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Predominance of core histones in formation of DNA–protein crosslinks in γ-irradiated chromatin

(radicals/gel electrophoresis)

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ABSTRACT **Chromatin and a subunit of chromatin containing a complex of DNA and the core histones—H2A, H2B, H3, and H4—have been prepared from cultured Chinese hamster cells. Comparison of the formation of radiation-induced DNA–protein crosslinks in whole chromatin with that in the DNA–core histone complex has demonstrated that the core histones are the specific proteins involved in crosslinking. γ irradiation of the chromatin subunit in the presence of radical scavengers has shown the hydroxyl radical to be the most effective aqueous radical intermediate for the promotion of crosslinking and the solvated electron and superoxide radical to be essentially ineffective.**

DNA-protein crosslinks are formed when whole cells or mixtures of DNA and cell protein *in vitro* are subjected to γ or UV irradiation (1, 2). Such nucleic acid–protein bonds are stable to salt and detergent treatment. These linkages may be of importance in cell killing, mutagenesis, and carcinogenesis. We have shown recently that γ irradiation of chromatin *in vitro* or *in vivo* induces such DNA–protein crosslinks (3). We now investigate the question of which chromosomal protein(s) is crosslinked to the DNA, and which radiation-induced radical(s) is responsible for the reaction.

It is generally accepted that, in eukaryotic cells, nuclear DNA is organized as nucleosomes, consisting of a core region, containing the histones H2A, H2B, H3, and H4, wrapped around with DNA; these core regions are joined by linker DNA, probably associated with H1 histone. Isolated together with the DNA and histones in purified chromatin are nonhistone chromosomal proteins (NHCP), probably consisting of as many as 100 structural, enzymic, and regulatory proteins, of which 15–20 are major constituents.

Chromatin and a subunit of chromatin containing a complex of DNA and the core histones have been isolated from cultured Chinese hamster cells. On the basis of comparing radiation-induced crosslinking in the whole chromatin and in the chromatin subunit, we have now identified the core histones as the specific chromosomal proteins predominantly involved in crosslinking to DNA. Furthermore, γ irradiation of the chromatin subunit in the presence of radical scavengers has confirmed the efficacy of the hydroxyl radical for the promotion of formation of crosslinks, and the inefficacy of the solvated electron and superoxide radical (3).

MATERIALS AND METHODS

Cell Culture and Labeling. Chinese hamster lung fibroblasts (V79-753 cell line from J. A. Belli, Harvard Medical School) were grown as described (4). Eagle's minimal essential medium was supplemented with 15% fetal bovine serum, 4% NCTC-109 medium (M. A. Bioproducts, Walkersville, MD), 2 mM L-glutamine, 0.1 mM of each nonessential amino acid, penicillin at 50 units/ml, and streptomycin at 50 μg/ml. Cells were grown as monolayers in 150-cm^2 plastic flasks at 37°C in an atmosphere of 5% CO_2. They were grown for two generations (18 hr) prior to harvesting in a medium containing [*methyl*-^{3}H]thymidine (New England Nuclear) at a concentration of 0.25 μCi/ml (1 Ci = 3.7×10^{10} becquerels) to label the DNA, and in some experiments, in a medium containing L-[*U*-^{14}C]lysine (New England Nuclear) at a concentration of 0.5 μCi/ml to label the proteins.

Preparation of Chromatin and a Chromatin Subunit. The isolation of chromatin from the cell pellet has been described in detail (4). Nuclei were prepared by the method of Hymer and Kuff (5), in which cells were suspended in hypotonic sucrose buffer (0.25 M sucrose/3 mM $CaCl_2$/50 mM Tris·HCl, pH 7, 10 ml per 10^8 cells), the nonionic detergent Triton X-100 was added (1 ml per 100 ml), and the nuclei were sedimented with centrifugation at 1000 × *g* at 4°C for 15 min. Chromatin was isolated from the nuclei by successive suspension and sedimentation in Tris·HCl buffers, pH 7, of decreasing ionic strengths: 50, 10, 5, and 1 mM (6).

