Preferential Cross-Linking of Matrix-Attachment Region (MAR) Containing DNA Fragments to the Isolated Nuclear Matrix by Ionizing Radiation†

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ABSTRACT: The sequences that anchor DNA, matrix-attachment regions (MARs), can be identified by their specific and preferential binding to the nuclear matrix. This microenvironment may be hypersensitive to the formation of ionizing radiation-induced DNA damage, including DNA−protein cross-links (DPC). To examine the induction of DPC at or near MARs, we developed an *in vitro* binding assay by using nuclear matrices isolated from murine erythroleukemia cells by high-salt extraction of DNase I-digested nuclei. The cross-linking of nuclear matrix protein to DNA fragments containing κ-immunoglobulin (κ-Ig) or an hsp70 MAR was studied. Fragments of pBR322 of similar size to the MAR-containing fragments served as non-MAR controls. Two types of experiments were conducted: type A in which nuclei were irradiated and nuclear matrices were isolated and assayed for the binding of exogenous ^{32}P-labeled DNA fragments, and type B in which mixtures of isolated nuclear matrices and [^{32}P]DNAs were irradiated and assayed for binding. Poly(dAT) served as a competitor in the binding assays, because it eliminated nonspecific binding of DNA to the nuclear matrix and revealed the radiation-induced increase in tightly bound DNA. When nuclear matrices were isolated from irradiated nuclei (0−200 Gy) and incubated with the κ-Ig MAR fragment in the absence of poly(dAT) (type A experiments), much nonspecific, non-dose-dependent binding was observed. With poly(dAT) in the incubation mixture, a dose-dependent decrease ($p < 0.001$) in the binding was revealed, indicating a radiation-induced loss of available binding sites, perhaps due to the cross-linking of endogenous sequences. The pBR322 fragment did not show a similar loss of binding sites. Irradiation of mixtures of isolated nuclear matrices and end-labeled fragments (type B experiments) allowed the study of radiation-induced cross-linking of exogenous fragments to the matrices. If poly(dAT) was present during irradiation, nonspecific binding was eliminated; however, no significant increase ($p = 0.5$) in the specific binding of the DNA to the nuclear matrix was observed. In contrast, if poly(dAT) was added after irradiation, in addition to the elimination of nonspecific binding, a radiation dose-dependent increase in binding was revealed for both the κ-Ig MAR and the hsp MAR ($p < 0.001$), but not for either of the pBR322 fragments. The results indicate that the specific interaction of MARs with proteins of the nuclear matrix provides a radiation-sensitive substrate for the formation of DNA−protein cross-links.

Eukaryotic DNA is organized into loops of 50−100 kb, which are constrained by attachment to a residual structure termed the nuclear matrix or scaffold (Cook & Brazell, 1976; Paulson & Laemmli, 1977). The DNA sequences that attach to these structures at specific points are called matrix-attachment regions (MARs)[1] or scaffold-attachment regions (SARs). For the sake of simplicity and convenience, the term MARs will be used throughout this document. MARs have been found in or near numerous eukaryotic genes [reviewed by Garrard (1990)]. They are ~300 bp long and often contain topoisomerase II consensus sequences (Sander & Hsieh, 1985; Cockerill & Garrard, 1986), AT-rich motifs [reviewed by Garrard (1990)], or other sequence characteristics (Neuer-Nitsche et al., 1988; Asano et al., 1989; Boulikas & Kong, 1993). MARs can coincide with the boundaries of gene domains (Mirkovitch et al., 1984; Bode & Maass, 1988; Mirkovitch et al., 1988) or replication origins (Amati & Gasser, 1988), or they may reside close to or within enhancer-like regulatory sequences (Udvardy et al., 1985; Izzaurralde et al., 1988; Phi-Van & Stratling, 1988). MARs allow position-independent activity of cloned genes (Stief et al., 1989; Eisenberg & Elgin, 1991) and mediate the relaxation of torsional stress introduced by transcription (Bode et al., 1992).

Initial evidence for the involvement of the nuclear matrix in the formation of DNA−protein cross-links (DPCs) was provided by the observation that transcriptionally active and replicating DNA sequences were found to be enriched in the DPCs of irradiated and unirradiated cells (Chiu et al., 1986a,b, 1989; Oleinick et al., 1986, 1987). Both transcriptionally active and replicating sequences have been found to be associated with the nuclear matrix (McCready et al.,

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[1] Abbreviations: ARBP, attachment region binding protein; BSA, bovine serum albumin; DSB, double-strand breaks; DPC, DNA−protein cross-links; EDTA, ethylenediaminetetraacetic acid; hsp, heat shock protein; κ-Ig, κ-immunoglobulin; LIS, lithium diiodosalicylate; MAR, matrix-attachment region; MELC, murine erythroleukemia cell line; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RSB, reticulocyte standard buffer; SAR, scaffold-attachment region; SDS, sodium dodecyl sulfate; TAE, Tris−acetate−EDTA.

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1980; Robinson et al., 1982; Ciejek et al., 1986), and DPCs were enriched in the proteins of the nuclear matrix (Cress, 1985; Chiu et al., 1986a). The compaction of chromatin within the nuclei by multiple levels of organization protects DNA from radiation-induced damage, including single-strand breaks (Ljungman et al., 1991) and double-strand breaks (DSBs) (Chiu et al., 1992; Warters & Lyons, 1992). However, the DNA associated with the nuclear matrix remains more prone to the formation of DPCs than bulk DNA (Chiu et al., 1986a; Xue et al., 1994). Thus, chromatin organization promotes a nonrandom distribution of radiation-induced strand breaks and DPCs within the cell (Oleinick et al., 1994; Chiu et al., 1995).

The presence of hydroxyl radical scavengers protects cells from the production of ionizing radiation-induced DPCs (Olinski et al., 1981; Mee & Adelstein, 1981; Cress & Bowden, 1983; Oleinick et al., 1987) and from radiation-induced cross-linking of bases and amino acids in solution (Simic & Dizdaroglu, 1985; Gajewski & Dizdaroglu, 1990), suggesting that hydroxyl radicals are responsible for the formation of ionizing radiation-induced DPCs. Radiation-induced DPC production is enhanced when nuclei are pretreated with copper during the preparation of the nuclear matrix (Chiu et al., 1993). Treatment of nuclei with copper is thought to stabilize nuclear structural organization (Lebkowski & Laemmli, 1982). The presence of copper in the nuclear matrix generates hydroxyl and/or metal−peroxyl radicals at the sites of its binding, and such radicals are likely agents for the enhancement of DPC production (Chiu et al., 1993, 1995). The linkage between DNA and protein in a DPC is thought to be covalent since it survives harsh treatments (Krauth & Werner, 1979; Cress & Bowden, 1983; Chiu et al., 1986a; Cress & Kurath, 1988). Evidence for covalent cross-links between DNA bases and amino acids (*e.g.*, thymine−tyrosine, thymine−lysine, and cytosine−tyrosine cross-links) has been provided by the series of studies by Dizdaroglu and co-workers (Gajewski et al., 1988; Dizdaroglu et al., 1989).

Nuclear matrix proteins are the predominant proteins found cross-linked to DNA in normal and irradiated cells (Olinski et al., 1981; Oleinick et al., 1986; Chiu et al., 1986a). However, the specific proteins involved in DPCs have not been identified. Several unique nuclear matrix proteins are known to be closely associated with MARs, *e.g.*, topoisomerase II (Cockerill & Garrard, 1986; Wolf & Stratling, 1990), a 26-kDa protein (Chou et al., 1990), lamin B1 (Ludérus et al., 1992), and SATB1 (Dickinson et al., 1992). It still is not clear whether the proteins that bind MARs are similar to the proteins that cross-link DNA in DPCs due to technical difficulties in isolating sufficient quantities of cross-linked proteins.

Since previous work in this laboratory has indicated the matrix and its associated DNA sequences to be more prone to radiation-induced cross-link formation than bulk cellular chromatin, we wished to evaluate more directly the importance of MARs in DPC formation. The present study was designed to compare the radiosensitivity with respect to DPC induction of two pairs of DNA fragments, one of each pair containing a known MAR and the other of similar size but lacking a MAR. It was found that the DNA fragments containing the κ-Ig MAR or the hsp MAR form significantly more cross-links to the nuclear matrix than do the corresponding non-MAR sequences.

Table 1: The Four DNA Fragments Studied in the *in Vitro* Binding Experiments

DNA fragment	size (kb)	restriction enzymes	parent plasmid
κ-Ig MAR	2.8	*Eco*RI−*Hin*dIII	pG 19/45
hsp MAR	1.15	*Bam*HI−*Hin*dIII	pDM110
pBR322-2.9	2.9	*Eco*RI−*Ava*I	pBR322
pBR322-745	0.745	*Ava*I−*Eco*RI	pBR322

EXPERIMENTAL PROCEDURES

Cell Culture. The murine erythroleukemia cell line DS19 (from Dr. Judith Christman, Michigan Cancer Foundation, Detroit, MI) was routinely grown in McCoy's 5A medium (Gibco) with 20 mM HEPES and 10% fetal bovine serum. The cells were labeled with [*methyl*-^{3}H]thymidine [^{3}H-TdR, 1.85 TBq/mmol (50 Ci/mmol), 0.1 μCi/mL] for 24 h, and the ^{3}H-TdR was chased with fresh medium containing unlabeled TdR (10^{-5} M) for 2 h or overnight prior to the collection of cells. Cells were in exponential growth at the time of harvest [(6−8) × 10^5 cells/mL].

