Differential Dependence on Chromatin Structure for Copper and Iron Ion Induction of DNA Double-Strand Breaks†

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ABSTRACT: The induction of DNA DSB (double-strand breaks) in isolated nuclear chromatin by Cu(II) or Fe(II)−EDTA in the presence of H_2O_2 and ascorbate has been compared to DSB induction by γ-radiation. V79 nuclei embedded in agarose plugs were treated with each agent on ice, and the resultant DNA fragments were analyzed by pulsed-field gel electrophoresis. In the absence of low molecular weight radical scavengers, both irradiation and treatment with iron ion induced random DSB, as judged by the size distribution of DNA fragments, and the yield of DSB in each case was enhanced by either the expansion of chromatin (~5-fold) or the removal of histones (21−25-fold) before treatment. In contrast, treatment with Cu(II) produced small DNA fragments of uniform size (~100−200 kbp), independent of the yield of DSB. In addition, neither the DNA fragment size nor the yield of DSB produced by Cu(II) was affected by the prior removal of histones from chromatin. Deproteinized DNA was degraded randomly by Cu(II) but at a slower rate than observed for chromatin. In the presence of ascorbate, H_2O_2 was found to be essential for DSB induction by Fe(II)−EDTA but not by Cu(II), possibly because H_2O_2 can be produced from ascorbate and Cu(II) in the presence of oxygen. Despite the above differences between the production of DSB by the two metal ions, DSB induction in native chromatin by either metal ion was blocked by 0.1 M EDTA or 0.25 M thiourea but was resistant to the hydroxyl radical scavengers 0.25 M DMSO and 0.25 M mannitol. In contrast, in the presence of 0.25 M DMSO, the production of DSB by γ-radiation was reduced about 3-fold. These observations suggest that DSB are induced at sites of metal ion binding and that these sites differ for iron and copper ions, with iron ion binding nonspecifically to exposed regions of DNA and copper ion binding to non-histone proteins at the nuclear matrix attachment sites of DNA loops. The mechanism(s) for the induction of DSB by the metal ions has (have) not been fully elucidated. However, three plausible candidates are the following: (a) the generation of hydroxyl radicals *via* site-specific Fenton reactions; (b) the formation of various copper−peroxyl and iron−peroxyl species at the site of metal binding; and (c) the activation of an endonuclease by either Cu(I) or Cu(II). While cleavage by an activated endonuclease is not likely at the ice-bath temperature at which the treatments were carried out, this mechanism was tested with the general endonuclease inhibitor aurintricarboxylic acid (ATA). Inhibition of DSB formation was most marked for iron ion treatment and considerably less so for either radiation or copper ion treatment, suggesting that ATA may act as a radical scavenger and a metal ion chelator as well as a nuclease inhibitor. ATA was not effective in resolving the possible participation of a nuclease in DSB formation. Regardless of the mechanisms of metal ion-induced DSB formation, the results indicate that histones play an important role in the protection of DNA against damage caused by iron ion or ionizing radiation. In contrast, the production of DNA damage caused by copper ion is not protected by histones, possibly because the damage forms at sites which are already relatively free of histones in untreated nuclei.

Hydroxyl radicals resulting from oxidative metabolism or exogenous sources such as ionizing radiation or redox-cycling drugs have been shown to induce base damage (Aruoma et al., 1989, 1991; Blakely et al. 1990; Gajewski et al., 1990), strand breakage (Bradley & Erickson, 1981; Kohen et al., 1986), DNA−protein cross-links (Mee & Adelstein, 1979, 1981; Lesko, 1982; Chiu et al., 1986, 1993; Gajewski et al., 1988; Nackerdien et al., 1991), and other types of DNA damage [reviewed by Hutchinson (1985) and von Sonntag (1987)]. The generation of hydroxyl radicals (•OH) by the Fenton reaction (Fenton, 1894):

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$$

has been widely used to footprint protein-binding sites on DNA, because •OH can be generated continuously in the presence of O_2^- or ascorbate (Samuni et al., 1983) to regenerate Fe(II), and the •OH formed produce DNA single-strand breaks (SSB)[1] indiscriminately except at sites of bound protein (Tullius & Dombroski, 1986). DNA is also protected from radiation-generated •OH damage at sites masked by proteins (Franchet-Beuzit et al., 1993). However, whether or not Fe(II)-dependent •OH can induce DSB in cellular DNA

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is not clear. Ward et al. (1985) concluded that, in comparison to γ-radiation, the single •OH resulting from decomposition of H_2O_2 are inefficient inducers of DSB: at isotoxic doses, H_2O_2 produced 3 orders of magnitude more SSB than did γ-radiation.

Enright et al. (1992) demonstrated that DNA within polynucleosomes is cleaved randomly by radicals from an Fe(II)−EDTA-dependent Fenton reaction, whereas linker DNA is preferentially attacked by Fe(II)−ADP, and both iron complexes cleave naked DNA randomly and with high efficiency. Furthermore, cleavage of polynucleosomes by Fe(II)−ADP was more resistant to scavenging by thiourea than was cleavage by Fe(II)−EDTA, suggesting the possibility of a site-specific [Samuni et al., 1983; Gutteridge, 1984; reviewed by Chevion (1988)] Fenton reaction due to the association of Fe(II)-ADP with DNA. Thus, histones not only protect DNA against lesion production but also influence the lesion site.

Copper ion, which can also participate in the Fenton reaction, has been suggested to play an important role in the maintenance of nuclear matrix organization and DNA folding (Lewis & Laemmli, 1982; Dijkwell & Wennink, 1986; George et al., 1987), presumably by stabilization of the association between DNA and topoisomerase II (Gasser et al., 1986), a major protein component of the nuclear matrix thought to be located at the base of DNA loops (Earnshaw & Heck, 1985; Earnshaw et al., 1985; Berrios et al., 1987). Consistent with this model, we have found (Chiu et al., 1992, 1993) that treatment of isolated nuclei with low levels of Cu(II) not only binds nuclear matrix-associated DNA to matrix proteins but also enhances the production of additional DPC upon subsequent γ-irradiation. Since the enhanced production of radiation-induced DPC is sensitive to •OH scavengers, EDTA, and catalase, we proposed that •OH is generated at Cu(II)-binding sites, presumably where chromatin loops are anchored to the nuclear matrix. Both iron and copper ions can also act through the intermediate formation of metal−peroxyl radicals, without the formation of free •OH (Masarwa et al., 1988; Sutton & Winterbourn, 1989; Yamamoto & Kawanishi, 1989).

