# Copper Ion-Mediated Sensitization of Nuclear Matrix Attachment Sites to Ionizing Radiation†

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ABSTRACT: Exposure of mammalian cells to ionizing radiation induces nuclear matrix proteins and their attached transcribing DNA sequences to form cross-links. To characterize the cellular and matrix components necessary for DNA–protein crosslink (DPC) formation, DPC yields have been examined in isolated nuclear matrices and in the intermediate steps during cell fractionation. It was found that, in both unirradiated and irradiated cells, all components of DPC are retained in isolated nuclei, and the formed DPC are retained as well during the cell fractionation procedure resulting in nuclear matrices. In contrast, nuclear matrices isolated from unirradiated cells are deficient in the ability to form DPC upon irradiation, indicating that elements necessary for DPC production have been disrupted or removed during the isolation procedure. When isolated nuclei were irradiated, the yield of radiation-induced DPC was about 2-fold higher than that for intact cells, presumably due to the removal of soluble cellular scavengers during the isolation procedure. Treatment of nuclei with $Cu^{2+}$ to stabilize nuclear structural organization during the preparation of the nuclear matrix caused additional DNA, especially the matrix-associated newly replicated DNA, to become bound to protein. Such treatment also enhanced radiation-induced DPC production which was sensitive to OH radical scavengers. Moreover, radiation-induced DPC production in $Cu^{2+}$-treated nuclei was more sensitive to EDTA and catalase than in untreated nuclei. It is therefore proposed that excess DPC induction in $Cu^{2+}$-treated nuclei occurs preferentially at the sites of $Cu^{2+}$ binding to chromatin where hydroxyl radicals are produced repeatedly through the Fenton reaction.

Eukaryotic chromatin is organized into loops of about 30–200 kbp of DNA by periodic anchorage to a proteinaceous framework called the nuclear matrix, cage, or scaffold (Cook & Brazell, 1975; Cook et al., 1976; Benyajati & Worcel, 1976; Paulson & Laemmli, 1977; Laemmli et al., 1978). Within each loop, DNA is arrayed by wrapping around the nucleosomal core histones to form nucleosomes and with histone H1 and other non-histone proteins to form the higher order structures of chromatin. Evidence has accumulated to show that certain nuclear functions, such as replication and transcription, take place in association with the nuclear matrix (Berezney & Coffey, 1975; McCready et al., 1980; Ciejek et al., 1983; Jackson & Cook, 1985). Anchorage of DNA to the nuclear matrix is a function of specific sequence domains within or flanking a variety of genes. These matrix attachment regions or MARs[1] contain about 300+ bp of DNA, are AT-rich, and contain motifs with homology to the topoisomerase II consensus sequence. In a limited number of cases, nuclear matrix proteins that bind to specific MARs with high affinity have been identified. A role has been proposed for trace elements such as copper ion in the maintenance of matrix structural organization and in the folding of the attached DNA (Lebkowski & Laemmli, 1982a; Dijkwel & Wenink, 1986).

Although the nature of the interaction between DNA and protein at the matrix attachment sites is not yet definitively established, various degrees of weak and strong associations appear to exist, as indicated by the sensitivity of some attachments to disrupting agents, such as mercaptoethanol, detergents, or guanidine hydrochloride (Razin et al., 1981; Lebkowski & Laemmli, 1982b). However, a small fraction of associations appears to be covalent, as indicated by its resistance to alkaline treatment and to extraction either by detergent and high ionic strength buffer or by phenol (Bodnar et al., 1983; Chiu et al., 1986; Krauth & Werner, 1979; Werner & Rest, 1987; Cress & Kurach, 1988). Attempts to identify the subset of proteins that bind tightly and directly to DNA have revealed many matrix proteins ranging in molecular weight from 25 000 to 200 000 (Nakayasu & Berezney, 1991). Two proteins have recently been identified which bind to specific MARs within the human myc and chicken lysozyme genes (Chou et al., 1990; von Kries et al., 1991). Analysis of the protein-bound DNA by hybridization has shown that it is enriched in transcribing DNA sequences as is matrix-associated DNA (Neuer & Werner, 1985; Chiu et al., 1986). However, the binding of MARs to the nuclear matrix does not appear to depend upon active transcription (Cockerill & Garrard, 1986; Mirkovitch et al., 1984).