Plasmids and End Labeling. Plasmid pDM110, obtained from Dr. Sherron Helms (Case Western Reserve University, Cleveland, OH), contains the major *Drosophila* hsp70 gene (derived from clone G3) and its promoter as a 2.88-kb insert at the *Bam*HI site of pUC13 (amp^r), with some deletions in the coding regions (Moran et al., 1979; Ingolia et al., 1980). The hsp promoter containing the MAR region (Mirkovitch et al., 1988) was excised as a 1.15-kb *Hin*dIII−*Bam*HI fragment. The plasmid pG 19/45 (amp^r), obtained from Dr. William Garrard (University of Texas Southwestern Medical Center, Dallas, TX), contains the κ-Ig MAR in a 2.85-kb fragment cloned at the *Bam*HI−*Hin*dIII site of pBR322 (Cockerill & Garrard, 1986). The plasmids were amplified in *Escherichia coli* HB101, and the MAR-containing DNA fragments were released using appropriate restriction enzymes. A 2.9-kb *Eco*RI−*Hin*dIII fragment of pBR322 and a 745-bp *Eco*RI−*Ava*I fragment of pBR322 were used as non-MAR controls of similar size to the MAR-containing fragments. The isolated DNA fragments were dephosphorylated and end labeled ([^{32}P]dATP, ~5000 Ci/mmol, Amersham). The final specific radioactivity in cpm/μg for each of the DNA fragments was (0.24−3.4) × 10^7 for κ-Ig MAR, (0.36−2.1) × 10^7 for pBR322-2.9, and (0.6−6) × 10^7 for hsp MAR and pBR322-745. Unincorporated nucleotides were removed by chromatography on Sephadex G-50 columns. The relevant properties of the four fragments are summarized in Table 1.

Isolation of Nuclei and Nuclear Matrices. The procedures described by Cockerill and Garrard (1986) were used with minor modifications. Nuclei were isolated from approximately 10^8 cells and used immediately. Isolated nuclei were resuspended in RSB−0.25 M sucrose containing 1 mM $CaCl_2$ and digested with 100 μg/mL DNase I (Sigma) for 90 min at 23 °C. After centrifugation for 10 min at 750*g* at 4 °C, pellets were resuspended in equal volumes of RSB−0.25 M sucrose and a solution containing 4 M NaCl, 20 mM EDTA, and 20 mM Tris-HCl (pH 7.4). The suspensions were kept on ice for 10 min and then centrifuged at 1500*g* for 15 min at 4 °C. The pellets were extracted twice by suspension in a solution containing 2 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, 0.5 mM PMSF (pH 7.4), and 0.25 mg/mL BSA followed by centrifugation at 4500*g* for 15 min at 4 °C. The resulting nuclear matrix preparation was washed

with RSB−0.25 M sucrose and 0.25 mg/mL BSA at 4 °C. The matrices either were used immediately or were suspended at 6×10^7/mL in the same solution, combined with an equal volume of glycerol, and stored for up to 2 months at −20 °C.

Irradiation of Nuclei or Nuclear Matrices. Exposure to γ-radiation was carried out in a Shepherd Mark I ^{137}Cs irradiator. The dose rate at the position of the samples during the course of these studies was 15.1 to 14.4 Gy/min, as determined by an ionization chamber. For type A experiments, nuclei were suspended in PBS (136 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.37 mM KH_2PO_4) at 6×10^7 nuclei/mL and irradiated on ice with doses of up to 200 Gy. The nuclear matrices were then isolated and assayed for the ability to bind [^{32}P]DNA fragments (see the following). For type B experiments, nuclear matrices were isolated as described earlier and suspended in assay buffer [50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.25 M sucrose, and 0.25 mg/mL BSA]. [^{32}P]DNA fragments were added, and the mixtures were irradiated and assayed for the binding of those fragments. In all cases, samples were irradiated in plastic 1.5-mL Eppendorf tubes and agitated several times during irradiation to maintain oxygenation.

Assay of DNA Binding to Nuclear Matrices. The procedure of Cockerill and Garrard (1986) for the assay of DNA binding to nuclear matrices was followed with a few minor modifications. The number of matrices used in each experiment was based on the number of cells from which the matrices were isolated. The protein content of the matrices for a few experiments was also determined. Matrices were washed three times in the washing solution, with centrifugation each time for 30 s at 10000*g* at 4 °C. Matrices prepared from $\sim 6 \times 10^6$ cells were suspended in each 100 μL of assay solution, and 2 ng of one of the end-labeled fragments was added to each reaction mixture. The final reaction mixture, with the exception of uncompeted controls, contained either 2−500 μg/mL sonicated *E. coli* DNA or 10 μg/mL poly(dAT) as competitor. After incubation with constant agitation on a shaker for 90 min at 23 °C, 1 mL of assay buffer was added, and the matrices were washed twice to remove unbound, labeled DNA and recovered by centrifugation. Matrices were solubilized in 0.5% SDS and digested overnight with 0.4 mg/mL proteinase K, and the DNA was resolved by electrophoresis on 1% agarose gels in TAE buffer (0.04 M Tris−acetate and 1 mM EDTA). The resolved DNA was transferred to a nylon membrane (Zetaprobe) in 0.4 N NaOH. The membrane was dried, and the presence of labeled DNA of appropriate size on the membrane was revealed by autoradiography. Quantitative evaluation of the autoradiograms was carried out by densitometric scanning on a Sci-scan 5000 laser densitometer (Bio-Rad). A region in one electrophoresis lane with no obvious bands was chosen as background and automatically subtracted from all scanned regions of that autoradiogram. Data are presented as the mean ± standard deviation of data from several experiments; p values were obtained by Student's t-test.

Two types of experiments were carried out (see flow chart in Figure 1a,b).

Type A Experiments: Binding of Exogenous DNA Fragments to Matrices from Irradiated Nuclei. The nuclear matrices isolated from irradiated or unirradiated nuclei were suspended in assay solution, and various ^{32}P-end-labeled DNA fragments were added. In the case of condition A2, poly(dAT) (10 μg/mL) was also present. The flow chart in Figure 1a gives details of the two experimental variations used for the type A studies.

Type B Experiments: Irradiation of DNA−Nuclear Matrix Mixtures. Nuclear matrices were suspended in the assay solution and irradiated (0−200 Gy) either in the presence of [^{32}P]DNA fragments followed by incubation in the absence of poly(dAT) (condition B1); in the presence of poly(dAT) and [^{32}P]DNA followed by incubation (condition B2); or in the presence of [^{32}P]DNA fragments followed by incubation with poly(dAT) (condition B3). The flow chart in Figure 1b gives additional detail concerning the three experimental variations used for these studies.

Electrophoretic Analysis of Nuclear Matrix Proteins. Nuclear matrices (10^6) were suspended in a sample-dissolving buffer [0.25 M Tris (pH 6.8), 10% glycerol, 10% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol blue], placed in a boiling water bath for 5 min, cooled to room temperature, and subjected to SDS−PAGE (Laemmli, 1970). The protein content of the nuclear matrices was determined by the method of Lowry et al. (1951), using BSA as standard.

Blotting Procedure and Detection of MAR Binding Proteins. After SDS−PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane in 25 mM Tris base and 190 mM glycine. The blots were either stored in sealed bags at 4 °C or used immediately. The procedure of Chou et al. (1990) was used with slight modifications to detect DNA binding proteins. All DNA binding reactions and washes were performed in DNA binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM β-mercaptoethanol, 0.1 mM Na_2EDTA, 1 mM magnesium acetate, and 50 mM NaCl]. Prior to the addition of [^{32}P]DNA, sonicated *E. coli* DNA (2500 μg/mL) was suspended in the DNA binding buffer and filtered through the nitrocellulose paper to reduce background binding. DNA binding reactions were performed by incubating a protein-blotted nitrocellulose strip in 50 mL of DNA binding buffer containing ^{32}P-labeled DNA fragment [200 ng, $(1-4) \times 10^5$ cpm/mL] and a 2000-fold weight excess of unlabeled, sheared carrier *E. coli* DNA at room temperature for 60 min. The binding reaction was terminated by washing the nitrocellulose strips in 500 mL of DNA binding buffer for 15 min with five changes of buffer, and the strips were washed overnight in the same buffer, air-dried, and subjected to autoradiography.

RESULTS

The nuclear matrices for all of our experiments were obtained by high-salt extraction of DS-19 nuclei. Izzauralde et al. (1988) showed that DNA fragments containing MARs bind with great specificity *in vitro* to nuclear scaffolds prepared by either LIS or high-salt extraction of nuclei. Initial experiments were designed to characterize the matrices of DS-19 cells by electron microscopy (Figure 2) and one-dimensional SDS−PAGE.