γ-Radiation produces DNA lesions mainly through the indirect reaction of •OH generated by the radiolysis of water. Irradiation of cells induces random strand breaks, although excess damage has been demonstrated in regions of chromatin active in transcription (Chiu et al., 1982; Bunch et al., 1992) or replication (Warters & Childers, 1982). In the present study, we have tested the ability of free radicals generated by metal-dependent reactions to produce DSB in chromatin, comparing DSB induction by copper or iron ions with that by γ-radiation. DSB were chosen as the test lesions for this study, because their induction requires multiple radical attack, and they are assumed to be the primary lesions for radiation-induced cell killing [Painter, 1980; Blocher & Pohlit, 1982; reviewed by Ward (1988)].

[1] Abbreviations: ATA, aurintricarboxylic acid; DPC, DNA−protein cross-links; Gy, Gray; DMSO, dimethyl sulfoxide; DSB, DNA double-strand breaks; EDTA, ethylenediaminetetraacetic acid; FAR, fraction of radioactivity released; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PFGE, pulsed-field gel electrophoresis; SDS, sodium dodecyl sulfate; SOD, copper−zinc superoxide dismutase; SSB, single-strand breaks; TE, Tris−EDTA; TBE, Tris−borate−EDTA.

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling. Exponential monolayer cultures of Chinese hamster V79-379 lung fibroblasts were grown in McCoy's 5A medium with 10% calf serum and were incubated overnight in medium containing 3.7 kBq/mL (0.1 μCi/mL) [^{3}H]thymidine to label nuclear DNA.

Preparation of Nuclei, Dehistonized Chromatin, and Expanded Chromatin. The method of Elia and Bradley (1992) was used with some modification (Xue et al., 1994). Briefly, trypsinized cells suspended in cold PBS (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) at 2×10^7 cells/mL were mixed with an equal volume of 1.5% low-melting-point agarose at 42 °C and poured into plastic molds (100 μL/plug). Solidified plugs were treated with buffer A (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, and 1 mM $MgCl_2$) containing 0.5% Triton X-100. Dehistonized chromatin was prepared by extraction of plugs overnight in buffer A containing 2.0 M NaCl. The plugs were washed and treated in PBS. Expanded chromatin was prepared by stepwise treatment of plugs with phosphate buffer (pH 7.4) of decreasing ionic strength from 50 to 1 mM (Chiu et al., 1992). EDTA (10 μM) was included in these buffers to prevent DNA degradation (Warters & Lyons, 1992).

Analysis of Nuclear Proteins. Proteins were analyzed as described (Xue et al., 1994). One plug from each treatment was mixed with 100 μL of 2× SDS−PAGE sample buffer and boiled for 5 min. An equal volume of each sample was loaded onto an 11.5% acrylamide minigel (Bio-Rad) for electrophoresis.

Irradiation. Agarose plugs containing native or dehistonized chromatin were irradiated on ice in PBS at a dose rate of 4.3 Gy/min using a ^{60}Co irradiator. Plugs with expanded chromatin were irradiated in low-salt buffer (1 mM sodium phosphate, pH 8.0, 5 mM NaCl, and 10 μM EDTA) on ice and then stored in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) at 4 °C bfore pulsed-field gel analysis.

Treatment with Cu(II) or Fe(II)-EDTA. Fe(II)-EDTA was freshly prepared by mixing equal volumes of 2 mM Fe-$(NH_4)_2(SO_4)_2$ and 4 mM EDTA. Agarose plugs were incubated on ice for 0−60 min in 200 μL of PBS or low-salt buffer (for expanded chromatin) containing ascorbic acid (0.2 mM) and either $CuSO_4$ or Fe(II)-EDTA (35 μM unless otherwise indicated in the figure legends), in the presence or absence of H_2O_2 (0.88 mM). The reaction was initiated by the addition of the metal ion. The final concentrations of scavengers used were as follows: EDTA (0.1 M), DMSO (0.25 M), mannitol (0.25 M), thiourea (0.25 M), catalase (100 units/mL), SOD (200 units/mL). After incubation, the plugs were treated for 15 s in a solution containing 1 M DMSO and 0.1 M EDTA to stop the reaction; no DSB production was observed in the presence of 0.1 M EDTA.

Pulsed-Field Gel Electrophoresis. Details of the measurement of DNA DSB have been described (Xue et al., 1994). Briefly, the plugs were incubated in buffer B (0.1 M EDTA, 10 mM Tris, 50 mM NaCl, pH 7.8, and 1 mg/mL proteinase K) at 50 °C overnight. After deproteinization, the plugs were washed in TE buffer. Electrophoresis was carried out in 0.5× TBE (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA) in an HG1000-Hula gel pulsed-field electrophoresis unit (Hoefer Scientific Instruments) at 14 °C and 200 V with

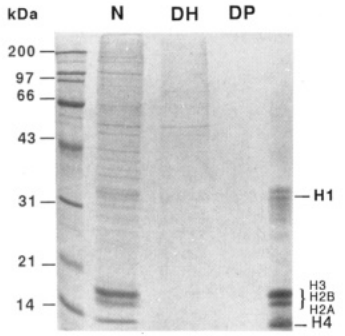

FIGURE 1: SDS−PAGE analysis of proteins from nuclei treated with NaCl or proteinase. V79 cells embedded in agarose plugs were lysed and incubated with 0.14 M (lane N, native chromatin) or 2.0 M NaCl (lane DH, dehistonized chromatin), or with proteinase K (lane DP, deproteinized DNA). Samples were prepared as described under Experimental Procedures, and equal volumes were loaded onto an 11.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue. The protein molecular mass markers (SDS−PAGE standard; Bio-Rad) are shown in the leftmost lane, and the histone standards (mixture of V-S and II-S, Sigma) are in the rightmost lane.

a reorientation angle of 120° and switch times linearly ramped from 50 to 100 s over a period of 24 h. Gels in Figures 9 and 10 are the results of electrophoresis in a CHEF-DRIII unit (Bio-Rad), under identical electrophoresis conditions, except that switch times were ramped from 50 to 90 s over 20 h. The gels were stained with ethidium bromide and photographed.