Because of the close association between DNA and protein in eukaryotic chromatin, exposure of cells to ionizing radiation results in the formation of DNA–protein cross-links (DPC) (Yamamoto, 1976), in addition to other types of damage, such as single- and double-strand breaks and base damage. Histones are quantitatively the most abundant protein components of chromatin. γ-Irradiation of isolated chromatin leads to production of cross-links between DNA and core histones in a reaction mediated by radiation-generated hydroxyl radicals (Mee & Adelstein, 1979, 1981; Gajewski et al., 1988). In contrast, our studies on the irradiation of intact mammalian cells show that cross-links are induced

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[1] Abbreviations: DPC, DNA–protein cross-links; Gy, Gray; DMSO, dimethyl sulfoxide; SOD, copper–zinc superoxide dismutase; LIS, lithium 3,5-diiodosalicylate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MAR, nuclear matrix attachment region; SAR, scaffold attachment region; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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mainly between a subset of proteins of the nuclear matrix and the associated DNA (Chiu et al., 1986). When nuclei were isolated and the chromatin was expanded in buffers of decreasing ionic strength, the yield of DPC was greatly increased (Chiu et al., 1992), suggesting that regions of chromatin which are protected from DNA damage within the cell become exposed to radiation-generated hydroxyl radicals in the expanded state. On the basis of these observations, we have hypothesized that the majority of chromatin which is removed from the nuclear matrix and is tightly condensed is a poor substrate for the formation of radiation-induced DPC and that radiation induces cross-links by stabilization of pre-existing interactions between matrix protein and DNA (Oleinick et al., 1986, 1987). The present study was undertaken to define further the essential substrates for DPC induction and the mechanism of the preferential cross-linking of DNA and protein in the nuclear matrix.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Irradiation.* Exponential monolayer cultures of Chinese hamster V79 fibroblasts were grown in McCoy's 5A medium containing 10% calf serum. Cultures were labeled with [$^3$H]thymidine (0.1 $\mu$Ci/mL) 18–24 h prior to experiments. Irradiation was carried out at 0 °C using a $^{60}$Co source at a dose rate of 7.4 Gy/min.

*Isolation of Nuclei and Nuclear Matrices.* The proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) was included at 0.1 mM in all solutions used for cell fractionation and irradiation. Nuclei were isolated as described previously (Chiu et al., 1986) except that the hypotonic solution contained 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, and 0.1% Triton X-100, as nuclei were more stable in this solution. In most cases, nuclear matrices were prepared according to the low-salt LIS procedure of Mirkovitch et al. (1984), as modified by Tsutsui et al. (1988). Nuclei were stabilized in a $Cu^{2+}$-containing solution (10 mM Tris-HCl, pH 7.4, 0.5 mM $CuSO_4$) on ice for 10 min followed by suspension in 5 mM HEPES (pH 7.4) at 30 °C for 5 min. LIS was then added to 25 mM, and the incubation was continued at 30 °C for another 10 min. The dehistonized nuclei (containing the nuclear matrix with attached DNA loops and termed "matrix") were washed several times in matrix buffer (10 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM $MgCl_2$) before irradiation. In some cases, nuclear matrices have been isolated by a high-salt extraction of histones from nuclei (Berezney & Coffey, 1974).

*Assay for DNA–Protein Cross-Links.* DNA–protein cross-links were determined by a nitrocellulose filter binding technique, as described in detail elsewhere (Chiu et al., 1986). Cells or nuclei or nuclear matrices were lysed in DPC assay buffer (1 M $NaClO_4$, 1% Sarkosyl, 5 mM EDTA, pH 7.2), incubated at 60 °C for 20 min, and cooled. The lysate was filtered through a nitrocellulose filter, and the percentage of input radioactivity retained on the filter was taken as a measure of DPC.