Figure 2 presents an electron micrograph of an unirradiated nuclear matrix, revealing structural features reported by others (Berezney & Coffey, 1977; Capco et al., 1982), including a residual pore complex lamina, nucleolus, internal network, ribonucleoprotein particles, and membrane components. The preparation contained mostly intact structures, and no morphologically identifiable differences could be

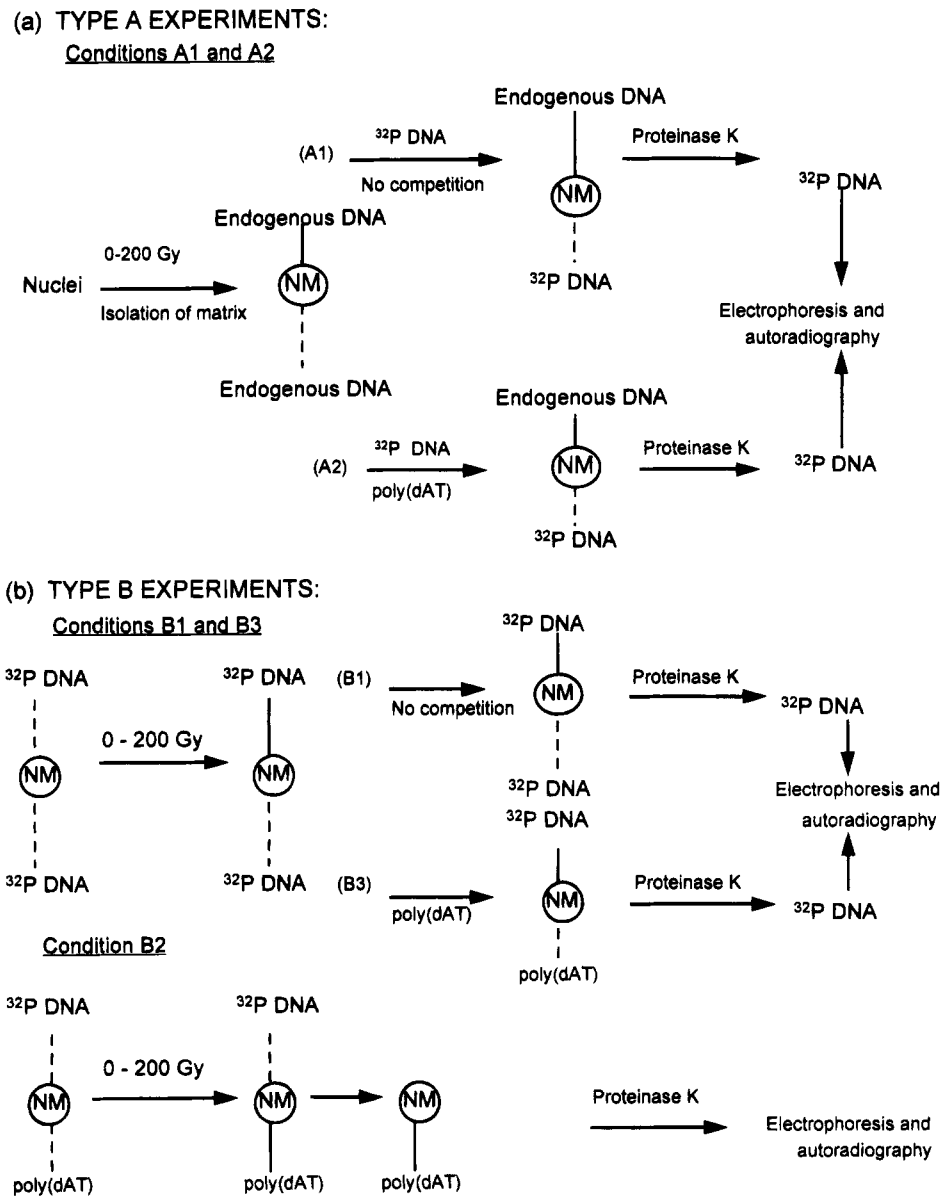

FIGURE 1: Flow chart outlining experiments to assay the effect of radiation on the binding of MAR-containing DNA fragments (κ-Ig MAR or hsp MAR) and two pBR322 DNA fragments to the nuclear matrices. The broken lines in the flow chart indicate noncovalent binding of DNA to the nuclear matrix, and the continuous lines indicate DNA presumed to be cross-linked to the matrix. Although the isolation procedure should remove noncovalently bound DNA from the matrices, for completion both covalent and noncovalent binding of endogenous DNA is indicated. The full description of the assay can be found in the text. (a) Type A experiments (Figures 4, 5): For these experiments, nuclei were irradiated (0−200 Gy), and the nuclear matrices (denoted NM) were isolated from the irradiated nuclei, following which *in vitro* binding assays were carried out under two conditions: condition A1, no competitor added; condition A2, poly(dAT) present during the *in vitro* binding assay with [^{32}P]DNA. (b) Type B experiments (Figures 6−9): This panel explains the assays and conditions used when [^{32}P]DNA and nuclear matrix (NM) mixtures were irradiated. The *in vitro* binding assays were carried out under three conditions: condition B1, no competitor added; condition B2, poly(dAT) present during irradiation; condition B3, poly(dAT) added after irradiation.

discerned between unirradiated and irradiated nuclear matrices (not shown). The preparations contained 2.7 ± 0.7 pg of protein per nuclear matrix (n = 3). Approximately 0.21 ± 0.05% (n = 3) of the chromosomal DNA remained in the isolated nuclear matrices, as determined after prelabeling the cellular DNA with [^{3}H]thymidine. The residual DNA consisted of fragments of 100−1000 bp, as revealed by electrophoresis on 1% agarose gels. The major protein components of the nuclear matrix determined by SDS−PAGE have apparent molecular masses of 45, 50, 57, 62, 67, and 70 kDa (Berezney & Coffey, 1974), as we have previously demonstrated (Chiu et al., 1986a). It has also been demonstrated that nuclear lamins and nuclear matrix proteins called matrins (M_r > 50 000) were the major components of the nuclear matrices isolated from rat hepatocytes (Hakes & Berezney, 1991). Our matrix preparations contained proteins of molecular weights similar to those described by Chiu et al. (1986a) and Berezney and Coffey (1974), as well as a variety of larger proteins.

The next experiments were designed to determine whether the conditions for the *in vitro* binding assays originally established by Cockerill and Garrard (1986) for plasmacytoma cells would be suitable for nuclear matrices from DS-19 erythroleukemia cells. Initially, 6 × 10^6 or 1 × 10^7 matrices per 100 μL of reaction mixture were incubated with 2 ng of end-labeled DNA, and the concentration of unlabeled *E. coli* DNA (Figure 3, competitor II) was varied from 0 to 0.5 mg/mL. Tested as competitor I (Figure 3) were unlabeled

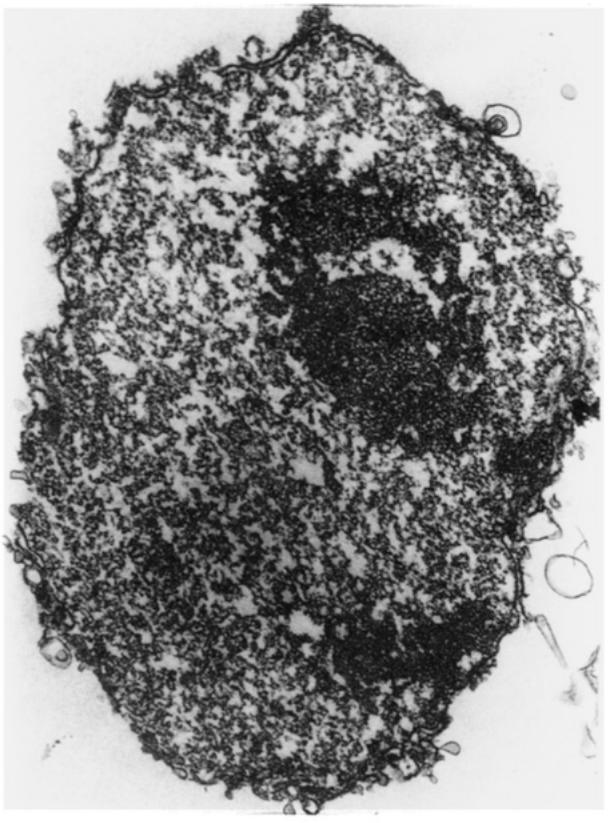

FIGURE 2: Transmission electron micrograph (20000×) of a nuclear matrix from an unirradiated DS-19 nucleus. The residual nuclear lamina and membrane, residual nucleoli, and network-like intranuclear structure containing other residual particles can be seen (cf. Pienta & Coffey, 1985; Berezney, 1991).

κ-Ig MAR and the non-MAR-containing DNA fragments, poly(dAT) and pBR322-2.9. Figure 3 shows the results of such an experiment to evaluate the binding of [^{32}P]κ-Ig MAR (Figure 3a) or [^{32}P]pBR322-2.9 (Figure 3b) to the DS-19 nuclear matrices.

The specific binding of κ-Ig MAR to the nuclear matrix was as expected from the experiments of Cockerill and Garrard (1986) (Figure 3a). The binding of the κ-Ig MAR to nuclear matrices was competed only by very high concentrations (0.5 mg/mL) of *E. coli* DNA. A similarly sized pBR322 fragment, pBR322-2.9, which does not contain a MAR, is competed by lower concentrations of *E. coli* DNA (Figure 3b). The difference in competition was confirmed by densitometry, which revealed that 0.5 mg/mL *E. coli* DNA inhibited 65 ± 10% of [^{32}P]κ-Ig MAR binding and 99 ± 3% of [^{32}P]pBR322-2.9 binding. Since similar results were obtained with both levels of nuclear matrices, for practical reasons the lower value of 6×10^6 nuclear matrices per 100 μL of reaction was chosen for further study. The addition of unlabeled κ-Ig MAR as competitor I markedly inhibited the binding of [^{32}P]κ-Ig MAR to the nuclear matrices with or without the simultaneous presence of sonicated *E. coli* DNA (Figure 3a), whereas only partial inhibition of the binding of [^{32}P]pBR322-2.9 occurred. Unlabeled pBR322-2.9 was an inefficient competitor of [^{32}P]κ-Ig MAR binding in the presence or absence of *E. coli* DNA (Figure 3a).

These results demonstrate that the nuclear matrices isolated from DS-19 cells bind large amounts of DNA, irrespective of the presence of a MAR. Although the sites to which nonspecific binding occurs are not known, the different efficiencies of competitors against the binding of MAR-containing and MAR-free DNA suggest that either different sites on the nuclear matrices are involved or the binding affinities differ or both. Poly(dAT) was also tested as a competitor of the binding of MAR-containing DNA fragments to the nuclear matrices because MAR regions are AT-rich. As shown in Figure 3, poly(dAT) eliminates most of the DNA binding, both MAR-specific and nonspecific, with or without the simultaneous presence of *E. coli* DNA.