Determination of DNA DSB Using PFGE. After photography, each lane of the pulsed-field gel was sliced into 2.5-cm segments, which were placed into scintillation vials containing 75 μL of 12 N HCl and heated to melt the agarose. Radioactivity was determined in a scintillation counter, and the *f*raction of DNA radio*a*ctivity *r*eleased (FAR) from the plugs into the gel was calculated. DSB yields have been demonstrated to be correlated with FAR (Stamato & Denko, 1990). For treatments with copper ion or high radiation doses, there was a variable loss of DNA from the plugs before PFGE, as estimated by the difference between radioactivity recovered from untreated and treated plugs. Recovery of DNA was nearly 100% for all samples receiving γ-radiation or iron ion treatment giving FAR values <0.6 but decreased to 95%, 90%, and 30%, for FAR of 0.6−0.7, 0.7−0.8, and >0.9, respectively. Much lower recovery of DNA was found in samples treated with copper ion; i.e., the recovery was about 80% for FAR of 0.3−0.6 but dropped to 40% for FAR >0.6. DNA loss during PFGE was negligible. The DNA lost during the deproteinization step following treatment with copper ion was found to migrate as a smear on conventional agarose gel electrophoresis, with all fragments having sizes <30 kbp. FAR was corrected to include this fraction of DNA as released DNA.

RESULTS

Intact nuclei embedded in agarose plugs were used to study the induction of DSB in order to avoid toxic effects of metal

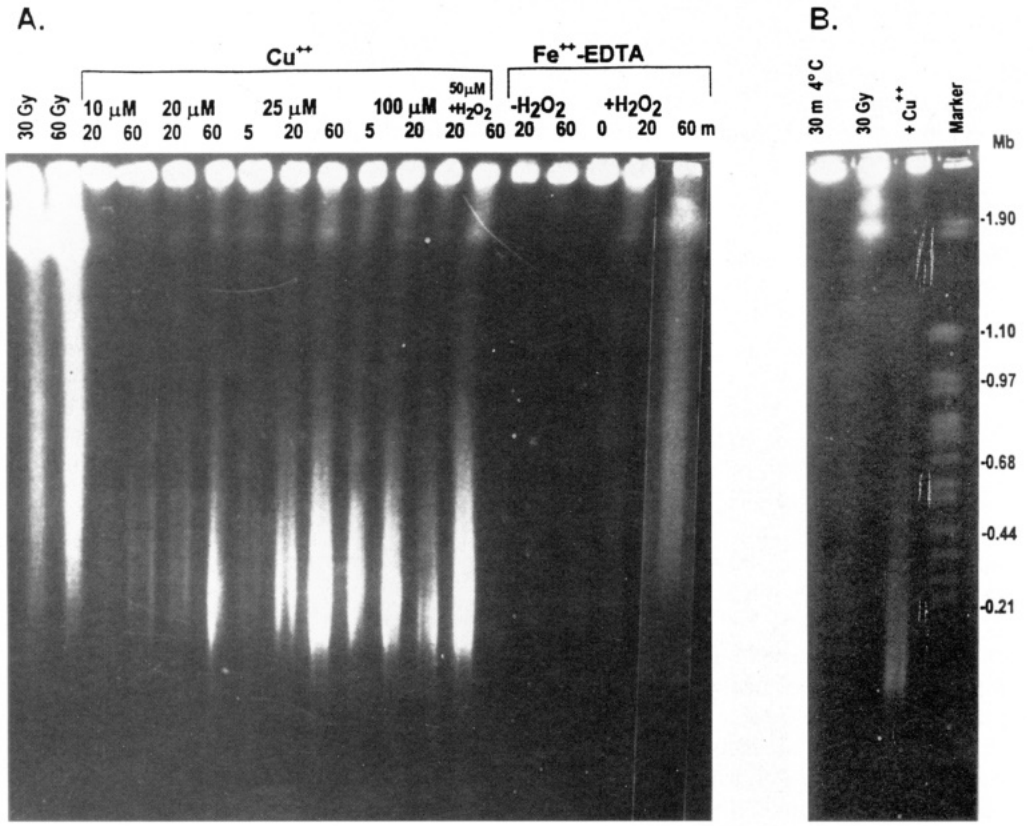

FIGURE 2: PFGE analysis of DNA fragments from γ-irradiated, Cu(II)-treated, or Fe(II)−EDTA-treated nuclei. V79 cells were embedded in agarose plugs, and nuclei were prepared as described under Experimental Procedures. In panel A, nuclei were exposed to 30 or 60 Gy (lanes 1 and 2 from the left) or various concentrations of $CuSO_4$ for 5−60 min (lanes 3−13), or Fe(II)−EDTA (2 μM) for 20−60 min (lanes 14−18), in the presence of ascorbic acid (0.2 mM) and in the presence or absence of H_2O_2 (0.88 mM) as indicated. Panel B compares nuclei incubated in PBS for 30 min on ice (lane 1), or exposed to 30 Gy (lane 2), or treated with 35 μM $CuSO_4$ plus ascorbic acid and H_2O_2 for 20 min. After treatment, all samples were deproteinized and subjected to PFGE, as described under Experimental Procedures. The gel was stained with ethidium bromide and photographed. *Saccharomyces cerevisiae* chromosomal DNA served as molecular weight markers (panel B, lane 4).

ions on cells and to minimize quenching by soluble cellular scavengers, such as glutathione. As analyzed by SDS–PAGE, the agarose-embedded nuclei contained both histone and non-histone proteins (Figure 1, lane N). Beginning with this preparation, chromatin was expanded in low ionic strength solutions and found to retain the full complement of histones and non-histone chromosomal proteins (Chiu et al., 1992). Treatment of agarose-embedded nuclei with 2.0 M NaCl removed all detectable histones from chromatin, leaving insoluble non-histone matrix proteins (Elia & Bradley, 1992; Xue et al., 1994), as shown in Figure 1 (lane DH). No proteins were detected after proteinase digestion (Figure 1, lane DP).

DSB production was first studied in intact nuclei exposed to γ-radiation or treated with Cu(II) or Fe(II)-EDTA in the presence of H_2O_2 and ascorbate. An example of DSB production analyzed by PFGE is presented in Figure 2. It reveals that the induction of DSB by Cu(II) in the presence of ascorbate is dependent on both the concentration of Cu-(II) and the incubation time at 0–4 °C. Although H_2O_2 is required for the induction of DSB by Fe(II)-EDTA treatment, it was found not to be essential for Cu(II) treatment (Figure 2A).