*Electrophoretic Analysis of Matrix Proteins.* For analysis of matrix proteins, matrices were suspended in nuclease buffer (20 mM Tris-HCl, pH 7.4, 20 mM KCl, 70 mM NaCl, 3 mM $MgCl_2$) and digested with DNase I. The matrix proteins were subjected to electrophoresis in a 7.75% acrylamide gel containing SDS (Oleinick & Evans, 1985). The gels were stained either with Coomassie blue or with silver to visualize the peptide bands.

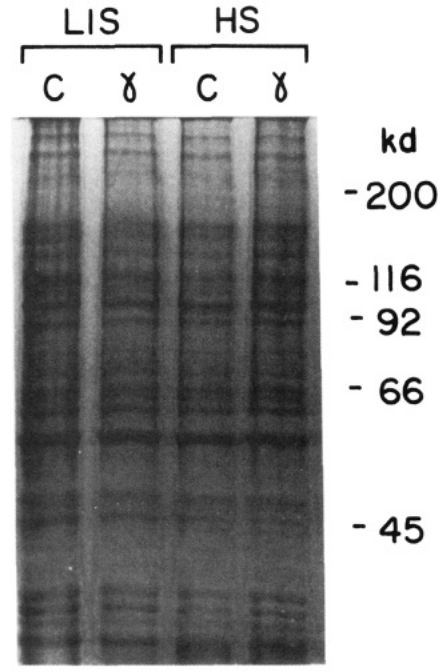


FIGURE 1: Electrophoretic analysis of V79 nuclear matrix proteins. Nuclear matrices were isolated from 100-Gy-irradiated ($\gamma$) or unirradiated (C) V79 cells either by the low-salt LIS extraction procedure (LIS) or by 2 M NaCl (HS). Proteins were analyzed on 7.5% acrylamide gels and stained with Coomassie blue.

## RESULTS

Since the protein composition of the nuclei and nuclear matrices is considerably simpler than that of whole cells, the initial goal was to exploit the ability to induce DPC by $\gamma$-irradiation of these preparations, in order to study the mechanism of DPC production as well as to investigate the interaction of DNA at matrix attachment sites. We have chosen the widely employed low-salt LIS extraction procedure (Mirkovitch et al., 1984) to prepare nuclear matrices, because of the ease with which nuclear matrices with large DNA loops attached may be handled. As a result of the gentle procedure for extraction of histones, greater than 80% of the DNA remains attached to the nuclear matrix, and the DNA fragment sizes are greater than 20 kb. As also demonstrated by Izaurralde et al. (1988), the same protein pattern was observed in matrices prepared by the low-salt LIS method and by extraction with high salt (Figure 1). Furthermore, irradiation of the cells prior to preparation of the nuclear matrices did not alter the recovery of nuclear matrix proteins, as we reported previously for the isolation of nuclear matrices by a high-salt procedure (Chiu et al., 1986).

It was important to establish whether or not DPC, generated when intact cells were $\gamma$-irradiated, would be stable during the subsequent preparation of low-salt matrices. Therefore, exponential Chinese hamster V79 cells were irradiated with 0–100 Gy, and the yield of DPC was measured by the filter-binding assay. After each dose, some of the irradiated cells were lysed and assayed directly, while others were used in the isolation of nuclei and nuclear matrices, which were then suspended in assay buffer and subjected to the filter-binding assay. As shown in Figure 2, a linear radiation dose response was found for the induction of DPC in whole cells. Furthermore, the same dose response was observed both for the assay of isolated nuclei and for the assay of nuclear matrices prepared from the irradiated cells. This indicates that essential components of DPC are retained in nuclei and nuclear matrices during the cell fractionation. The overall elevated level of