By using a Polytron homogenizer (Brinkmann), chromatin was sheared in a buffer containing 0.45 M NaCl, 10 mM sodium bisulfite, 10 mM EDTA, 25 mM Tris·HCl at pH 7.0 (final concentrations); under these conditions H1 histone and NHCP are dissociated, but the core histones—H2A, H2B, H3, and H4—remain associated with the DNA. Fractionation was accomplished on a 2.5 × 55 cm column of Sepharose 4B (Pharmacia), using the same buffer as eluent; DNA and the associated core histones elute in the void volume and the dissociated H1 histone and NHCP elute later in a separate peak. Fractions identified as containing DNA, by UV absorbance at 260 nm and by tritium counting were pooled and concentrated in an Amicon filtration apparatus. In some experiments, fractions containing H1 histone and NHCP, identified by ^{14}C radioactivity, were concentrated in a similar manner.

Irradiation Conditions. The radiation sources were a cobalt-60 unit (International Chemical and Nuclear, Irvine, CA) having a dose rate of 8 krad/min (1 rad = 0.01 gray) and a cesium-137 unit (Gamma-cell, Atomic Energy of Canada, Ottawa) having a dose rate of 133 rad/min.

All solutions for irradiation were prepared by using water distilled three times in quartz. Whole chromatin was diluted in 5 mM sodium perchlorate with brief homogenization in a Polytron homogenizer, dialyzed against the same buffer, and adjusted to a final DNA concentration of 100 μg/ml. The concentrated DNA–core histone complex fraction was also dialyzed against 5 mM sodium perchlorate, except in one experiment in which 5 mM sodium formate was used, and also adjusted to a

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Abbreviations: NHCP, nonhistone chromosomal proteins.

final DNA concentration of 100 μg/ml. Samples were contained in Wheaton Hopkins tagging vials (7) and kept at 4°C during and after irradiation. Which radicals were present was controlled by irradiation in the presence of appropriate radical scavengers (Table 2).

Filter Assay for the Detection of DNA–Protein Crosslinks. The technique utilizes the differential behavior of protein and DNA on filtration through Millipore filters; double-stranded DNA passes through the filter, whereas protein remains bound to the filter. After treatment of the chromatin with high concentrations of salt and detergent, any DNA that is covalently linked to the protein is trapped by the filter.

The procedure has been described by Strniste and Rall (8). Chromatin samples (50 μl containing 5 μg of DNA) were mixed with 2 ml of a solution containing 3 M NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 0.5% sodium lauroyl sarcosinate, and incubated at 37°C for 20 min. The samples were filtered through Millipore filters (type HA) and washed under gentle suction (about 3 ml/min) with 50 ml of a solution containing 3 M NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA (high-salt wash), followed by 10 ml of a solution containing 1 mM Tris·HCl (pH 7.5), 1 mM EDTA (low-salt wash). The filters were oven dried and placed in scintillation vials with 10 ml of Aquasol (New England Nuclear), and their radioactivities were measured in a liquid scintillation system (Beckman LS 8000). For total radioactivity measurement, 50-μl samples of chromatin were spotted on filters, dried, and counted. Hence, the radioactivity retained on the filter expressed as a percentage of the total gives a measure of the formation of DNA–protein crosslinks.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Chromatin proteins were examined with polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ according to the procedure of Laemmli (9), as modified by Thomas and Kornberg (10). The 18% acrylamide slab gels (0.15 × 24 cm) with 3% stacking gels were run at 4 W for 15 hr. Gels were fixed in acetic acid/methanol and stained with Coomassie blue (10).

Autoradiography of the gels was performed with Kodak SB5 film; gels were pretreated with En^{3}Hance (New England Nuclear) to increase sensitivity. For the measurement of radioactivity, gels were sliced (≈2 mm thickness), solubilized in 3% Protosol in Econofluor (New England Nuclear), and counted in a Beckman LS 8000 system.

RESULTS

Separation and Characterization of the DNA–Core Histone Complex. Conditions were established for the preparation of the chromatin subunit. The proteins from whole chromatin and the separated fractions were examined with $NaDodSO_4$/polyacrylamide gel electrophoresis to determine that the subunit contained only core histones in addition to DNA.