Also shown in Figure 2 are the striking differences in size distribution of DNA fragments released from plugs during PFGE following treatments with γ-radiation or Fe(II)-EDTA on the one hand and Cu(II) on the other. While the majority of released DNA from γ-irradiated or iron-treated nuclei formed a compression band close to the sample wells and therefore was larger than 2 Mbp, the majority of DNA fragments from Cu(II)-treated nuclei migrated further from the plugs (Figure 2, panels A and B) and could be sized at ~100–200 kbp (Figure 2, panel B). The differences in fragment size distributions were quantified by counting the radioactivity in slices of the gel lanes, examples of which are shown in Figures 3 and 4. When the three different treatments were compared at approximately equal but low levels of FAR (16–23%), it was apparent that only Cu(II) treatment resulted in a predominance of small (~100–200 kbp) DNA fragments, appearing as a peak at slice 4 in Figure 3. As shown in Figure 4, the production of small DNA fragments was apparent after low levels of DSB induction in Cu(II)-treated nuclei (FAR = 11%), and this size distribution remained unchanged when higher DSB levels were induced (FAR = 22 or 33%). In contrast, the production of small DNA fragments by radiation or Fe(II) treatment occurred only after very high radiation doses or extensive Fe(II) treatment, and in these cases, most of the DNA had entered the gel, i.e., very high FAR (data not shown).

A variety of controls has been carried out for which the data are not shown: (a) Incubation of nuclei with 0.2 mM ascorbate alone generated <8% FAR after 40 min on ice; ascorbate also had little effect on DSB induction by 40 Gy γ-radiation (FAR of 45% vs 48% for irradiation in the absence vs the presence of ascorbate). (b) Treatment of either unirradiated or irradiated DNA samples with 1 mM $CuSO_4$ prior to PFGE affected neither the FAR nor the size distribution of the DNA released from the plugs; thus, the fast migrating DNA in lanes from Cu(II)-treated nuclei was not due to artifactual migration of DNA in the presence of Cu(II). (c) Incubation of nuclei in buffer alone or with Cu-(II) or Fe(II)–EDTA in the absence of H_2O_2 and ascorbate

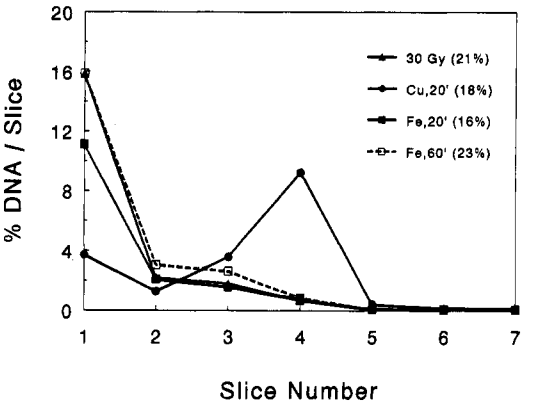

FIGURE 3: Size distribution of DNA fragments from irradiated and metal ion-treated nuclei which were released from plugs and entered the body of the gel during PFGE. V79 nuclei embedded in plugs were either irradiated or treated with Cu(II) or Fe(II)–EDTA, as for Figure 2. After deproteinization and electrophoresis, individual gel lanes were cut into 2.5-cm slices, and DNA radioactivity in each slice was determined by liquid scintillation counting. Plotted is the percentage of the total DNA radioactivity (radioactivity of plug plus gel lane) found in each slice of the gel lane. (▲) 30 Gy, 21% FAR; (●) 25 μM Cu(II), 20 min, 18% FAR; (■) 35 μM Fe-(II)–EDTA, 20 min, 16% FAR; (□) 35 μM Fe(II)–EDTA, 60 min, 23% FAR. The recovery of DNA in the plugs before PFGE was >98% for samples treated with iron ion or radiation and 87% for the copper ion-treated sample. The data are from a single experiment; the trends of the DNA size distributions were repeated in 5 additional experiments.

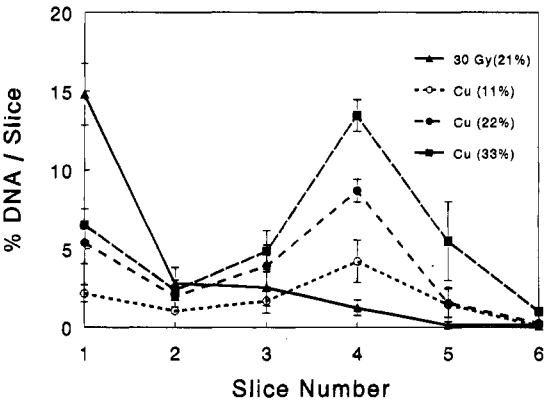

FIGURE 4: Size distribution of DNA fragments from irradiated and Cu(II)-treated nuclei which entered the gel during PFGE. V79 nuclei embedded in plugs were either irradiated with 30 Gy or treated with 35 μM Cu(II) for 5–30 min and analyzed as described in Figures 2 and 3. (▲) 30 Gy, 21.7 ± 3.6% FAR; (○) Cu(II), 5 min, 10.7 ± 2.3% FAR; (●) Cu(II), 20 min, 22.4 ± 2.9% FAR; (■) Cu(II), 30 min, 32.6 ± 1.0% FAR. Data are presented as mean ± standard deviation of data from 3–5 independent experiments. Retention of DNA in the plugs before PFGE was 86%, 82%, and 75% for samples treated with Cu(II) for 5, 20, and 30 min, respectively.

did not induce significant levels of DSB. However, in order to make the two metal ion systems comparable, the reaction mixtures in all subsequent experiments (Figures 5–10) contained 0.2 mM ascorbate, 0.88 mM H_2O_2, and 35 μM of either metal ion.