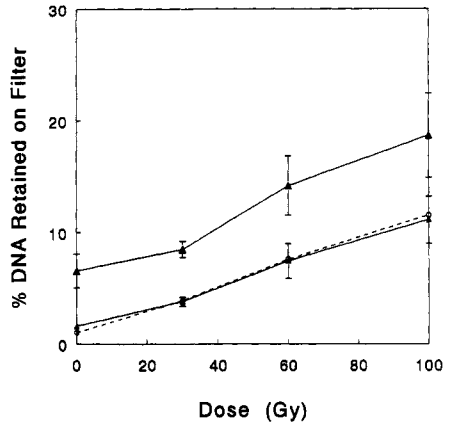


FIGURE 2: Production of DPC by irradiation of intact V79 cells. V79 monolayers, prelabeled with [$^3$H]thymidine, were exposed to 0–100 Gy and immediately harvested. Some of the cells were lysed in DPC assay buffer (○), and the remaining cells were used to isolate nuclei (△) or nuclear matrices (▲) prior to assay for DPC.

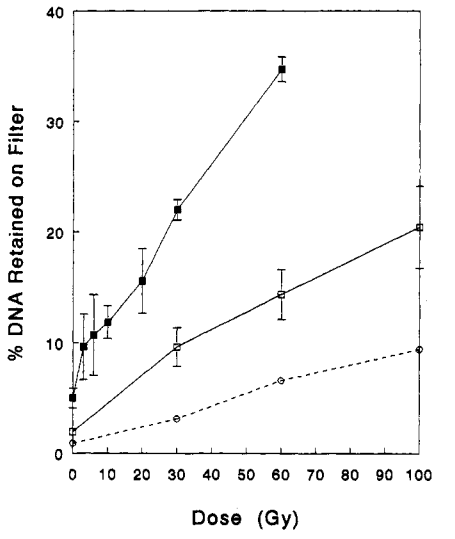


FIGURE 3: Production of DPC by irradiation of isolated nuclei. Nuclei isolated from exponentially growing V79 cells were either treated with 0.5 mM $CuSO_4$ (■) or not treated (□) before irradiation with 0–100 Gy, after which they were lysed and assayed for DPC. The dotted line represents the dose response for DPC produced in intact cells and is taken from Figure 2.(○). Values are means ± SD from three to eight independent experiments.

DPC observed for the assay of the nuclear matrix was due to the treatment of nuclei with $Cu^{2+}$ prior to LIS extraction (see below).

When isolated nuclei were irradiated and assayed for DPC, the production of DPC was dose-dependent, although the yield was about 2 times greater than that observed for irradiation of intact cells (Figure 3). This result indicates that the substrates for DPC formation are preserved in isolated nuclei. The increase in the dose response for the induction of DPC in isolated nuclei is presumably due to the removal of soluble sulfhydryl and other cellular scavengers during the isolation procedure (Ljungman et al., 1991). Mirkovitch et al. (1984) demonstrated that to prepare nuclear matrices by the low-salt extraction procedure, it is necessary to include a step to stabilize nuclear matrix structural organization prior to extraction. Stabilization was accomplished either by treatment of nuclei with a low concentration of $CuSO_4$ or by incubation of nuclei at an elevated temperature. We have chosen to use the $CuSO_4$ treatment for matrix stabilization, because of greater consistency of the results. As shown in Figure 3, treatment of nuclei with 0.5 mM $Cu^{2+}$ results in a higher background level