It is generally acknowledged that radiation induces DPCs by the covalent linkage of DNA to protein (Cress & Bowden, 1983; Cress, 1985; Dizdaroglu et al., 1989; Oleinick et al., 1987, 1990). Although the assay employed in this study measures the binding of [^{32}P]DNA to nuclear matrices, it is inferred that the binding that is resistant to reversal by poly(dAT), particularly radiation-induced binding, is covalent. Since poly(dAT) is a highly efficient competitor of noncovalent binding, it was used in all subsequent experiments to test whether DPCs were formed between nuclear matrix proteins and DNA upon irradiation. To examine the induction of DPCs by irradiation, the binding of nuclear matrix protein to the four DNA sequences listed in Table 1 was studied in two types of experiments.

Type A Experiments (Figures 4 and 5). Nuclei were suspended in PBS and irradiated with 0−200 Gy. Nuclear matrices were subsequently isolated and tested for their ability to bind exogenous [^{32}P]DNA. It was hypothesized that, upon irradiation of the nuclei, some of the endogenous MAR-containing DNA would become cross-linked to closely associated nuclear matrix proteins, forming DPCs. If DPCs were formed at the endogenous MAR regions and the yields were dose-dependent, then nuclear matrices isolated from the irradiated nuclei would be deficient in unoccupied MAR binding sites. Loss of the capacity to bind exogenous [^{32}P]κ-Ig MAR in a specific manner might also occur through radiation-induced changes in the structure of MAR binding sites. In either case, it would be expected that the specific binding of [^{32}P]κ-Ig MAR to the nuclear matrices would decrease in a radiation dose-dependent manner. If, on the other hand, DPCs were not formed preferentially between MARs and their nuclear matrix-attachment sites in irradiated nuclei, but rather were formed in a nonspecific manner, then the dose-dependent *in vitro* binding of κ-Ig MAR and pBR322-2.9 to the matrices would be similar to one another. An example of results from the type A experiments is shown in Figure 4.

When nuclear matrices were isolated from irradiated nuclei and incubated with either [^{32}P]κ-Ig MAR or [^{32}P]pBR322-2.9 in the absence of poly(dAT) (condition A1, see Figure 1), a large amount of either DNA became bound, and the extent of binding was little affected by prior irradiation of the nuclei (Figure 4). In contrast, when the *in vitro* binding assays were carried out in the presence of a 500-fold excess of poly(dAT) (condition A2, see Figure 1), the majority (~90−98%) of the binding of [^{32}P]DNA was eliminated, and the residual binding of [^{32}P]κ-Ig MAR, but not that of [^{32}P]-pBR322-2.9, was reduced further in nuclear matrices from irradiated nuclei. As the radiation dose to the nuclei was increased to 200 Gy, the specific binding of [^{32}P]κ-Ig MAR to the nuclear matrices was further reduced (Figure 4). In order to normalize for interexperimental variations in the binding of the fragments, and to quantify the apparent differences in the responses of the two [^{32}P]DNA fragments, it was necessary to combine data from several independent

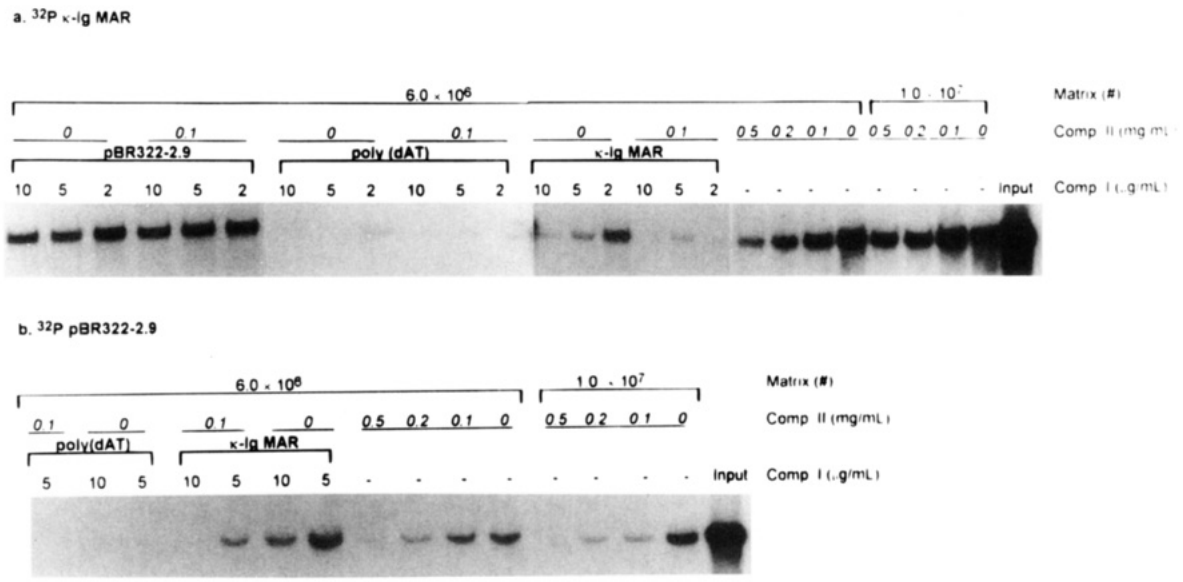

FIGURE 3: Effect of competitor DNAs on the binding of [^{32}P]κ-Ig MAR (a) or [^{32}P]pBR322-2.9 (b) to the nuclear matrix. The *in vitro* binding assays were carried out in the presence of competitor I (unlabeled κ-Ig MAR, pBR322-2.9, or poly(dAT) at 0−10 μg/mL) and/or competitor II (*E. coli* DNA at 0−0.5 mg/mL), as indicated. The lanes marked input represent 10% of the total input of the [^{32}P]DNA used in the binding assays. Each of the other lanes is labeled with the number of nuclear matrices in the 100-μL reaction mixture, the concentration of competitor II (*E. coli* DNA), and the identity and concentration of competitor I, where present. Additional details are described in Experimental Procedures.

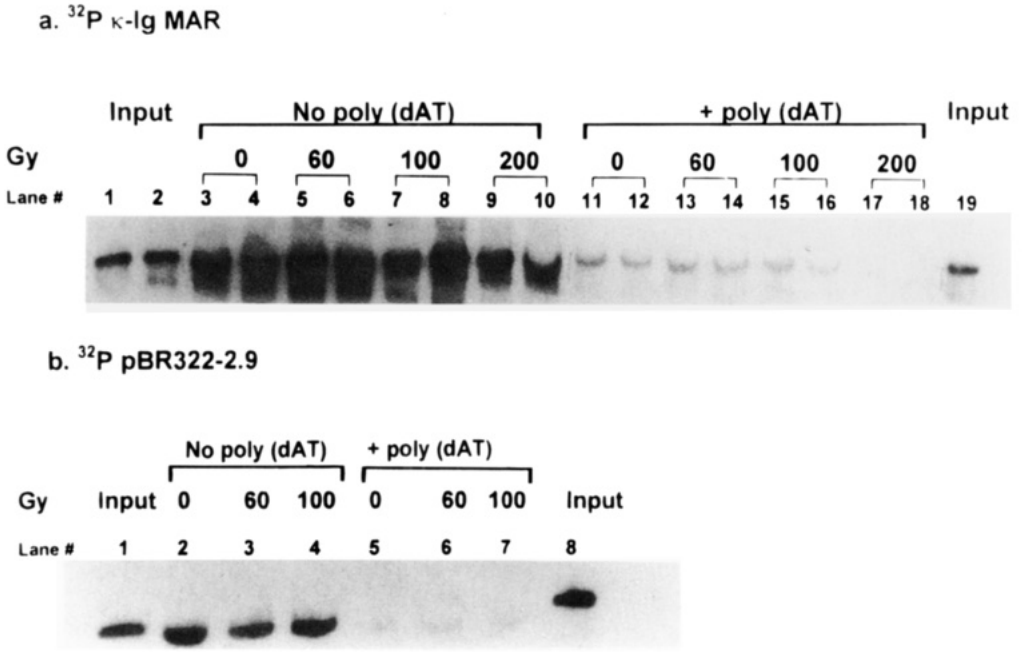

FIGURE 4: Binding of [^{32}P]DNA to nuclear matrices isolated from unirradiated and irradiated nuclei (type A experiments). The upper panel shows the results of an *in vitro* binding assay for 2 ng of [^{32}P]κ-Ig MAR and 6 × 10^6 matrices in 100 μL of reaction mixture. Lanes containing 10% of the total input of [^{32}P]DNA are labeled input. Each of the remaining lanes is labeled with the conditions used for their respective reactions, including the presence or absence of 10 μg/mL poly(dAT) and the radiation dose (Gy) administered to the nuclei from which the nuclear matrices were isolated. The lower panel shows a similar experiment performed with [^{32}P]pBR322-2.9. For this experiment, the average levels of fragment bound to matrices exposed to 0 and 100 Gy (in arbitrary units, as determined by densitometry) are, respectively, for [^{32}P]κ-Ig MAR, condition A1, 8.9 and 6.5; condition A2, 0.87 and 0.13; for [^{32}P]pBR322-2.9, condition A1, 29.7 and 17.8; condition A2, 0.98 and 1.36. The results of a series of such experiments are summarized in Figure 5.

experiments. Therefore, for each experiment, the autoradiograms were scanned in a densitometer, and data, in arbitrary densitometry units, for the binding of each [^{32}P]DNA fragment in irradiated samples were normalized to data for the binding of that fragment to nuclear matrices from unirradiated nuclei. The data for 100 Gy are shown in Figure 5.