Chromatin, prelabeled in the DNA with [^{3}H]thymidine and in the proteins with [^{14}C]lysine, was treated with a buffer containing 0.45 M NaCl and fractionated on a column of Sepharose 4B (Fig. 1). Essentially all the ^{3}H was associated with peak I, which eluted in the void volume, whereas ^{14}C was associated with both peak I and peak II.

Gel electrophoretic patterns of the proteins contained in whole chromatin, peak I, and peak II are shown in Fig. 2. Chromatin contains a full complement of five histones—H1, H2A, H2B, H3, and H4—as well as NHCP; peak I contains the four core histones but lacks H1 histone and NHCP; and peak II contains only H1 histone and NHCP. Thus, treatment of whole chromatin with a buffer containing 0.45 M NaCl effectively dissociates H1 histone and NHCP (peak II) from the DNA, and a complex of DNA and core histones (peak I) can be separated with Sepharose chromatography.

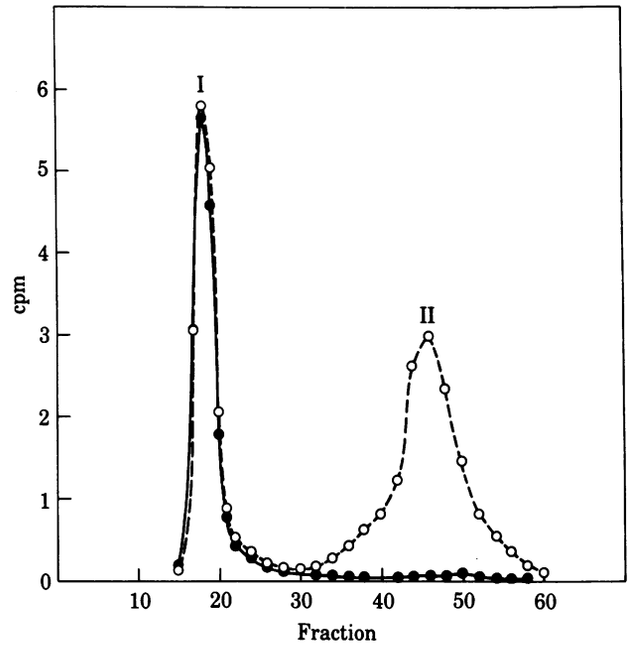

FIG. 1. Sepharose 4B chromatography of whole chromatin. Peak I, DNA–core histone complex; peak II, H1 histone and NHCP. ●, ^{3}H cpm × 10^{-5}; ○, ^{14}C cpm × 10^{-4}.

Formation of DNA–Protein Crosslinks. Whole chromatin and the chromatin subunit were examined with the filter assay for the formation of radiation-induced DNA–protein crosslinks. Both entities were irradiated in nitrous oxide-saturated solutions, so that the predominant radical species present was the hydroxyl radical, and the formation of DNA–protein crosslinks

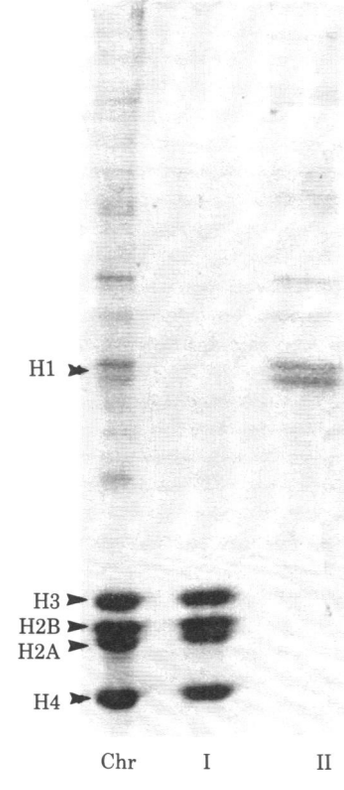

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of chromosomal proteins from whole chromatin, peak I, and peak II (see Fig. 1). The gel was stained with Coomassie blue.