Histones have been observed to be the major DNA radioprotectors (Ljungman, 1991; Elia & Bradley, 1992; Xue et al., 1994). To examine their role in protection against DSB induction by metal ions, histones were removed from chromatin by extraction of agarose-embedded nuclei with 2 M NaCl. Figure 5 shows the same size distribution of DNA released from Cu(II)-treated dehistonized nuclei as had been

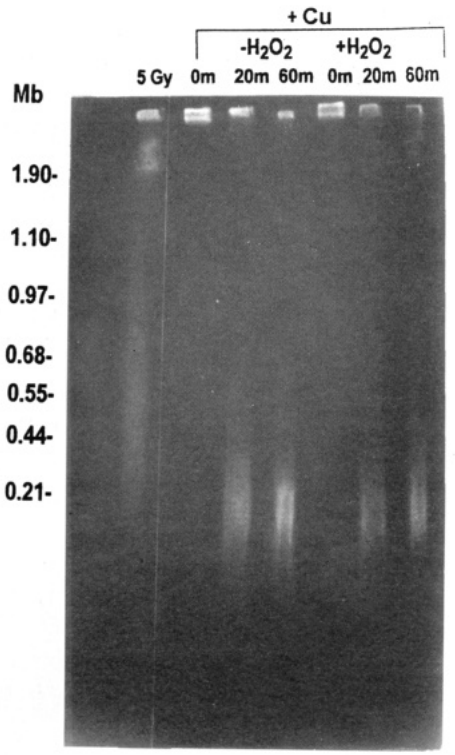

FIGURE 5: PFGE analysis of DNA fragments from dehistonized nuclei exposed to radiation or Cu(II). V79 nuclei embedded in agarose plugs were extracted with 2.0 M NaCl to remove histones, as described under Experimental Procedures. Dehistonized nuclei were either irradiated with 5 Gy or treated with 35 μM Cu(II) plus 0.2 mM ascorbate for 0, 20, or 60 min in the presence or absence of H_2O_2. Samples were deproteinized and analyzed by PFGE as described in Figure 2. Molecular size markers were *S. cerevisiae* chromosomal DNAs.

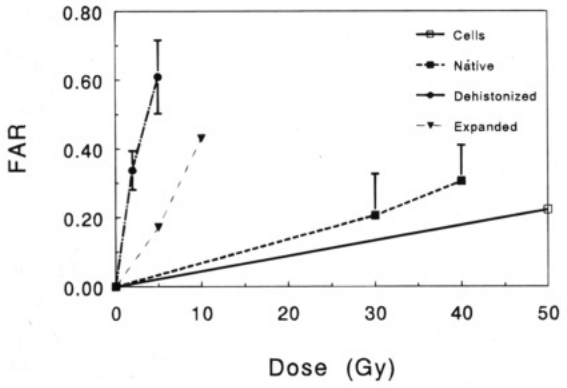

FIGURE 6: Comparison of the yield of DSB induced by γ-irradiation of cells (□), native nuclei (■), dehistonized nuclei (●), or expanded chromatin (▼). PFGE was performed as in Figure 2, and FAR was calculated as described under Experimental Procedures. The mean ± standard deviation of data from 3 experiments is plotted, except for expanded chromatin and cells, for which the average of 2 experiments is shown. Samples with FAR of 0.6−0.7 lost 5−10% of their DNA before PFGE, whereas there was negligible loss of DNA from the other plugs.

observed in Cu(II)-treated intact nuclei (cf. Figures 2−4). Removal of histones also had no effect on the size distribution of the DNA fragments from irradiated or iron-treated dehistonized nuclei, as revealed by PFGE (data not shown). However, dehistonization markedly enhanced the overall yield of DSB per Gray of γ-radiation (Figure 6) or per minute of exposure to Fe(II)-EDTA (Figure 7). On the basis of the initial slopes of the curves for dehistonized and native chromatin in Figures 6 and 7, it was calculated that

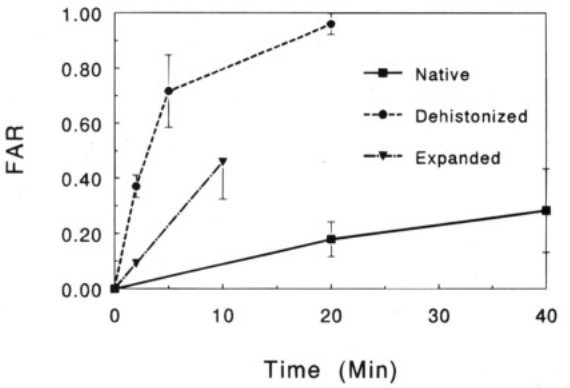

FIGURE 7: Estimation of the yield of DNA DSB induced by Fe(II)−EDTA in nuclei (■), dehistonized nuclei (●), or expanded chromatin (▼). PFGE was performed as in Figure 6. The nuclear samples were exposed to 35 μM Fe(II)−EDTA plus ascorbate and H_2O_2 for 0−40 min. The mean ± standard deviation of data from 3−5 independent experiments is plotted, except that the 2-min point for expanded chromatin is an average of 2 experiments. Recovery of DNA before PFGE was about 100%, 95−100%, 90−95%, and 30% for samples with FAR of <0.5, 0.5−0.6, 0.6−0.8, and 0.9−1.0, respectively.

Table 1: Relative Yields of DNA DSB in Native, Expanded, and Dehistonized Chromatin[a]

	relative yield of DSB		
chromatin	Cu(II)	Fe(II)−EDTA	γ-radiation
native	1.0	1.0	1.0
expanded	ND	5.1	4.5
dehistonized	1.0	20.6	24.6

[a] Data were calculated from the initial slopes of curves in Figures 6−8. In each vertical column, the yield of DSB production in expanded or dehistonized chromatin was normalized to the yield for native chromatin.

dehistonization resulted in a 21-fold and 25-fold increased yield of DSB in response to Fe(II)−EDTA and γ-radiation, respectively (Table 1).

Expansion of chromatin by suspension of nuclei in low ionic strength buffers has been shown to greatly increase the radiosensitivity of DNA (Ljungman, 1991; Chiu et al., 1992; Warters & Lyons, 1992). The production of DSB was about 5-fold higher in expanded than in native chromatin irradiated in the plugs (Figure 6, Table 1). A similar enhancement in DSB yield was observed in Fe(II)-treated expanded chromatin (Figure 7, Table 1). The induction of DSB by Cu(II) was not estimated in expanded chromatin, because the presence of EDTA inhibited its activity (see below).

