactivity and O----O----O, A.S. nucleotides after digestion with 2.5 μg micrococcal nuclease. Each point is the average of at least three determinations. Data for 2.5 μg concentration of the nuclease, reported earlier[14] are included for the sake of comparison only.

additional nuclear DNA is converted to an acid-soluble form, indicating that the chromatin has been digested to the nucleosome core particle which resists further nuclease digestion. However, at this end-point of digestion 70-80% of the total radioactivity due to DNA repair synthesis has been converted to an acid-soluble form, indicating that the chromatin fraction digested during the 30 min incubation with micrococcal nuclease constitutes the main site for DNA repair synthesis after MMS treatment. Since micrococcal nuclease preferentially digests the linker portion of the nucleosome[19-21], the above results thus provide evidence that the linker portion of the nucleosome is the primary site for DNA repair synthesis after MMS treatment.

Polyacrylamide gel electrophoretic analysis of DNA fragments formed after micrococcal nuclease digestion of nuclei isolated from control, MMS, and MNU-treated tissue are shown in figure 3. The DNA fragments produced are similar both in size and distribution in all three cases, suggesting that treatment of the mammary cells with the alkylating agents has not altered the specificity of micrococcal nuclease for chromatin digestion. This finding is not unexpected, because previous studies have shown that under the conditions of

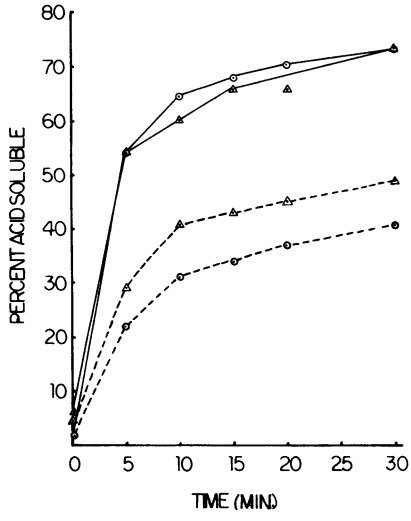

FIGURE 2. Micrococcal digestion of "repair labeled" nuclei or "repair labeled" chromatin from MMS treated fragments in culture. Digestion with 2.5 μg micrococcal nuclease. O——O——O, A.S. radioactivity and O----O----O, A.S. nucleotides after digestion of "repair labeled" nuclei; Δ——Δ——Δ, A.S. radioactivity and Δ----Δ----Δ, A.S. nucleotides after digestion of "repair labeled" chromatin. Each point is the average of at least three determinations. Data on nuclear digestion were reported earlier[14] and these are shown only for comparison.

treatment MMS introduces 4 methyls/10^5 bases[24], but methylation of the histones may produce some alteration in chromatin accessibility to the nuclease. This then confirms that the 2-fold higher release of acid-soluble radioactivity compared to acid-soluble nucleotides shown in figures 1 and 2 is not due to an alteration of chromatin structure caused by alkylation. Moreover, earlier experiments have also shown that the above results are not due to preferential recognition and digestion of the repair patches by micrococcal nuclease[14].

The results of micrococcal nuclease digestion of "repair labeled" nuclei isolated from MNU-treated tissue pulse-labeled with ^{3}H-Tdr, immediately (0 hr), 6 hr and 12 hr after treatment with MNU is shown in figure 4. Micrococcal nuclease digestion of 0 hr "repair labeled" nuclei for 5 and 30 min results in 57% and 69% of the total radioactivity becoming acid-soluble. This result is similar to those in figures 1 and 2 indicating that for tissue fragments pulse-labeled immediately after treatment with MNU there is a nonuniform distribution of DNA repair synthesis in the chromaitn, and the linker portion of the nucleosome is the primary site for MNU-induced DNA repair synthesis.

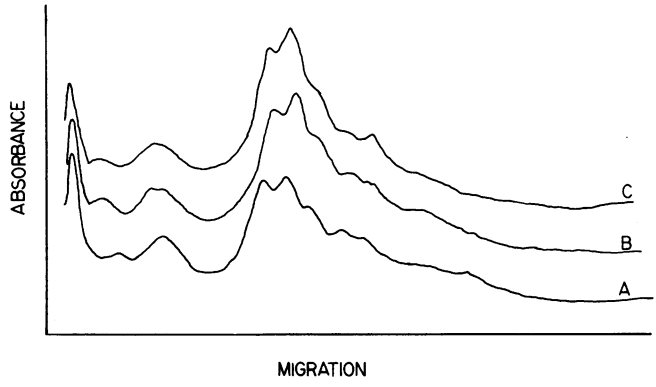

FIGURE 3. Polyacrylamide gel analysis of DNA fragments produced by micrococcal nuclease digestion of control nuclei (A), nuclei from MNU-treated tissue (B), and nuclei from MMS-treated tissue (C). The levels of A.S. nucleotides were 16%, 18% and 18% respectively.