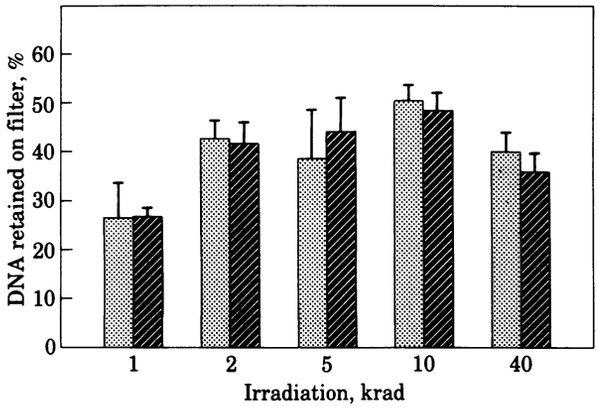

FIG. 3. Formation of radiation-induced DNA–protein crosslinks. Stippled bars, whole chromatin; hatched bars, DNA–core histone complex. Error bars are SD.

was determined (Fig. 3). Comparison of the amount of crosslinking measured in the whole chromatin with that measured in the DNA–core histone complex shows no significant differences over the dose range 1–40 krad. The absence of NHCP in the DNA–core histone complex with very little change in the formation of DNA–protein crosslinks indicates that NHCP are not predominantly involved in the crosslinking process and it can be inferred that the core histones are the specific proteins crosslinked to DNA in irradiated chromatin.

Further evidence for the predominance of the core histones in DNA–protein crosslinking in irradiated chromatin was obtained from experiments in which crosslinks were measured before and after Sepharose chromatography (Table 1). Whole chromatin was irradiated with doses of 1 and 2 krad, the chromatin was fractionated with Sepharose chromatography, and the formation of DNA–protein crosslinks was measured in the concentrated chromatin before fractionation and in the isolated recoverable DNA–core histone complex after fractionation. At least 80% of the crosslinking observed in whole chromatin is found in the DNA–core histone complex, supporting the conclusion that the core histones are primarily involved in the crosslinking process.

Fractionation of Chromatin. Irradiated whole chromatin was examined with chromatography on Sepharose 4B, to determine if any changes were apparent in the separation of the DNA–core histone complex from the H1 histone and NHCP. Whole chromatin, adjusted to a DNA concentration of 100 μl/ml in 5 mM sodium perchlorate, was irradiated in nitrous oxide-saturated solution so that the hydroxyl radical was the predominant radical species present; a dose of 1 krad was used. After concentration to $\approx$1 mg of DNA per ml, unirradiated and irradiated chromatin samples were fractionated with chromatography on Sepharose 4B (Fig. 4). Comparison of the two elution profiles showed no

Table 1. Formation of DNA–protein crosslinks in irradiated chromatin

Sample	DNA retained on filter, %		
	0 krad	1 krad	2 krad
Whole chromatin	(4.1)	16.6	34.7
DNA–core histones	(5.5)	12.7	28.2

Crosslinking was determined before Sepharose separation for whole chromatin and after Sepharose separation for DNA–core histones (peak I). Numbers in parentheses are the backgrounds found in the absence of radiation; this background has been subtracted in calculating the radioactivity retained after irradiation.