In the absence of competition by poly(dAT) (Figure 5, condition A1), values for the relative binding of [^{32}P]κ-Ig MAR to the nuclear matrices from 100-Gy-irradiated nuclei varied widely, but the mean was not significantly different from the mean of values for binding to unirradiated matrices. Similarly, binding of [^{32}P]pBR322-2.9 did not differ for unirradiated and 100-Gy-irradiated matrices (Figure 5). When poly(dAT) was present, the binding of either fragment was much reduced, i.e., the ratio of binding in condition A2 to that in condition A1 for unirradiated samples was 0.09 for [^{32}P]κ-Ig MAR and 0.03 for [^{32}P]pBR322-2.9. Although

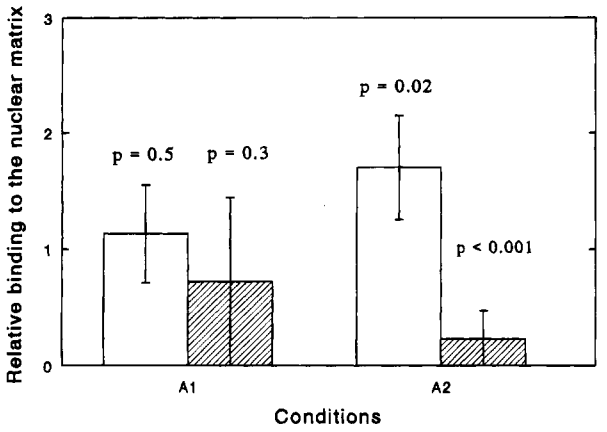

FIGURE 5: Summary of the data from type A experiments. For each experiment of the type described in Figure 4, data for the binding of [^{32}P]DNA (κ-Ig MAR, hatched, or pBR322-2.9, □) to nuclear matrices from irradiated (100 Gy) nuclei were normalized to the binding of the same DNA fragment to nuclear matrices from unirradiated nuclei (=1.0 on the ordinate). The panels show the following: condition A1, DNA binding to nuclear matrices in the absence of a competitor; condition A2, DNA binding to matrices in the presence of poly(dAT). Error bars signify the standard deviation about the mean value. p values are given for the differences in relative binding at 100 versus 0 Gy for four (pBR322-2.9) or eight (κ-Ig MAR) independent determinations.

the binding was reduced by poly(dAT), it was possible to compare the ability of matrices from irradiated and unirradiated nuclei to bind exogenous DNA under these conditions (condition A2). Thus, in the presence of poly(dAT), irradiated (100 Gy) matrices bound only 36 ± 12% as much [^{32}P]κ-Ig MAR as unirradiated matrices ($p < 0.001$), whereas the binding of [^{32}P]pBR322-2.9 was more efficient (170 ± 45%) to irradiated than to unirradiated matrices ($p = 0.02$). The reason for the small but consistent elevation in the binding of [^{32}P]pBR322-2.9 after irradiation is not known, but it may reflect the opening of additional nonspecific binding sites following irradiation or the promotion of binding of the pBR322 fragment by the poly(dAT).

These experiments confirm the ability of the nuclear matrix to bind high levels of DNA in a nonspecific manner and further demonstrate that up to 200 Gy of γ-radiation does not interfere with nonspecific binding. The data also show that poly(dAT) is an effective competitor of nonspecific binding, essentially eliminating all but a small fraction of the binding. Furthermore, the MAR-containing fragment is distinguished from the non-MAR fragment by its residual binding in the presence of poly(dAT). Only the specific binding of the exogenous MAR-containing fragment to the nuclear matrices is reduced by prior irradiation.

Type B Experiments (Figures 6−9). The conditions for type B experiments are discussed in detail in Experimental Procedures and diagrammed in Figure 1b. The purpose of these experiments was to analyze whether the irradiation of mixtures of nuclear matrices and end-labeled DNA would show preferential binding of MAR-containing DNA, indicative of radiation-induced cross-linking. Hence, if the specific binding of [^{32}P]κ-Ig MAR to the nuclear matrices increased as a function of radiation dose when nonspecific binding was eliminated by poly(dAT), the dose response could be attributed to the specific cross-linking of MAR-containing DNA fragments. As before, the presence of labeled fragments on the matrices was detected by autoradiography after gel electrophoresis. A representative experiment for κ-Ig MAR and pBR322-2.9 is shown in Figure 6, and the data of several independent experiments are summarized in Figure 7.

In the absence of competition (condition B1), both [^{32}P]κ-Ig MAR and [^{32}P]pBR322-2.9 bound at high levels to the nuclear matrices, and the binding was independent of radiation dose (Figures 6 and 7). Since the binding of all DNA fragments to the nuclear matrices in the absence of poly(dAT) is very high, the small fraction of DPCs that might form after 100 Gy would not be large enough to be distinguished from the background of nonspecific binding under this condition. Upon irradiation (0−100 Gy) of DNA fragments with the isolated nuclear matrices in the presence of poly(dAT) (condition B2), little if any binding of either [^{32}P]κ-Ig MAR or [^{32}P]pBR322-2.9 was revealed (Figure 6). In contrast, when matrix−DNA fragment mixtures were irradiated in the absence of poly(dAT) and subsequently incubated with poly(dAT) (condition B3), a radiation-induced increase in the specific binding of [^{32}P]κ-Ig MAR was observed (2.6 ± 0.6-fold at 100 Gy). This increase was significantly different from the binding at 0 Gy ($p < 0.001$). Under the same conditions, there is no evidence of dose dependence for the binding of [^{32}P]pBR322-2.9 to the nuclear matrices (condition B3; binding at 100 Gy was 1.1 ± 0.8-fold relative to binding at 0 Gy, $p = 0.5$) (Figures 6 and 7). Therefore, it appears that the MAR-specific binding of DNA to the nuclear matrix-anchorage sites predisposes the DNA to the formation of DPCs.

To determine whether this property is characteristic of another MAR-containing fragment, the hsp MAR and a non-MAR-containing pBR322 fragment of similar size were also assayed by a type B experiment. Figures 8a and 9a show representative experiments and Figures 8b and 9b summarize the normalized data for several independent experiments. Irradiation of [^{32}P]hsp MAR and nuclear matrices in the absence of competitor resulted in a high level of DNA binding to the matrices, which was not altered by irradiation (Figure 8, condition B1). When poly(dAT) was present during irradiation (condition B2), little [^{32}P]hsp MAR binding was observed. As previously observed for [^{32}P]κ-Ig MAR (Figures 6 and 7), irradiation of [^{32}P]hsp MAR and nuclear matrices caused a 4.2 ± 0.9-fold increase ($p < 0.001$) in specific binding, which was revealed by postirradiation competition with poly(dAT) (Figure 9, condition B3). In contrast, the binding of [^{32}P]pBR322-745 in irradiated (100 and 200 Gy) samples never exceeded that in unirradiated samples; instead the binding decreased to 69 ± 25% (100 Gy, $p < 0.01$; 200 Gy, $p = 0.07$) of the binding at 0 Gy. A decrease in binding ($p = 0.1$) was also observed for 200 Gy in condition B1 for pBR322-745.

Oleinick et al. (1986), Chiu et al. (1986a), and Cress (1985) have identified a subset of nuclear matrix proteins as the dominant species cross-linked to DNA in normal and irradiated cells. In order to identify candidate DNA binding proteins of the nuclear matrix, nuclei were irradiated (0−200 Gy), nuclear matrices were isolated, and matrix proteins were recovered and subjected to electrophoresis on triplicate SDS−PAGE gels. One of the gels was stained with Coomassie Blue to visualize the protein bands (not shown). The proteins on the other gels were transferred to nitrocellulose membranes and allowed to bind to [^{32}P]κ-Ig MAR or [^{32}P]pBR322-2.9. The southwestern blots were then washed and visualized by autoradiography. Figure 10 shows