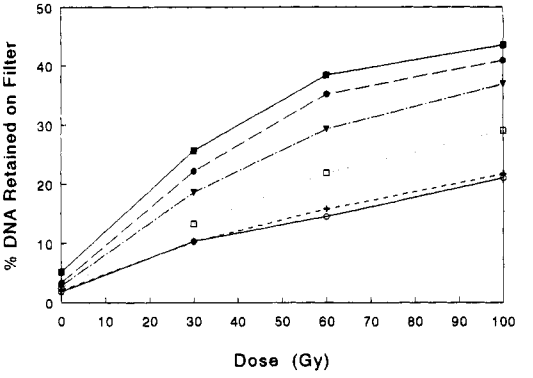


FIGURE 4: Effect of increasing concentrations of copper ion on the production of DPC by γ-radiation in isolated nuclei. Isolated nuclei were suspended in nuclear buffer and were treated with 0–$10^{-3}$ M $CuSO_4$ prior to irradiation: (+) untreated control; (○) $10^{-7}$ M; (□) $10^{-6}$ M; (▼) $10^{-5}$ M; (●) $10^{-4}$ M; (■) $10^{-3}$ M. DPC were then assayed.

Table I: Effect of Hydroxyl Radical Scavengers, EDTA, Catalase, and SOD on DPC Production Induced by Radiation in Isolated V79 Nuclei[a]

| | $-Cu^{2+}$ | | $+Cu^{2+}$ | |
|---|---|---|---|---|
| | net % DPC | % of control | net % DPC | % of control |
| 60 Gy | 12.75 ± 1.45 (4) | 100 | 36.90 ± 3.24 (5) | 100 |
| 60 Gy + cysteine | 6.37 ± 1.82 (3)[b] | 51.8 | 6.50 ± 4.40 (3)[b] | 17.6 |
| 60 Gy + mannitol | 7.09 ± 1.82 (5)[b] | 55.6 | 32.53 ± 6.22 (6) | 88.2 |
| 60 Gy + DMSO | 6.59 ± 1.41 (5)[b] | 51.7 | 12.17 ± 6.38 (3)[b] | 32.9 |
| 60 Gy + EDTA | 7.38 ± 1.06 (4)[b] | 57.9 | 6.88 ± 3.90 (6)[b] | 18.7 |
| 60 Gy + catalase | 7.98 ± 1.45 (4) | 62.6 | 12.10 ± 0.56 (4)[b] | 32.8 |
| 60 Gy + SOD | 8.93 ● 1.33 (5) | 70.0 | 22.36 ± 6.87 (5) | 60.6 |

[a] Nuclei were suspended in nuclear buffer supplemented with cysteine (50 mM), mannitol (0.25 M), DMSO (0.25 M), EDTA (0.1 M), catalase (100 units/mL), or SOD (200 units/mL). The net DPC was calculated by subtracting the DPC found for unirradiated nuclei [$-Cu^{2+}$, 3.24 ± 1.27 (7); $+Cu^{2+}$, 7.70 ± 2.80 (7)] from the DPC produced under each condition. The number in parentheses is the number of determinations. The background DPC was not significantly affected by any of the additives at the levels used. [b] Significantly different from respective controls ($P < 0.05$).

of DPC in unirradiated nuclei and a further 2-fold increase in the dose response for radiation-induced DPC production. Treatment of nuclei with $Cu^{2+}$ after irradiation, on the other hand, has no effect on the frequency of radiation-induced DPC, indicating the absence of long-lived radicals generated by radiation that would react with $Cu^{2+}$. When nuclei were treated before irradiation with either $Fe^{2+}$ or a reducing agent, sodium tetrathionate, the degree of enhancement of DPC production was less than the enhancement produced by $Cu^{2+}$ (data not shown).

Further characterization of the effect of $Cu^{2+}$ was obtained by varying the concentration during the stabilization procedure. Figure 4 shows that the degree of enhancement of DPC production depends on the concentration of $Cu^{2+}$. No enhancement was observed for irradiation after treatment with $10^{-7}$ M $Cu^{2+}$, whereas increasing enhancement occurs for concentrations between $10^{-6}$ and $10^{-3}$ M $Cu^{2+}$.