Digestion of "repair labeled" nuclei isolated from tissue fragments, pulse-labeled with ^{3}H-Tdr at 6 hr and 12 hr after treatment with MNU shows that the release of acid-soluble radioactivity more closely corresponds to the release of acid-soluble nucleotides. This finding does not appear to be due to a significant change in the specific activity of the samples, because the specific activity for 0 hr, 6 hr, and 12 hr samples was 35 cpm/μg, 29 cpm/μg and 31 cpm/μg, DNA respectively. Therefore it is reasonable to suggest that at early periods after treatment DNA repair synthesis is primarily located in the linker portion of the nucleosome, but with advancing time it is more evenly distributed throughout the linker and core particle portion of the nucleosome.

The results of DNase I digestion of "repair labeled" nuclei isolated from MMS- and MNU-treated tissue fragments are shown in figure 5. Under the conditions of the assay used DNase I is known to selectively digest transcriptionally active fraction of the chromatin[6-8]. After 30 min of digestion, while only 18% of the nuclear DNA becomes acid-soluble the total radioactivity rendered acid-soluble is 36%. Thus the acid-soluble radioactivity after DNase I digestion is 2-fold greater than cellular DNA released into the acid-soluble fraction. This suggests an increased repair activity in the transcriptionally active regions of chromatin. Moreover, in contrast to the results after micrococcal nuclease digestion, the level of acid-soluble radioactivity after DNase I digestion remains similar for cells pulse labeled either immediately or 12 hours after treatment with the alkylating agent.

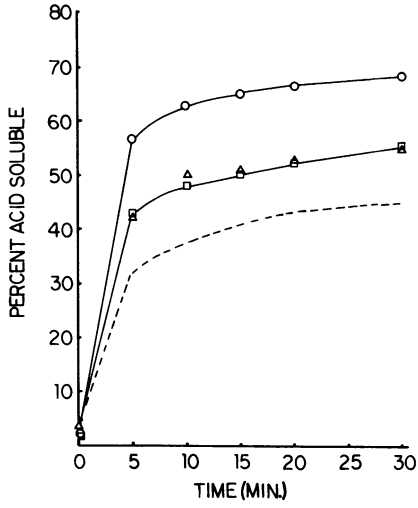

FIGURE 4. Digestion of "repair labeled" nuclei with 5.0 µg micrococcal nuclease from MNU-treated cultures which were pulse-labeled immediately (0 hr), 6 hr and 12 hr after treatment. O——O——O, A.S. radioactivity after digestion of 0 hr "repair labeled" nuclei; □——□——□, A.S. radioactivity after digestion of 6 hr "repair labeled" nuclei; Δ——Δ——Δ, A.S. radioactivity after digestion of 12 hr "repair labeled" nuclei. Dashed line shows the release of A.S. nucleotides during digestion. Each point is the average of at least three determinations.

Micrococcal nuclease sensitivity of UV-induced DNA repair synthesis also has been reported to decrease with time after exposure of the cells to irradiation, but that with DNase I there is little difference between cells labeled at early or late periods of time after treatment[46].

DISCUSSION

Consistent with our earlier observations[14] the results presented here indicate that there is a nonuniform distribution of DNA repair synthesis in "repair labeled" nuclei isolated from MMS- and MNU-treated mammary fragments pulse-labeled immediately after treatment in culture. The DNA digested during the 30 minute incubation with micrococcal nuclease is the primary site for DNA repair synthesis immediately after treatment. This has been shown to be 1) independent of nuclease concentration, 2) similar in both "repair labeled" nuclei and chromatin, and 3) not due to an alteration in the specificity of micrococcal nuclease digestion caused by alkylation. Based on current understanding of nucleosome structure these results suggest that the linker