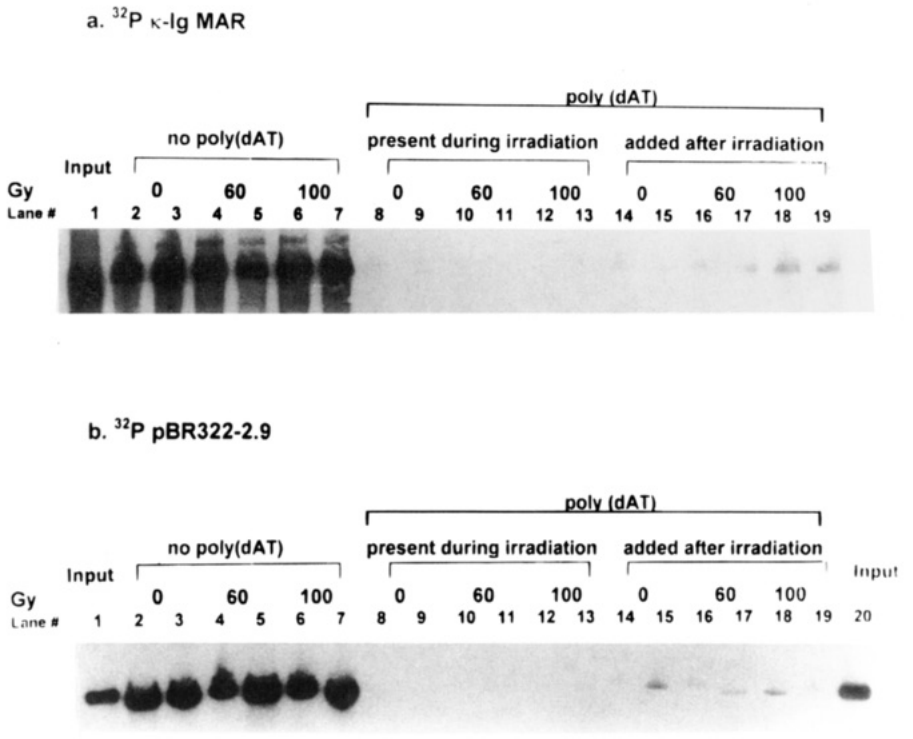

FIGURE 6: Effect of irradiating [^{32}P]DNA and nuclear matrix mixtures under the three conditions for type B experiments (see flow chart, Figure 1): upper panel, [^{32}P]κ-Ig MAR; lower panel, [^{32}P]pBR322-2.9. Lanes marked input contain 10% of the total [^{32}P]DNA per reaction mixture. The remaining lanes show the matrix-bound [^{32}P]DNA following irradiation (0−100 Gy) of DNA−nuclear matrix mixtures lacking poly(dAT) or with poly(dAT) present during or added after irradiation. For this experiment, the average levels of fragment bound to matrices exposed to 0 and 100 Gy (in arbitrary units, as determined by densitometry) are, respectively, for ^{32}P-κ-Ig MAR, condition B1, 16.8 and 15.3; condition B3, 0.23 and 1.1; for [^{32}P]pBR322-2.9, condition B1, 10.6 and 14.6; condition B3, 1.38 and 1.24. Values for condition B2 were too low for accurate determination. The results from a series of such experiments are summarized in Figure 7.

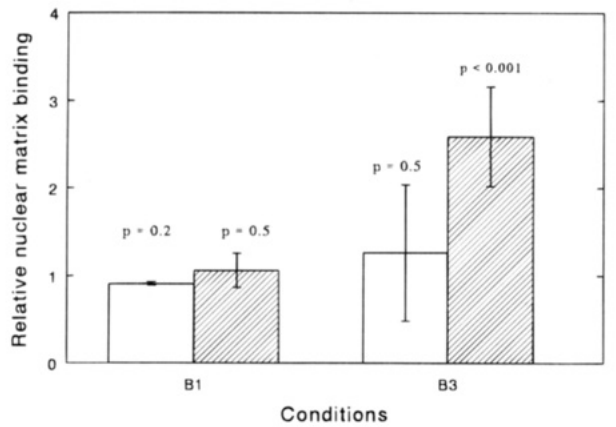

FIGURE 7: Summary of data from type B experiments with [^{32}P]κ-Ig MAR or [^{32}P]pBR322-2.9. For each experimental condition of the types described in Figure 6, data for the binding of [^{32}P]DNA (κ-Ig MAR, hatched, or pBR322-2.9, □) to nuclear matrices after irradiation (100 Gy) were normalized to the binding of the same DNA fragment to unirradiated nuclear matrices (=1.0 on the ordinate): condition B1, DNA binding to nuclear matrices in the absence of a competitor; condition B3, poly(dAT) added after irradiation. Error bars signify the standard deviation about the mean value. p values are given for the differences in relative binding at 100 versus 0 Gy for three (pBR322-2.9) or six (κ-Ig MAR) independent determinations.

that, under the binding conditions used, both DNA fragments bound predominantly to three proteins of M_r 40 000, 107 000, and 158 000. Neither the identity nor the intensity of the protein bands was affected by the radiation dose. The similarity in the DNA binding proteins and the absence of a radiation dose response indicate that the identified proteins may be responsible for the nonspecific binding of DNA to the matrix.

DISCUSSION

An *in vitro* system capable of detecting specific MAR−matrix interactions (Cockerill & Garrard, 1986) has been modified to study the formation of radiation-induced cross-links between the nuclear matrix and MAR-containing DNA sequences. High-salt extraction of DNase I-digested nuclei from murine erythroleukemia cells produced a morphologically recognizable nuclear matrix (Figure 2), which maintained its capacity to bind MAR-containing DNA fragments in a specific manner (Figure 3). The nuclear matrices retained 0.21 ± 0.05% (n = 3) of the chromosomal DNA present as fragments of ~100−1000 bp. Neuer and Werner (1985) and Werner and Rest (1987) have demonstrated very tight DNA−polypeptide complexes remaining in the nuclear matrix after high-salt extractions; the complexes were stable to treatments with SDS, β-mercaptoethanol, and alkali, indicating that they may be covalent and could represent the anchorage sites of DNA in the nuclear matrix. Assays with nuclear matrices from other cell types have been used previously to identify the MAR sequences residing in or near a variety of genes (*e.g.*, Cockerill & Garrard, 1986; Jarman & Higgs, 1988; Phi-Van & Stratling, 1988; Chou et al., 1990). By using this system, the present study has provided evidence for the cross-linking of matrix-attachment sites and MARs by radiation. This was demonstrated by the preferential conversion of specific MAR−matrix interactions into attachments not removable by competition with poly(dAT) (Figures 6−9) and by the preferential radiation-induced loss

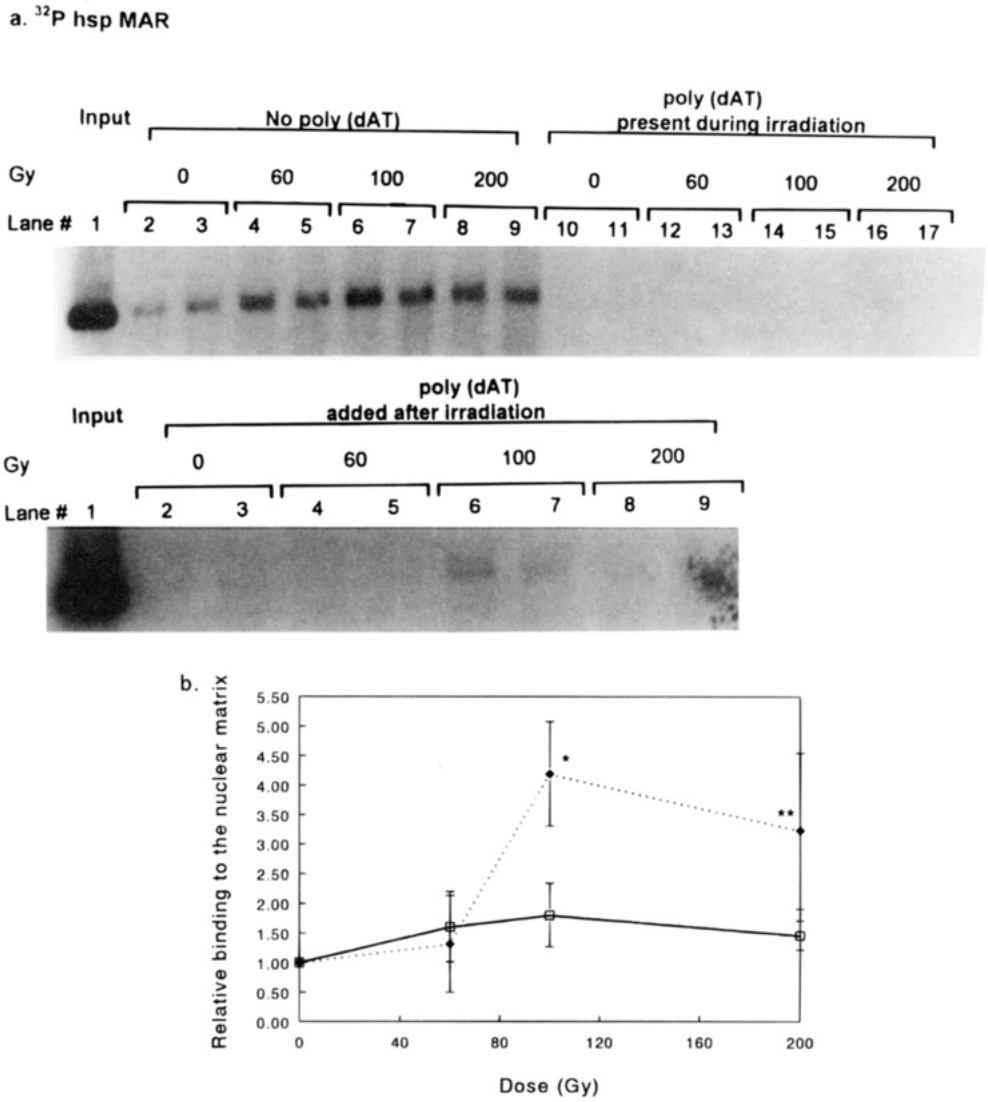

FIGURE 8: Effect of irradiating [^{32}P]hsp MAR and nuclear matrix mixtures under the three conditions for type B experiments. (a) [^{32}P]hsp MAR bound to nuclear matrices after irradiation (0−200 Gy) under conditions B1, B2, and B3 (as in Figure 6), as indicated. Input: 10% of the total input of [^{32}P]hsp MAR in this experiment. For this experiment, the average levels of fragment bound to matrices exposed to 0 and 100 Gy (in arbitrary units, as determined by densitometry) are, respectively, condition B1, 8.6 and 14.9; condition B3, 1.5 and 10.0. Levels of binding under condition B2 were too low to be determined accurately. (b) Summary of the type B experiments for [^{32}P]hsp MAR. All of the data for irradiated samples were normalized to binding under the same conditions at 0 Gy: □, condition B1; ◆, condition B3. p (*p < 0.001, **p < 0.005) values are given for the differences in relative binding at 100 versus 0 Gy for four independent determinations with two or three repeats within each experiment.

of nuclear sites capable of specific MAR binding (Figures 4 and 5).