To test whether the enhancement in DPC production in $Cu^{2+}$-treated nuclei was mediated through the formation of hydroxyl radicals, several hydroxyl radical scavengers were included in the nuclear suspension during irradiation (Table I). It was found that the formation of radiation-induced DPC, in untreated and $Cu^{2+}$-treated nuclei, was at least partially blocked by these scavengers. Interestingly, the induction of DPC by irradiation of $Cu^{2+}$-treated nuclei was significantly more sensitive to the effects of cysteine, EDTA, catalase, and

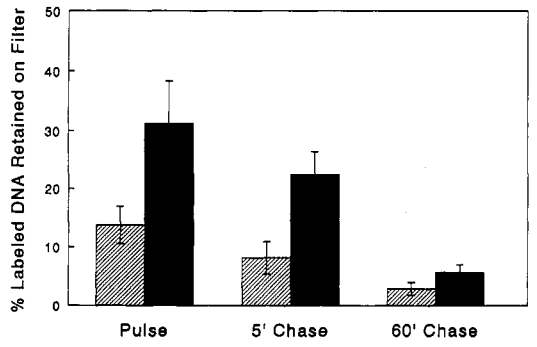


FIGURE 5: Effect of pretreatment of nuclei with $CuSO_4$ on the cross-linking of pulse-labeled DNA to protein. Exponentially growing cultures were pulse-labeled for 90 s with [$^3$H]thymidine and chased with cold thymidine for 0, 5, or 60 min. Nuclei were then isolated from the cultures, and some were treated with 0.5 mM $CuSO_4$ (solid bar) and others were not treated with $CuSO_4$ (striped bar). The percentage of labeled DNA cross-linked to protein was assayed. Error bars indicate the standard deviation of four or five determinations.

DMSO than was the induction in untreated nuclei. Conversely, mannitol was a more effective scavenger in nuclei not treated with $Cu^{2+}$.

We have previously found that $Cu^{2+}$ induces many nuclear matrix-associated proteins to become bound to DNA (Chiu et al., 1992). To test whether the fraction of chromatin DNA that is closely associated with the matrix is preferentially induced to bind to matrix protein when irradiation is carried out after $Cu^{2+}$ treatment, we took advantage of the localization of DNA replication on the nuclear matrix (Berezney & Coffey, 1975; McCready et al., 1980; Pardoll et al., 1980). Cultures were pulse-labeled with [$^3$H]thymidine to label the newly replicating DNA. Some of the cultures were chased for various periods in medium containing cold thymidine to allow the pulse-labeled DNA to mature and migrate various distances from the matrices. As shown in Figure 5, the recovery of pulse-labeled DNA from the assay filters (13.74 ± 3.19) was 4–8-fold greater than the recovery of the pulse-labeled DNA after a 60-min chase period (2.86 ± 1.09), which is similar to the recovery of uniformly labeled bulk DNA (Chiu et al., 1989). Treatment of the nuclei with $Cu^{2+}$ induced more pulse-labeled DNA to form DPC, regardless of the chase time. However, the effect of $Cu^{2+}$ treatment was greatest immediately or shortly after the pulse, inducing 17.5 or 14.3%, respectively. After a chase period of 60 min, the $Cu^{2+}$ treatment caused an increase of only 2.8%. These data are consistent with a preferential effect of $Cu^{2+}$ on the matrix-associated replicating DNA.

In contrast to the results with nuclei, the induction of DPC in isolated nuclear matrices is more limited (Figure 6). Maximum DPC production is reached after 30–60 Gy. The deficiency in DPC production in isolated nuclear matrices is not due to a loss of DNA during nuclear matrix preparation. About 80–90% of nuclear DNA remains attached to the matrix, and there is no correlation between the recovery of DNA on the nuclear matrix and the extent of DPC formation by radiation. Neither is the deficiency of DPC production in isolated nuclear matrix associated with a selective removal of specific matrix proteins, since an identical pattern of the major matrix proteins was found for nuclear matrices isolated from unirradiated or 100-Gy-irradiated nuclei that had been treated with $Cu^{2+}$ either before or after irradiation (Figure 1).