When DSB induction was studied in Cu(II)-treated native and dehistonized nuclei (Figure 8, top panel), no significant effect of histone removal was observed. During the course of this study, it was discovered that some 20−60% more DNA was lost during washing and deproteinization of agarose plugs containing Cu(II)-treated samples than from plugs containing untreated samples or samples subjected to the other treatments. The DNA exiting the plugs during deproteinization produced a smear during conventional gel electrophoresis indicating random breakage into small pieces of <30 kb (data not shown). These fragments would have entered the gel during PFGE had they been retained in the plugs. Thus, for the estimation of FAR, the lost fraction of DNA should be included as part of the fraction of released DNA. Figure 8 (bottom panel) shows the dose response for

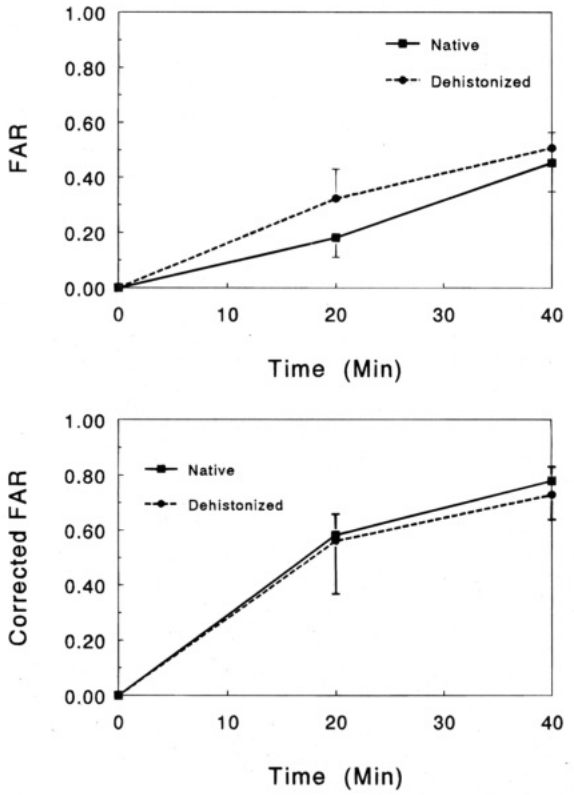

FIGURE 8: Estimation of the yield of DNA DSB induced by Cu(II) in native (■) or dehistonized nuclei (●). Nuclei were exposed to 35 μM Cu(II) in the presence of ascorbate and H_2O_2 for 0−40 min. For the top panel, FAR was determined as in Figure 6. The mean ± standard deviation of data from 3−4 independent experiments is plotted. For the bottom panel, FAR was recalculated from the data of the top panel but including the fraction of DNA lost prior to electrophoresis. This fraction, as estimated from the difference between the radioactivity recovered prior to electrophoresis in untreated plugs and those treated for 20 or 40 min, varied from 15 to 48% for both native and dehistonized samples.

DSB production by Cu(II) treatment of native and dehistonized chromatin, corrected for the fraction of DNA lost from the plugs prior to electrophoresis. These data show clearly that there is no increase in the yield of Cu(II)-induced DSB after dehistonization (Figure 8, bottom panel, and Table 1).

Deproteinized DNA has been shown to be extremely susceptible to DSB induction by radiation (Xue et al., 1994) due to the absence of radioprotection by protein. A similar high yield of DSB was found for deproteinized DNA treated with Fe(II)−EDTA in the presence of ascorbate and H_2O_2 (FAR = ~0.7 after 5 min), and the DSB were induced in a random manner. In contrast, deproteinized DNA was highly resistant to DSB induction by Cu(II) in the presence of ascorbate and H_2O_2 (FAR <0.04 after 60 min on ice). Increasing the concentration of Cu(II) to 100 μM and the incubation temperature to 37 °C produced a higher FAR and, interestingly, random induction of DSB (data not shown).

The ability of various free radical scavengers and enzymes to interfere with DSB production by the two metal ions was also studied in native chromatin (Figure 9). Despite differences in their patterns of DSB induction, iron and copper ions have similar responses to the scavengers tested. Both metal ion systems were resistant to the •OH scavengers DMSO and mannitol and the enzyme SOD, but were sensitive to EDTA and thiourea. The same concentration of DMSO (0.25 M), however, reduced the yield of radiation-induced DSB by a factor of about 3 (Oleinick et al., 1994). The sensitivity of Fe(II)-dependent but not Cu(II)-dependent DSB production to catalase is consistent with the observation that H_2O_2 is essential for the former but not the latter reaction. Similar results were found with dehistonized chromatin.

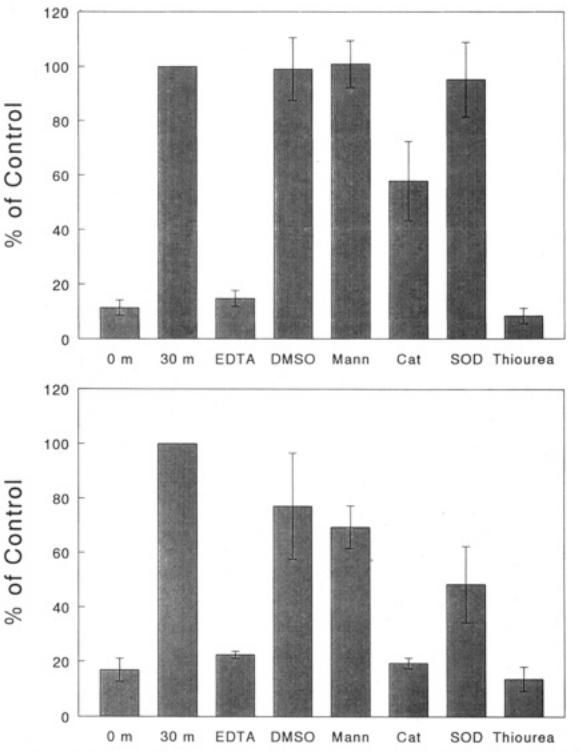

FIGURE 9: Effects of scavengers on the yield of DNA DSB induced in nuclei by Cu(II) or Fe(II)−EDTA in the presence of ascorbate and H_2O_2. Top panel: Nuclei were treated with 35 μM Cu(II) for 30 min as described in Figure 8 in the absence or presence of various scavengers. The corrected FAR from nuclei treated with Cu(II) for 30 min in the absence of scavenger was 69.7 ± 14.9%, and this value (set equal to 100%) was used to normalize all other data. Nuclei treated for 0 min were also included for comparison. The error bars represent the mean ± standard deviation of data from 4−6 experiments. Bottom: Nuclei treated with Fe(II)−EDTA for 0 or 40 min as described in Figure 7. The 40-min incubations were conducted in the absence or presence of various scavengers. The FAR from nuclei subjected to a 40-min treatment in the absence of scavenger was 40.9 ± 6.8% , and all data are normalized to this value. FAR for untreated nuclei are also included. Error bars represent the mean ± standard deviation of data from 3 experiments. The concentrations of scavengers used were EDTA (0.1 M), DMSO (0.25 M), mannitol (0.25 M), thiourea (0.25 M), catalase (100 units/mL), and SOD (200 units/mL).