An important component of the *in vitro* binding assay, especially for the radiation studies, is the competitor DNA. In studies by Izzauralde et al. (1988) and Cockerill and Garrard (1986), the presence of a MAR within a test DNA sequence was inferred from its increased capacity to resist competition by sonicated *E. coli* DNA, and this competitor was evaluated for the present binding assays (Figure 3). The mechanisms responsible for the strong competition by *E. coli* DNA against the nonspecific binding of DNA in general and the reduced competition against the specific binding of MAR-containing DNAs to the nuclear matrix remain unclear. In living *E. coli*, the DNA exhibits torsional tension that is relaxed by γ-radiation-induced DNA strand breaks, consistent with the presence of independent supercoiled domains (Sinden & Pettijohn, 1981). Among the compatible models to explain the domain structure of *E. coli* DNA, one proposes direct DNA−membrane interactions. Such a model implies the presence of DNA sequences in *E. coli* having a function similar to those of the MARs of eukaryotic DNA. Hence, *E. coli* DNA may contain MAR-like sequences that compete for MAR binding sites on the matrix in a manner similar to a eukaryotic DNA (*e.g.*, salmon sperm DNA). Furthermore, the unlabeled, sonicated DNA of either *E. coli* or salmon sperm is a mixture of DNA sequences and fragment sizes. Competition for nonspecific binding sites on the nuclear matrix may result from the presence of DNA ends or unknown sequences or structures scattered throughout the DNA.

In the absence of a competitor, the nuclear matrices bind a vast amount of DNA independent of the presence of a MAR. At 20 ng/mL [^{32}P]DNA, 159 ± 7 (n = 3) and 620 ± 33 (n = 3) fragments of pBR322-2.9 and pBR322-745, respectively, were bound per matrix, which are levels comparable to those of the κ-Ig MAR (167 ± 8, n = 6) and the hsp MAR (400 ± 20, n = 5), respectively. Much of this binding appears to be at sites other than specific matrix-

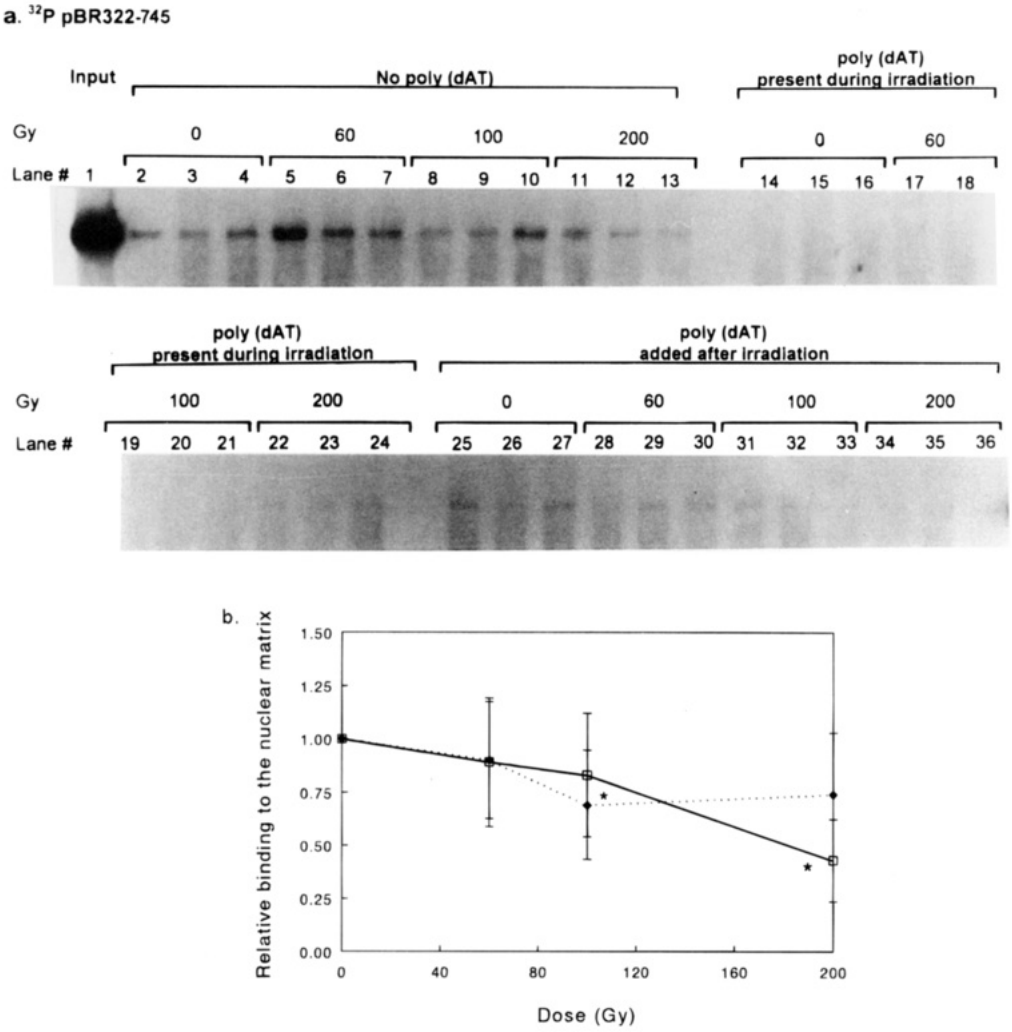

FIGURE 9: Effect of irradiating [^{32}P]pBR322-745 and nuclear matrix mixtures under the three conditions for type B experiments. (a) [^{32}P]pBR322-745 bound to nuclear matrices after irradiation (0−200 Gy) under conditions B1, B2, and B3 (as in Figure 8). Input: 10% of the total input of [^{32}P]pBR322-745 in this experiment. For this experiment, the average levels of fragment bound to matrices exposed to 0 and 100 Gy (in arbitrary units, as determined by densitometry) are, respectively, condition B1, 6.9 and 4.9; condition B3, 4.1 and 4.3. Values for condition B2 were too low for accurate determination. (b) Summary of the type B experiments for [^{32}P]pBR322-745. Data for irradiated samples were normalized to binding under the same conditions at 0 Gy: □, condition B1; ◆, condition B3. Error bars signify the standard deviation about the mean value. *$p < 0.01$ for the relative binding of irradiated samples versus 1.00 for three independent determinations.

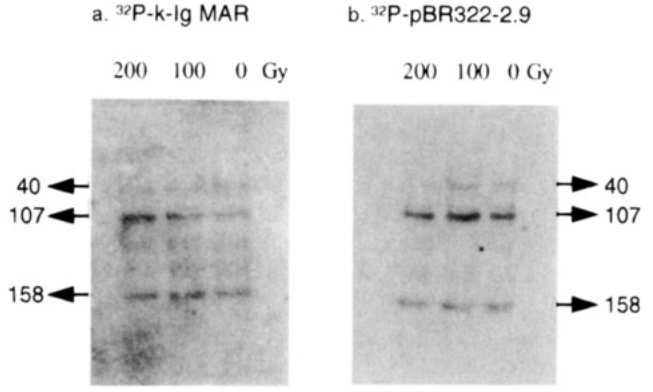

FIGURE 10: Southwestern blot showing the binding of [^{32}P]κ-Ig MAR and [^{32}P]pBR322-2.9 to nuclear matrix proteins. After one-dimensional SDS−PAGE, proteins from unirradiated (0 Gy) and irradiated (100 or 200 Gy) nuclear matrices were transferred to nitrocellulose membranes, and end-labeled [^{32}P]κ-Ig MAR and [^{32}P]-pBR322-2.9 were allowed to bind to the blotted proteins, as described in Experimental Procedures.

attachment sites because a MAR is not required, and the binding is more easily prevented or removed by competitors than is the low level of binding found uniquely for MAR-containing fragments. Although the structural requirements for the nonspecific binding are not known, the possibility that it is directed by DNA ends is consistent with the greater binding of the smaller test fragments (pBR322-745 and hsp MAR). There are ~100 000 potential MAR binding sites per nuclear matrix (Cockerill & Garrard, 1986), only a few of which will be occupied by [^{32}P]DNA at the subsaturation levels of MAR fragments chosen here for the purpose of distinguishing between unirradiated and irradiated nuclear matrices. The occupancy level in the intact cell is not known, but is likely to be less than 100% as it has been found that only a fraction of MARs are matrix-bound at any particular time and dynamic, as evidenced by the exchange of exogenous with endogenous MAR-containing fragments (*e.g.*, Cockerill & Garrard, 1986; Gasser & Laemmli, 1986a,b).

We found the heteropolymer poly(dAT) to be an efficient competitor against both nonspecific (*e.g.*, pBR322) and specific (*e.g.*, κ-Ig MAR) DNA binding to the nuclear matrix. MAR sequences are AT-rich [reviewed by Garrard (1990)], and mutations in certain AT-rich motifs have led to reduced

affinity of MARs for the nuclear matrix (Bode et al., 1992). Since poly(dAT) removes most of the binding of MAR-containing fragments in the absence of irradiation, cross-linking after the irradiation of mixtures of nuclear matrix and MARs can be identified as an increase in poly(dAT)-resistant binding. Alternatively, cross-linking of endogenous sequences in irradiated nuclei or destruction of MAR sites can be deduced by a decrease in the poly(dAT)-resistant binding of exogenous MARs to nuclear matrices derived from the irradiated nuclei. Poly(dAT) provides strong competition for the nonspecific binding, possibly by virtue of fragment ends, as well as competition for the specific binding of MAR fragments by virtue of the presence of AT-rich sequences.