## DISCUSSION

In our previous studies, we have shown that radiation induces DPC preferentially between proteins of the nuclear matrix

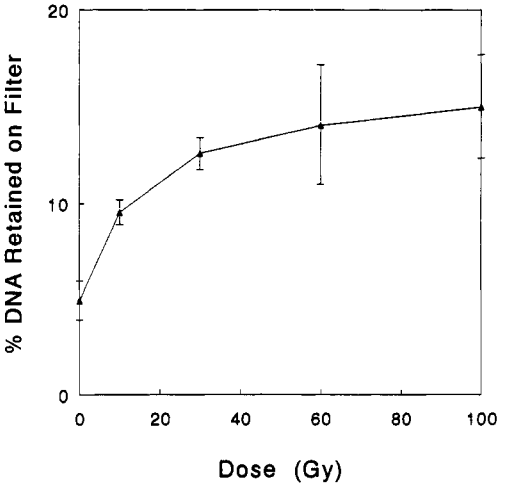


FIGURE 6: Production of DPC by irradiation of isolated nuclear matrices. Nuclear matrices isolated from exponentially growing V79 cells were irradiated with 0–100 Gy and assayed for DPC. Error bars represent the standard deviation of four or five determinations.

and the associated DNA sequences (Chiu et al., 1986). Because the isolated matrix (dehistonized nuclei or nuclear haloes) contains a simpler protein composition than intact cells, yet retains the attachment of nearly all chromosomal DNA, it seemed a suitable model system to investigate the mechanism of DPC induction by radiation. However, we found that the nuclear matrices prepared by the low-salt method are limited in their ability to form DPC (Figure 6) and therefore are not satisfactory for this purpose. The deficiency in radiation-induced DPC is not due to the actual loss of either the DNA attached to the matrix or the matrix proteins but probably results from the removal of an essential factor for DPC formation or from the disruption of the association between DNA and matrix protein. Since limited amounts of DPC can still be induced when the matrix preparations are irradiated (Figure 6), these preparations may provide a good system to study the mechanism of DPC formation at putative matrix attachment sites. Moreover, from the observation that once DPC are formed they are preserved during the matrix extraction procedure, it would be more straightforward to analyze the cross-linked proteins in this subnuclear fraction than in whole cells or nuclei because of the simpler protein composition.

Our results indicate that the isolated nuclei, which are intermediate in complexity between cells and nuclear matrices, maintain all of the components for DPC production found in intact cells yet are independent of the influence of cytoplasmic scavengers or metabolites. That the induction of DPC is about 2-fold more efficient in isolated nuclei than in intact cells is in agreement with reports on radiation-induced DNA strand breaks (Ljungman et al., 1991; Elia & Bradley, 1992; Warters & Lyons, 1992). The higher yield of DNA damage by radiation in isolated nuclei as compared to intact cells appears to be a general phenomenon and probably results from the removal of soluble cellular scavengers, such as glutathione and other sulfhydryl compounds, during the isolation procedure. Soluble cellular scavengers have been estimated to contribute less than 2% to the total intrinsic protection of cellular DNA against $\gamma$-radiation-induced DNA single-strand break formation, as compared to the much greater protection (98%) afforded by DNA-bound proteins (Ljungman et al., 1991). However, in normal cells, the radioprotection provided by intracellular scavengers must be very important for the small component of chromatin which is in an open confor-

mation and thus not protected by tight packing with chromatin proteins.

Topoisomerase II is a major component of the nuclear matrix (Berrios et al., 1987; Earnshaw et al., 1985) and therefore a likely candidate for DPC formation. Nelson et al. (1986) have reported that covalent complexes of pulse-labeled DNA with topoisomerase II can be trapped by the topoisomerase II inhibitor teniposide (VM-26), implying that newly replicated DNA is associated with topoisomerase II. Our previous studies (Chiu et al., 1992) have shown that treatment of nuclei with $Cu^{2+}$ induces numerous matrix proteins to form DPC, among which is one prominent protein of about $M_r$ 170 000, presumably topoisomerase II. In the current study, the pulse–chase experiment (Figure 5) shows the preferential induction by $Cu^{2+}$ of DPC involving the matrix-associated newly replicated DNA. Together these observations suggest that $Cu^{2+}$ mimics the effect of topoisomerase II inhibitors by trapping the covalent linkage between replicating DNA and topoisomerase II. If DPC formation were the major damage to $Cu^{2+}$-treated cells, it would be expected to be more cytotoxic to proliferating cells than to nonproliferating cells, as demonstrated by topoisomerase II poisons (D'Arpa et al., 1990).