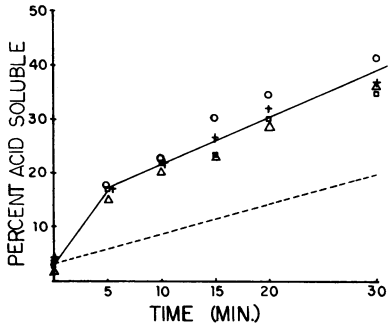

FIGURE 5. Digestion of "repair labeled" nuclei with DNase I. O——O——O, A.S. radioactivity after digestion of "repair labeled" nuclei from MMS-treated cultures; Δ——Δ——Δ, A.S. radioactivity after digestion of 0 hr "repair labeled" nuclei from MNU-treated tissue fragments; □——□——□, A.S. radioactivity after digestion of 6 hr "repair labeled" nuclei; +——+——+, A.S. radioactivity after digestion of 12 hr "repair labeled" nuclei. Dashed line shows the release of A.S. nucleotides. Each point is the average of at least three determinations.

portion of the nucleosome is the primary site for DNA repair synthesis immediately after treatment with the alkylating agent. However, with advancing time, DNA repair synthesis appears to be more evenly distributed throughout the linker and core particle portion of the nucleosome. A similar pattern of intranucleosomal distribution of DNA repair synthesis also has been observed in UV-irradiated cells[15,46].

"Replicating labeled" chromatin has been shown to be sensitive to both micrococcal nuclease and DNase I, but this sensitivity decreases rapidly with the length of labeling time[25-27]. This change in sensitivity to enzymatic digestion probably reflects the time required for replicating chromatin to be reassembled into the nucleosome structure. Since the labeling time used in the present study was 2 hr, the increased susceptibility of the "repair labeled" nuclei to micrococcal nuclease is probably not due to incomplete assembly of the DNA repair patches into nucleosomes.

"Repair labeled" nuclei were digested with DNase I under conditions known to selectively digest transcriptionally active chromatin[6-8]. Digestion of "repair labeled" nuclei with DNase I resulted in a two-fold greater release of acid-soluble radioactivity compared to acid-soluble nucleotides. This indicates that while DNA repair occurs in both transcriptionally active and inactive chromatin, the rate of repair may be different in the two chromatin fractions. Autoradiographic analysis of WI-38 cells treated with UV and

alkylating agents has shown a two-fold higher level of DNA repair synthesis in the euchromatic portion of the cell nucleus[17].

The results thus suggest that chromatin structure influences the distribution of DNA repair synthesis both at the nucleosome level and at higher levels of chromatin organization. This may be due to 1) nonuniform distribution of DNA damage in chromatin and/or 2) differences in the accessibility of the DNA damage to the repair enzymes. Chromatin digestion studies have revealed that the "linker" portion of the nucleosome is the primary site of alkylation in chromatin after treatment with N-hydroxy-2-aminofluorene[28], benzo[a]pyrene[29], and trimethylpsoralen[30,31]. Evidence also indicates that chromosomal proteins can influence enzymatic removal and recognition of DNA damage. Histones have been reported to mask pyrimidine dimers from a UV endonuclease[32,33]. Early experiments with xeroderma pigmentosum (XP) indicated that these cells were defective for a UV endonuclease[34], however, recent work has shown that XP cells carry the enzymes, which can remove pyrimidine dimers from purified DNA but not from chromatin[35].

Studies on DNA repair have shown that a cell's or a tissue's capacity to remove DNA alkylation products or pyrimidine dimers is inversely related with the tissue or cells susceptibility to the mutagenic or carcinogenic effect of the agent[36-39]. However, most cells or tissues appear to sustain the carcinogenic effects of these agents as evidenced by delayed appearance of the malignant disease, even though DNA excision repair can be demonstrated to occur. A large fraction of both DNA alkylation products[40-42] and pyrimidine dimers[33,43-45] are known to be present in cellular DNA long after DNA repair synthesis has stopped, and the persistence of these DNA lesions may be influenced by a chromatin structure. These unrepaired DNA lesions may remain as a potential source for delayed expression of the action of the carcinogenic and/or mutagenic agents. Further work is required to elucidate the relationship between chromatin structure and unrepaired DNA lesions.

ACKNOWLEDGEMENTS

The authors would like to thank Rosanne Chloupek for preparation of the figures, Arvilla Kirchhoff for secretarial assistance and Dr. Manfred Kröger for his critical reading and many helpful suggestions during preparation of this manuscript. This research was supported by DHEW grant CA-11058 and research contract NO1-CP-33289 from the National Cancer Institute.

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