Since DSB induction by Cu(II)/ascorbate was equally efficient in the absence and presence of H_2O_2, we considered the possibility that reduction of Cu(II) to Cu(I) by ascorbate activated a Cu(I)-dependent nuclease. This possibility was tested by measuring the formation of DSB by radiation or metal ions in the presence of the general endonuclease inhibitor ATA. The results presented in Figure 10 indicate that DSB production by all three treatments could be at least partially inhibited by ATA and, in contrast to our expectation, the iron ion-dependent reaction was the most susceptible, with 0.5 mM ATA producing nearly complete inhibition.

DISCUSSION

Differences in DSB Induction by Cu(II)/H_2O_2/Ascorbate and Fe(II)−EDTA/H_2O_2/Ascorbate. Our results indicate that DSB can be induced in nuclei treated with either copper or

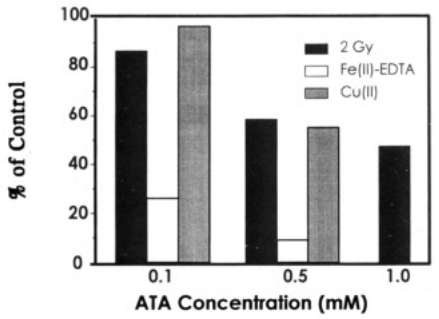

FIGURE 10: Effects of ATA on DSB induction in dehistonized nuclei by γ-radiation, Cu(II), or Fe(II)−EDTA. Dehistonized nuclei were exposed to 2 Gy γ-radiation, Cu(II)/ascorbate/H_2O_2, or Fe(II)−EDTA/ascorbate/H_2O_2 in the presence of 0−1.0 mM ATA. The recovery of DNA from these treatments prior to PFGE in the absence of ATA was 98%, 87%, and 86%, and the corrected FAR was 0.65, 0.34, and 0.82 for γ-radiation, Cu(II), and Fe(II)−EDTA treatments, respectively.

iron ions in the presence of hydrogen peroxide and ascorbic acid. However, marked differences in the pattern of DSB induction by the two metal ions were revealed in studies of the size distribution of the resultant DNA fragments and the response to the removal of histones prior to treatment. The induction of DSB by Fe(II) was random, similar to that of γ-radiation, as revealed by the appearance of large (generally >2 Mbp) fragments and no bands of unique size DNA upon PFGE (Figures 2 and 3). In addition, histones and chromatin condensation protected nuclear DNA from DSB induction by Fe(II), and dehistonization or chromatin expansion prior to Fe(II) exposure markedly enhanced the induction of DSB (Figure 7, Table 1), as also observed for γ-radiation (Figure 6, Table 1). In contrast, the induction of DSB by Cu(II) occurred at close and constant intervals, since the size of the DNA fragments observed on PFGE (~100−200 kbp) remained unchanged with increasing exposure (Figures 2−4). Furthermore, prior removal of histones had no effect on either the rate of DSB induction by Cu(II) (Figure 8) or the size distribution of the resultant DNA fragments (Figure 5).

In a study of the induction of SSB by Fe(II), Ljungman and Hanawalt (1992) found a 116-fold increase in dehistonized as compared to native chromatin, whereas we observed only a 21-fold increase in the yield of DSB after removal of histones. In addition, Ljungman and Hanawalt (1992) found a 2-fold greater effect of dehistonization on induction of SSB by iron ions than by radiation, whereas in our studies, dehistonization increased the yield of DSB by radiation ~20% more than by Fe(II)−EDTA. In their system, the production of SSB occurred in the absence of H_2O_2, and an 8-fold reduction in the yield of SSB was observed when dehistonized chromatin was treated in isotonic rather than hypotonic buffer. In our system, DSB production by Fe(II) required the presence of H_2O_2 and was independent of ionic strength. Since their earlier data on the effect of histone removal on radiation-induced SSB (Ljungman, 1991) are in close agreement with our data on DSB, the discrepancy may be due to their use of $FeSO_4$ and our use of $Fe(NH_4)_2(SO_4)_2$ as iron source rather than differences in the types of cell lines or DNA damage studied.

Although Cu(II) has been shown to bind to DNA (Kohen et al., 1986) and preferentially to GC-rich regions (Goldstein & Czapski, 1986; Kazakov et al., 1988; Sagripanti & Kraemer, 1989), our observations argue that copper ion is bound to nuclear matrix proteins for the following reasons: (1) Removal of histones affected neither the yield of DSB nor the size distribution of DNA fragments. (2) In contrast to the extensive degradation of deproteinized DNA by iron ion, in the absence of proteins DNA was degraded inefficiently by copper ion and in a random manner. Although the location of bound copper ion in the nuclei has not been determined, based on previous data on the role of copper ion in chromatin folding (Lewis & Laemmli, 1982; Dijkwell & Wennink, 1986; George et al., 1987) as well as the nonrandom cleavage of DNA at close and constant intervals (Figures 2−5), we propose that copper ion is bound to nonhistone nuclear matrix proteins at sites where DNA loops anchor. A similar hypothesis was proposed by George et al. (1987). Treatment with $CuSO_4$ is a common method of stabilizing the nuclear matrix. Reversal of the stabilization by chelators but not reducing agents supports a mechanism involving complex formation between Cu(II) and matrix proteins rather than disulfide bond formation between matrix proteins (Lewis & Laemmli, 1982), and recent studies by Vassetzky et al. (1994) reveal that Cu(II) can enhance the formation of insoluble multimeric complexes of the nuclear matrix protein topoisomerase II and the enzyme's decatenation activity. The hypothesis of Cu(II) binding to nuclear matrix protein predicts that loop size DNA should be produced. Indeed, the observed DNA fragment size of ~100−200 kbp is within the range generally estimated for DNA loops (Pienta & Coffey, 1984; Luchnik et al., 1988), and the recent demonstration of 20−500 kbp loop size DNA in cells treated with the topoisomerase II inhibitor VM-26 confirms the earlier esimates (Razin et al., 1993). However, it is to be noted that smaller (<30 kbp) DNA fragments are also produced by copper treatment but not retained in the agarose plugs, suggesting that the actual mean size of DNA products from copper treatment is smaller than that measured with PFGE. The failure to find large DNA intermediates in copper-treated nuclei suggests that the induction of one DSB at the end of a loop may result in the rapid production of additional DSB at the other end of the same loop or in a neighboring loop.