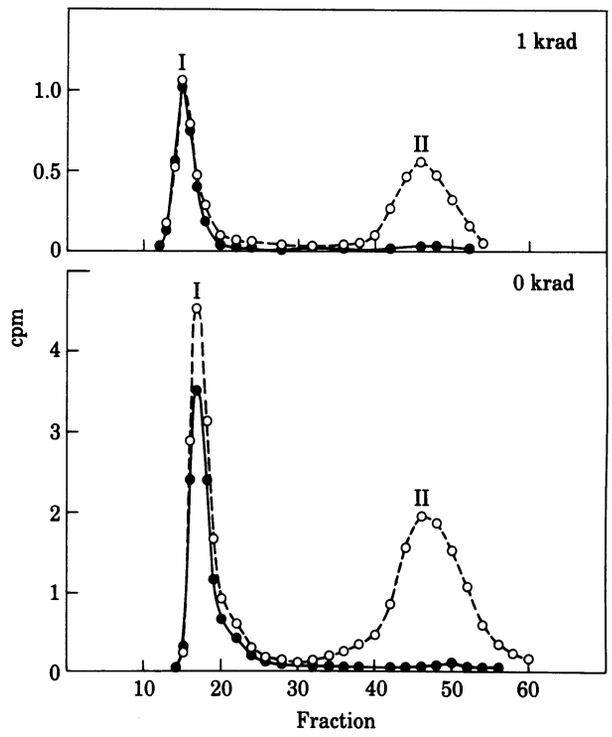

FIG. 4. Sepharose 4B chromatography of whole chromatin (a smaller sample of irradiated chromatin was chromatographed). Peak I, DNA–core histone complex; peak II, H1 histone and NHCP. ●, ^{3}H cpm $\times 10^{-5}$; ○, ^{14}C cpm $\times 10^{-4}$.

significant changes in the separation of the irradiated chromatin; the distributions of ^{3}H and ^{14}C between peak I and peak II were essentially the same. This lack of change in the elution profile is consistent with the conclusion of the previous experiments that the core histones are primarily involved in crosslinking in irradiated chromatin.

The proteins in whole chromatin and in the separated DNA–core histone complex (peak I) were examined for radiation damage with NaDodSO$_4$/polyacrylamide gel electrophoresis. No changes were detected in the distribution of proteins in the irradiated compared with the unirradiated samples.

Examination of Histones from Irradiated DNA–Core Histone Complex. The core histones from irradiated samples of the chromatin subunit were examined for radiation damage with NaDodSO$_4$/polyacrylamide gel electrophoresis. Samples of the subunit, prelabeled in the DNA and histones, were irradiated in nitrous oxide-saturated solutions with doses of 1, 2, 5, 50, and 100 krad. In the 1-, 2-, and 5-krad-irradiated samples, autoradiographs of the gels showed no changes in the core histones and, in particular, no formation of dimers or higher polymers of histones, which would be retarded in the upper part of the gel (Fig. 5). In addition, a band was visible at the top of the gel, which was particularly obvious in the 5-krad sample; judging from the radioactivity obtained in the gel slices this band contains ^{3}H and probably represents DNA that is slightly degraded, enabling it to penetrate the high-percentage acrylamide gel. The distribution of ^{14}C in the gel slices of the four core histones showed no significant differences between the unirradiated and 1-, 2-, and 5-krad-irradiated samples. In 50- and 100-krad-irradiated samples, again no dimers or higher polymers of histones were detected on the autoradiographs of the gels, but some decrease in the intensity of the histone bands was apparent. This decrease was paralleled by decreases in the ^{14}C in the gel slices; in addition, significant amounts of ^{3}H were

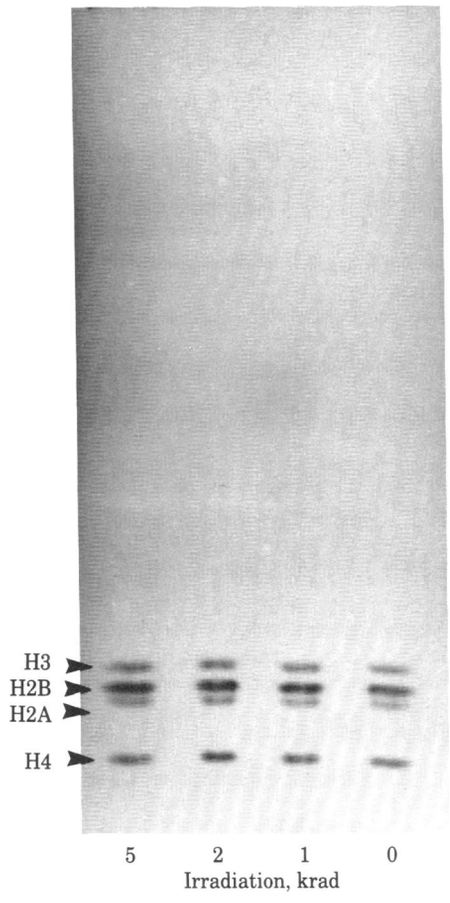

FIG. 5. Autoradiography of core histones from irradiated chromatin subunits separated by $NaDodSO_4$/polyacrylamide gel electrophoresis.

apparent throughout the gel, but they were not associated with any specific histones, and probably represent very degraded DNA.