Ionizing radiation-induced DPCs occur preferentially at or near nuclear matrix-attachment sites. Therefore, our first approach to study the radiation sensitivity of the MAR–matrix interaction was to cross-link endogenous DNA sequences to proteins of the nuclear matrix (or alter the structure of these sites) by irradiation of nuclei and subsequently to determine the ability of the matrices isolated from those irradiated nuclei to bind DNA. The number of potential MAR sites that were actually occupied in the nuclei during irradiation is not known, nor is it known whether or not a MAR site must be occupied to be subject to the radiolytic alteration of structure. The results of the type A experiments (Figures 4 and 5) show that the binding of κ-Ig MAR to nuclear matrices from irradiated (100 Gy) nuclei, in the presence of poly(dAT) (condition A2), was reduced to 23 $\pm$ 24% ($p < 0.001$) of that to matrices from unirradiated nuclei. In contrast, the binding of pBR322-2.9 was marginally increased to 170 $\pm$ 45% ($p = 0.02$) by prior irradiation of the nuclei. These results are consistent with the selective radiation-induced cross-linking of endogenous MARs to their binding sites or the selective loss of MAR sites, thereby reducing the number of attachment sites available for binding exogenously added MAR fragments. The large number of nonspecific binding sites on the nuclear matrix was not affected by γ-radiation (condition A1; κ-Ig MAR, $p = 0.3$; pBR322-2.9, $p = 0.5$). Therefore, the difference in binding of exogenous MAR sequences involved only a small fraction of the total amount of bound DNA and was apparent only in the presence of poly(dAT) (Figures 4 and 5). The increase in binding of pBR322-2.9 to matrices from irradiated as compared to unirradiated nuclei in the presence of poly(dAT) was unexpected, but might result from radiation-induced alteration of a subset of nonspecific binding sites such that they become less susceptible to competition by poly(dAT). Further investigation will be required to test this possibility.

As a second and more direct approach, mixtures of DNA fragments and nuclear matrices were irradiated. We assume that there are two forms of binding of the DNA fragments to the isolated nuclear matrices. Before irradiation, the binding is noncovalent and may arise in part from a dynamic exchange at MAR sites between endogenous MAR-containing DNA sequences and an exogenous [^{32}P]DNA containing a MAR [Cockerill & Garrard, 1986; reviewed by Garrard (1990)]. Preirradiation binding is primarily nonspecific and removable by poly(dAT). After irradiation, those DNA fragments that have been covalently cross-linked to proteins of the nuclear matrix can no longer be removed by poly(dAT). Hence, if MAR binding sites are more prone to cross-linking than nonspecific sites, then after irradiation of the mixtures, MAR sequences should be preferentially retained with the matrix compared to non-MAR sequences.

In the absence of competition by poly(dAT) (condition B1), all four DNA fragments bound efficiently to the matrices, and no significant effect of radiation was detected for the binding of any fragment (with the exception of a slight increase in binding of hsp MAR at 100 Gy but not at 200 Gy) (Figures 6–9). When the mixtures contained poly(dAT) during irradiation (condition B2), very little [^{32}P]DNA was bound, so that levels of binding were not quantified. The extremely low binding demonstrates the effectiveness of competition by poly(dAT). When poly(dAT) was added after irradiation (condition B3), the levels of binding, although low, were greater than those for condition B2, and evidence for the formation of DPCs involving MARs was obtained. Irradiation (100 Gy) produced 2.7 $\pm$ 0.6-fold ($p < 0.001$) and 4.2 $\pm$ 0.9-fold ($p < 0.001$) increases in binding of κ-Ig MAR and hsp MAR to the nuclear matrix, respectively (Figures 7 and 8). In contrast, the binding of the control non-MAR fragments to the matrix was unchanged after 100 Gy (pBR322-2.9, 1.1 $\pm$ 0.8-fold, $p = 0.5$) or decreased (pBR322-745, 0.43 $\pm$ 0.19-fold, $p < 0.01$) as compared to the binding of those fragments at 0 Gy (Figures 7 and 9). Therefore, it appears that poly(dAT), which can replace or remove most MARs from the specific binding sites before irradiation, is unable to do so after cross-linking and that preformed complexes between MARs and matrix proteins are susceptible to radiation-induced cross-linking under conditions in which DNA fragments lacking MARs are not.

Numerous purified nuclear matrix proteins have been found to bind MARs in a specific manner. These include ARBP (attachment region binding protein; von Kries et al., 1991), p25 (Chou et al., 1990), lamin B1 (Ludérus et al., 1992), and SATB1 (Dickinson et al., 1992). Kas et al. (1989) showed that MARs direct highly specific binding to purified histone H1 due to the presence of oligo(dA)·oligo(dT) tracts, which in turn are a target for the drug distamycin. The interaction of distamycin with MAR sequences resulted in complete suppression of binding to either matrices or histone H1. The authors proposed that both high-salt- and low-salt-extracted nuclear matrices contain residual histones, including histone H1. However, in a study of the correlation of histone extraction with the ability of radiation to induce DPCs, Xue et al. (1994) demonstrated that matrices extracted by 1.0–1.2 M NaCl had lost all detectable histone H1, but retained their sensitivity to formation of radiation-induced DPCs. Therefore, proteins other than histone H1 are preferred substrates for DPC formation. Matrices efficiently depleted of all detectable histones with 2 M NaCl were able to form 8% as many DPCs as unextracted matrices (Xue et al., 1994). Therefore, the low efficiency of DPC formation in the present study may be partially explained by our use of high-salt-extracted matrices for the binding assays.

The proteins of DPCs are predominantly nuclear matrix proteins (Oleinick et al., 1986; Chiu et al., 1986a). Analysis of nuclear matrix proteins isolated from unirradiated and irradiated MELC nuclei, by gel electrophoresis and binding to [^{32}P]DNA fragments, indicates that both MAR-containing and non-MAR fragments recognize three proteins of apparent molecular mass 40 000, 107 000, and 158 000 Da (Figure 10). As revealed by molecular sizing, these proteins differ

from all of the MAR binding nuclear matrix proteins identified in other laboratories (von Kries et al., 1991; Chou et al., 1990; Ludérus et al., 1992; Dickinson et al., 1992). Because the binding of DNA to these proteins does not appear to require a MAR, they could be proteins to which both specific and nonspecific DNA can bind; furthermore, these potential nonspecific binding sites are not affected by prior irradiation of the nuclei.

The observed cross-linking at MAR sites by radiation has not yet been linked to cellular radiosensitivity. However, a correlation between altered nuclear matrix organization and radiation sensitivity has been observed by electron microscopy (Yasui et al., 1994) and by studying the sedimentation properties or fluorescence of dehistonized nuclear matrices [Cook & Brazell, 1976, 1978; Roti Roti & Wright, 1987; Taylor et al., 1991; reviewed by Roti Roti et al. (1993)]. These latter studies have shown that the presence of radiation-induced DNA breaks interferes with the ability of unwound DNA loops to rewind upon the addition of an intercalating agent. To explain the defective loop rewinding, Roti Roti and colleagues have proposed a model invoking differences in the strength of the anchorage sites between the radioresistant and radiosensitive cells. Differences in protein content between the nuclear matrices of sensitive and resistant cell pairs have provided evidence for the role of the nuclear matrix in determining loop-rewinding characteristics (Malyapa et al., 1995).

The nuclear matrix is prone to the formation of chromium-induced DPCs, as well as to those produced by radiation. Upon treating CHO cells with chromium, Xu et al. (1994) found a 3.8-fold enrichment of chromium in the nuclear matrix as compared to bulk chromatin, a 4-fold enrichment of chromium−DNA adducts in the nuclear matrix as compared to total nuclear DNA, and chromium-induced DPCs exclusively in the nuclear matrix fraction. Similarly, treatment of nuclei with $CuSO_4$ in the presence of ascorbate and hydrogen peroxide leads to the formation of DPCs; the preferential location of the copper-induced DPCs in the nuclear matrix was revealed by the inability of histone extraction from the nuclei to affect DPC formation (Xue et al., 1994).

Dizdaroglu and co-workers have identified a variety of specific DNA base−amino acid covalent cross-links in chemical model systems and in cellular chromatin (Gajewski et al., 1988; Dizdaroglu et al., 1989). More commonly, DPCs have been detected in cells by alkaline filter elution assays. These assays detect the presence of single-strand breaks by the increased elution rate of broken DNA and infer the presence of DPCs when omission of the proteolysis step retards the elution rate. DPCs are detected by elution assays much more efficiently when the cells have been irradiated in the absence of oxygen, leading to the conclusion that DPC formation depends on radiobiological hypoxia (e.g., Meyn et al., 1987; Xue et al., 1988). However, a more direct assay for DPCs, nitrocellulose filter binding, has revealed efficient DPC formation upon the irradiation of well-oxygenated cells (Xue et al., 1988). Thus, the DPCs measured by elution and by filter binding may be different lesions. In the present study, small (100 μL) samples were irradiated in plastic tubes and with agitation at frequent intervals during irradiation; under these conditions, in spite of some radiolytic loss of oxygen, radiobiological hypoxia could not have been reached. Therefore, the DPCs formed between MAR-containing DNA and nuclear matrices resemble those formed in irradiated oxygenated cells and detected by filter binding.

In summary, the present study has demonstrated that the complexes formed by MAR-containing DNA fragments at nuclear matrix-attachment sites differ from nonspecific complexes and are prone to cross-linking by radiation under oxic conditions. Little is known concerning the structural characteristics of the MAR binding sites or their complexes with DNA, but the cross-linking of MARs to nuclear matrix proteins may help in the isolation of the complexes for elucidation of the DNA−protein interaction.

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