The dramatic increase in the yield of DSB which accompanied the expansion of chromatin or the removal of histones in γ-irradiated and iron ion-treated nuclei indicates the importance of chromatin compaction and chromatin proteins, and histones in particular, in the protection of DNA against free radical damage. Hence, the regions of chromatin that are more sensitive to ionizing radiation, such as actively transcribing and replicating DNA, would be expected to be hypersensitive to radicals produced by metal ions. In contrast, the absence of effect of histone removal on Cu(II)-induced DSB production is reminiscent of radiation-induced DPC, whose induction occurs preferentially at matrix-attachment sites (Chiu et al., 1986) and is not altered by the removal of the majority of histones (Xue et al., 1994). Although the contribution of site-specific reactions by bound copper ion may or may not be significant for ionizing radiation-induced DNA damage, the sites of bound metal ion, because of a paucity of protective histones, would be sensitive targets for generating DNA damage from oxidative stress. In addition, because important nuclear functions, such as DNA replication and transcription, take place at the nuclear matrix, copper ions bound at the matrix attachment

sites could disrupt nuclear functions through formation of DSB or other detrimental damage.

Although the data for DSB induction suggest that Fe(II)−EDTA binds to DNA in a non-sequence-specific manner, the random DSB production and its enhancement upon dehistonization can be explained equally well if Fe(II) is not bound to DNA and DSB are induced by •OH generated *via* an iron-catalyzed Fenton reaction in solution. The notion that iron ion from Fe(II)−EDTA is bound to DNA contradicts the conclusion drawn by Enright et al. (1992), based on differences in the location of induced strand breaks and in responses to thiourea, that Fe(II)−ADP but not Fe(II)−EDTA is bound to DNA. Although we and Enright et al. (1992) have observed similar sensitivity of Fe(II)-induced strand breakage to thiourea, it may not be possible to support DNA binding with these data, because the specificity of thiourea as a hydroxyl radical scavenger may be in question (see below).

Effects of Scavengers and Enzymes on Metal Ion Induction of DSB. The Fe(II)−EDTA-dependent induction of DSB required the presence of H_2O_2. In contrast, DSB induction by Cu(II)/ascorbate was not dependent on addition of H_2O_2, possibly because H_2O_2 can be generated from ascorbate under oxic conditions, as previously suggested by Chiou (1983). Despite a difference in the requirement for H_2O_2, DSB production by the two metal ions responded similarly to the scavengers tested: the induction of DSB was resistant to the •OH scavengers DMSO and mannitol but was blocked by EDTA and thiourea (Figure 9, top and bottom panels). Relative rate constants for the reactions of mannitol, thiourea, and DMSO with •OH are $(1.0-1.8) \times 10^9$, 4.7×10^9, and 5.8×10^9 M^{-1} s^{-1}, respectively (Reuvers et al., 1973; Gutteridge, 1987). Thus, inhibition was not correlated with the rate of reaction of the scavengers with •OH.

The inability of DMSO to inhibit DSB production contrasts with a previous report on DPC induction by Fe(III)−NTA, which induced 3 times more DPC than Fe(III)−EDTA, in the presence of ascorbate and H_2O_2 (Nackerdien et al., 1991). However, in the same study, DPC induction by a copper ion-catalyzed reaction was only partially inhibited by DMSO. In the face of resistance of DSB induction to DMSO and mannitol, the observed sensitivity to EDTA indicates that the generation of •OH, if it occurs, must be site-specific, permitting the highly reactive •OH formed at the sites of bound metal ions to react with DNA before being scavenged. Moreover, the multiple radicals required for DSB induction could readily be formed through repeated reactions at sites of bound metal ions. SOD produced a 50% inhibition of iron-dependent DSB formation, suggesting the possible participation of O_2^- in the process. The failure of SOD to block DSB production by Cu(II) suggests that O_2^- does not cause strand breakage directly, and in the presence of ascorbate, it would not be needed for the production of Cu(I). Inefficient inhibition by SOD of the formation of DNA damage by H_2O_2/metal ion combinations was also observed for the induction of DPC (Nackerdien et al., 1991). The most effective scavenger tested was thiourea, whose marked inhibitory effect on DSB production may be due to its ability (a) to chemically repair DNA radicals by hydrogen donation from the thiol tautomer (Reid, 1963), (b) to bind metal ions or react with H_2O_2 (Wasil et al., 1987), or possibly (c) to quench metal−peroxyl radicals as well as •OH. Chemical repair of a DNA radical by thiourea would effectively block DSB induction by bound or unbound metal ions acting through •OH or other radicals.

Possible Mechanism(s) of DSB Induction by Metal Ions. Since previous studies with a similar metal ion/H_2O_2/ascorbic acid system demonstrated the induction of DNA base damage mediated by •OH (Aruoma et al., 1991), it was initially assumed that these radicals were the main causative agents for DSB induction in our system. However, an alternative mechanism that includes the formation of metal−peroxyl complexes composed of tetravalent iron or trivalent copper ion has been suggested (Masarwa et al., 1988; Sutton & Winterbourn, 1989; Yamamoto & Kawanishi, 1989); these complexes exhibit a reactivity similar to that of hydroxyl radicals, and the scavengers tested might not distinguish between hydroxyl and peroxyl radicals in DSB induction.

Although all of our treatments were carried out on ice, the possibility that DSB production by Cu(II)/ascorbate might be due to reduction of Cu(II) to Cu(I) by ascorbate and Cu(I) activation of an endonuclease was investigated with the endonuclease inhibitor ATA. ATA has been shown to bind to a variety of cellular proteins and to chelate metal ions (Smith et al., 1949; Bina-Stein & Tritton, 1976). Our data (Figure 10) indicate that it is also a •OH scavenger, since DSB production by radiation was also inhibited. The resistance of half of the Cu(II)-dependent DSB induction to 1 mM ATA may indicate that at least that portion of the action of copper ion does not involve a nuclease. However, ATA did not fully resolve the possible participation of a nuclease in DSB formation as it does not act only as an endonuclease inhibitor.

In conclusion, whether DSB induction resulted from the production of •OH, the formation of metal−peroxyl complexes, or other mechanisms, it is clear that copper and iron ions act at different nuclear loci and that copper ion binds to non-histone proteins at chromosomal loop anchorage sites on the nuclear matrix.

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