Radical Effects. The DNA–core histone complex was irradiated under various environmental conditions to evaluate the effects of the individual radical intermediates, OH·, solvated electrons (e^-_{aq}), and O_2^-, for the formation of DNA–protein crosslinks (Table 2). The greatest degree of crosslinking was observed in nitrous oxide-saturated solutions. In particular, when the formation of crosslinks in nitrous oxide-saturated and nitrogen-saturated solutions (rows 1 and 2) are compared at doses of 0.5 and 1 krad to obtain initial yields, the crosslinking is found to double under conditions in which the hydroxyl radical yield is doubled and the solvated electron is eliminated from the solution. The ineffectiveness of the solvated electron was confirmed with irradiations carried out in the presence of the hydroxyl radical scavenger *t*-butyl alcohol; under these conditions negligible crosslinking was observed.

The effectiveness of the superoxide radical was evaluated with irradiations carried out in air. In air-saturated 5 mM sodium formate solutions, superoxide radicals are the only radical species present (11); under these conditions essentially no crosslinking was detected. When irradiations were carried out in air-saturated solutions under conditions such that both hydroxyl radicals and superoxide radicals were present (radical yields: 2.7 for OH· and 3.2 for O_2^-), relatively little crosslinking was observed. The relative ineffectiveness of the hydroxyl radical for crosslinking under these conditions is probably due to termination of protein and DNA radicals by the oxygen present in the solution.

DISCUSSION

Damage to DNA induced by ionizing radiation includes deamination and ring sission of the purine and pyrimidine bases, base elimination, and strand breakage (12). In addition, crosslinking between DNA and other substances, including proteins, and also intra- and intermolecular crosslinking of DNA occur (1). Which, if any, of these molecular lesions is responsible for cell death and for mutagenesis or carcinogenesis in irradiated cells remains speculative. For instance, the formation of DNA–protein covalent linkages within the cell may interfere with DNA transcription and, ultimately, cell division.

In the present investigation the core histones have been identified as the specific proteins involved in the formation of DNA–protein crosslinks in irradiated chromatin. The removal of the H1 histone and NHCP, which together represent ≈45% of the proteins in chromatin, produced no significant reduction in crosslinking. These results might have been predicted from the close proximity of the DNA to the core histones in the nucleosome structure of chromatin, in which DNA is wrapped around an octamer of two each of the H2A, H2B, H3, and H4 histones. Apparently the volume for interaction of the radiation-induced radicals of DNA and proteins is very small and the H1 histone and NHCP are outside the sensitive volume that is closely associated with the DNA in the chromatin structure. However, an alternative explanation for the predominance of histones in the crosslinking process could be that the specific amino acids involved in crosslinking occur predominately in the histones—e.g., the basic amino acids.

Four types of interactions have been recognized in protein–DNA linkages (13): electrostatic interactions between positively charged amino acid side chains (Lys, Arg, His^+) and phosphate groups; stacking interactions between aromatic amino acid side chains (Trp, Tyr, Phe, His) and nucleic acid

Table 2. Formation of DNA–histone crosslinks by OH·, e^-_{aq}, and O_2^-

Irradiation environment		Radical yield				DNA retained on filter, %		
Buffer	Atmosphere, radical scavengers	OH·	e^-_{aq}	H·	O_2^-	0.5 krad	1.0 krad	2.0 krad
$NaClO_4$	N_2	2.7	2.7	0.6		6.7	15.7 ± 1.3	34.4 ± 2.2
	N_2O	5.4		0.6		15.8	29.6 ± 3.3	42.8 ± 3.8
	N_2, *t*-butyl alcohol		2.7	0.6		—	1.0 ± 0.3	2.5 ± 1.6
	Air	2.7			3.2	2.4 ± 0.8	3.4 ± 0.8	6.2 ± 0.8
HCOONa	Air				6.0	—	0.2	0.4