It has been proposed that $Cu^{2+}$ plays an important role in the stabilization and maintenance of nuclear matrix organization and DNA folding, acting through matrix metalloproteins (Lebkowski & Laemmli, 1982a,b; Dijkwell & Wenink, 1986). Our results indicate that such treatment also induces high levels of DPC and more specifically the matrix-associated newly replicated DNA bound to protein (Figure 5). We have previously shown that this treatment induces more matrix proteins to become bound to DNA (Chiu et al., 1992). These observations imply that some artifacts may be generated when copper is used to stabilize nuclear organization for nuclear matrix preparations. Therefore, caution should be taken in interpreting the data especially with regard to DNA sequences at matrix attachment sites, SAR or MAR. It is interesting that stabilization of nuclear structure with $CuSO_4$ also results in an enhancement in DPC production by radiation and that the levels of copper required by these two processes are similar. Moreover, the induction of DPC by radiation in copper ion-treated nuclei as in untreated nuclei is very sensitive to OH scavengers, supporting previous conclusions (Mee & Adelstein, 1981; Lesko et al., 1982) that DPC are induced mainly by OH radicals. Our data described above indicate that copper ion induces matrix proteins and their attached DNA to form DPC. Therefore, according to our previous hypothesis (Oleinick et al., 1987) the enhancement of DPC induction in copper ion-treated nuclei may occur either by strengthening the pre-existing association between matrix proteins and their attached DNA to become more favorable for DPC induction or by increasing the number of new potential DPC formation sites on the matrices. However, the fact that a large portion of DPC induction in copper ion-treated nuclei was blocked by the OH scavenger DMSO suggests that the enchancement of DPC induction by copper ion is mainly caused by OH generated from a Fenton-type reaction mediated by copper ion. More significantly, the induction of DPC in copper ion-treated nuclei was very sensitive to agents such as EDTA, which chelates copper ion (Aruoma et al., 1991; Nackerdien et al., 1991), and catalase, which eliminates $H_2O_2$. It is assumed, therefore, that DPC are induced by OH radicals generated repeatedly through Fenton reaction at sites where copper ion binds to DNA or metalloprotein (Samuni et al., 1981, 1983; Ward et al., 1985; Higson et al., 1988). In the analysis of DPC induced in isolated chromatin by OH radical generated from metal ion-mediated Fenton reaction, Nackerdien et al. (1991) have shown that the types of cross-links induced in this system are similar to those induced by $\gamma$-irradiation under oxic conditions but differ from those induced by radiation under anoxic conditions. Whereas under oxic conditions only thymine–tyrosine-type cross-links are induced, under anoxic conditions additional types of cross-links such as thymine–lysine and cytosine–tyrosine are also induced (Gajewski et al., 1988; Gajewski & Dizdaroglu, 1990; Dizdaroglu & Gajewski, 1989; Dizdaroglu et al., 1989). Because copper ion-treated nuclei and untreated nuclei respond differently to agents such as OH radical scavengers, catalase, and EDTA, it is likely that the presence of copper ion influences not only the frequency but also the type of DPC induced by radiation. The present study suggests that, in addition to its role in the maintenance of structural organization of chromatin, copper ion may also play another role in determining the sites of DNA damage induced by OH radicals generated from a Fenton-type reaction either as a result of radiation or from oxidative stress of physiological processes. The DNA sequences that are bound either directly to copper ion or indirectly to metalloproteins (such as MAR or SAR) would be the targets most susceptible to such damage.

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