Buffers were 5 mM, pH 7.5. Radical yields are *g* values ($g_{OH}, g_{e^-_{aq}}, g_H, g_{O_2^-}$) for the production of the respective radical species in molecules per 100 eV energy absorbed by the solution as given in ref. 11. DNA retained values are given ±SD.

bases; hydrogen bonding between amino acids (Glu, Asp, Gln, Asn, His, Arg) and phosphate, deoxyribose, or bases; and hydrophobic interactions between aliphatic amino acid side chains and nucleotide bases. Which, if any, of these interactions is the basis for radiation-induced histone–DNA crosslinks is not known. Identification of the specific amino acids involved in the process might help to distinguish among them.

No changes in the chromosomal proteins of irradiated chromatin could be demonstrated, except at very high doses. This lack of change is surprising in view of the formation of radiation-induced DNA–histone crosslinks. However, it is in agreement with the studies of Ramakrishnan *et al.* (14); no alterations were detected in the gel electrophoretic patterns of the histones in irradiated chromatin.

Electron spin resonance studies of dry and frozen chromatin include evidence for the transfer of electrons from proteins to DNA (15, 16). These results suggest that the DNA in chromatin is the site of radiation damage, with the proteins acting as electron donors, thus leaving the chromosomal proteins undamaged.

The formation of DNA–protein crosslinks as a result of UV irradiation of isolated chromatin has been observed and some specificity in the process has been deduced (8, 17–19). Sperling and Sperling (17), using light of wavelengths >290 nm, reported that the H2A and H2B histones were preferentially crosslinked, whereas Kunkel and Martinson (18), using light <290 nm, reported that H1 and H3 histones were most readily crosslinked. The results of Mandel *et al.* (19), using unfiltered 254-nm light, implicated all the histones and also some NHCP in crosslinking. It was suggested that these results may be related to the differential absorption of different wavelengths of UV light in the DNA.

γ irradiation of the DNA–core histone complex in the presence of radical scavengers has confirmed and elaborated the results of an earlier study (3) on the effectiveness of the individual radical intermediates for the formation of DNA–protein crosslinks in whole chromatin; the hydroxyl radical is the most effective radical, whereas the superoxide radical and the solvated electron are essentially ineffective for crosslink formation. Oxygen appears to terminate the nucleic acid and histone radicals prematurely, preventing the formation of hydroxyl radical-induced crosslinks. Similar radical efficiencies have been reported for DNA strand breakage (20–22) and for the inactivation of DNA as measured by its ability to produce phage particles (23). That the hydroxyl radical may play a major role in radiation-induced damage in cultured mammalian cells has been demonstrated by irradiation of cells in the presence of radical scavengers (3, 20).

At the present time, the experimental evidence relating the formation of DNA–protein crosslinks to cell survival is inconclusive. Fornace and Little (24), using human diploid fibroblasts, reported that x-ray-induced DNA–protein crosslinking was increased under hypoxic irradiation. This observation is supported by the results of this investigation, which has shown that oxygen inhibits DNA–histone crosslinking, presumably by terminating the DNA and protein radicals. In our previous paper (3), we demonstrated that fewer crosslinks were formed in Chinese hamster cells irradiated in air than in an atmosphere of nitrogen. Fornace and Little (24) suggested that because the formation of crosslinks increased under hypoxia while cell killing decreased, other types of damage occur that are also responsible for the lethal effects of radiation. In the case of UV irradiation of a bacterial strain (*Escherichia coli*), Smith (2) reported that DNA–protein crosslinks played a significant role in cell killing.

The observation in this paper that the core histones are crosslinked to DNA in irradiated chromatin demonstrates that the structure of the nucleosome is altered in irradiated cells and that such change might interfere with DNA transcription and replication. The decrease of DNA–protein crosslinking observed under aerobic irradiation, however, suggests that the oxygen enhancement of cell killing is related to another kind of damage.

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