Modification of DNA Bases in Mammalian Chromatin by Radiation-Generated Free Radicals†

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ABSTRACT: Modification of DNA bases in mammalian chromatin in aqueous suspension by ionizing radiation generated free radicals was investigated. Argon, air, N_2O, and N_2O/O_2 were used for saturation of the aqueous system in order to provide different radical environments. Radiation doses ranging from 20 to 200 Gy ($J \cdot kg^{-1}$) were used. Thirteen products resulting from radical interactions with pyrimidines and purines in chromatin were identified and quantitated by using the technique of gas chromatography/mass spectrometry with selected-ion monitoring after acidic hydrolysis and trimethylsilylation of chromatin. The methodology used permitted analysis of the modified bases directly in chromatin without the necessity of isolation of DNA from chromatin first. The results indicate that the radical environment provided by the presence of different gases in the system had a substantial effect on the types of products and their quantities. Some products were produced only in the presence of oxygen, whereas other products were detected only in the absence of oxygen. Products produced under all four gaseous conditions were also observed. Generally, the presence of oxygen in the system increased the yields of the products with the exception of formamidopyrimidines. Superoxide radical formed in the presence of air, and to a lesser extent in the presence of N_2O/O_2, had no effect on product formation. The presence of oxygen dramatically increased the yields of 8-hydroxypurines, whereas the yields of formamidopyrimidines were not affected by oxygen, although these products result from respective oxidation and reduction of the same hydroxyl-adduct radicals of purines. The yields of the products were much lower than those observed previously with DNA.

Free radicals generated in vivo by cellular processes have been implicated to play an important role in a number of human diseases [for a review see Halliwell and Gutteridge (1985)]. Excess generation of free radicals in vivo by endogenous sources (e.g., oxidant enzymes, phagocytic cells) or exogenous sources (e.g., redox-cyclic drugs, ionizing radiation) can result in damage to cellular DNA. Thus, free radicals may be mutagenic and may act as promotors of carcinogenesis (Brawn & Fridovich, 1985; Cerutti, 1985). Much of the toxicity of free radicals in vivo is though to arise from reactions of the highly reactive hydroxyl radical ($^{\bullet}OH$)[1] produced by metal ion dependent conversion of superoxide radical (O_2^{-}) and H_2O_2 (Halliwell & Gutteridge, 1985). As an endogenous source, ionizing radiation can also cause formation of $^{\bullet}OH$ among other radical species [H atom, hydrated electron (e_{aq}^{-})] in reactions with cellular water [for a review see von Sonntag (1987)]. In the past, radiation chemists have studied extensively the reactions of these free radicals with DNA and its constituents and chemically characterized the resulting products. Free radicals, especially $^{\bullet}OH$, produce a large number of sugar and base products in DNA and DNA–protein cross-links in nucleoprotein [for reviews see von Sonntag (1987) and Oleinick et al. (1987)]. Lesions produced in cellular DNA are subject to cellular repair processes and, unless repaired, may have detrimental biological consequences [for a review see Friedberg (1985)]. For an understanding of the biological consequences of free-radical-induced lesions in DNA and nucleoprotein, it is essential to identify and quantitate such lesions under oxic and anoxic conditions of free-radical formation.

In the present work, we investigated the modification of DNA bases in mammalian chromatin exposed to ionizing radiation in aqueous suspension. In living cells, DNA is not free but is complexed with histones to form chromatin. This complex forms a variety of substructures such as nucleosome, solenoid, loop domains, miniband, and chromatid, which are present during the different phases of the cell cycle [for a review see Nelson et al. (1986)]. Thus, mammalian chromatin in aqueous suspension represents a biologically more relevant model system than DNA alone in aqueous solution for studies of free-radical-induced damage to cellular DNA. Histones that are closely associated with DNA in nucleosomes may also react with free radicals, and DNA bases may participate in formation of DNA–protein cross-links in chromatin. Furthermore, if DNA of living cells exposed to ionizing radiation or to any other free-radical-producing systems were to be

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[1] Abbreviations: $^{\bullet}OH$, hydroxyl radical; O_2^{-}, superoxide radical; e_{aq}^{-}, hydrated electron; BSTFA, bis(trimethylsilyl)trifluoroacetamide; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PBS, phosphate-buffered saline; 5,6-diHThy, 5,6-dihydrothymine; 5-OHMeUra, 5-(hydroxymethyl)uracil; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 8-OH-Gua, 8-hydroxyguanine; Gy, gray ($J \cdot kg^{-1}$); GC/MS, gas chromatography/mass spectrometry; SIM, selected-ion monitoring; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine; 5-OH-6-HCyt, 5-hydroxy-6-hydrocytosine; 5,6-diOH-Cyt, 5,6-dihydroxycytosine.

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analyzed for modification of DNA bases, it would be desirable to analyze the chromatin isolated from exposed cells rather than DNA alone. This is because DNA, which is covalently cross-linked to proteins, may not be extracted efficiently (Smith, 1976; Yamamoto, 1976; Mee & Adelstein, 1979), and thus one may loose a significant portion of modified DNA bases. For these reasons, we believe, it is important to study the free-radical chemistry of mammalian chromatin under oxic and anoxic conditions of free-radical production. Furthermore, it is important to use in such studies a selective and sensitive analytical technique, which permits a direct analysis of the free-radical-induced products of all four DNA bases in chromatin without the necessity of isolation of DNA from chromatin first. In this work, four different gases (argon, air, N_2O, and N_2O/O_2) and low radiation doses ranging from 20 to 200 Gy were used to study the dependence of the types of products in chromatin and their quantities on the various radical environments provided by the presence of the gases.

Experimental Procedures

Materials.[2] Sucrose and histones H1, H2A, H2B, H3, and H4 were purchased from Boehringer Mannheim. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were from Pierce Chemical Co. Reagents for electrophoresis were obtained from Bethesda Research Laboratories. Phenylmethanesulfonyl fluoride (PMSF), Triton X-100, dithiothreitol (DTT), Tris, 5,6-dihydrothymine (5,6-diHThy), 5-(hydroxymethyl)uracil (5-OHMeUra), isobarbituric acid (5-hydroxyuracil), 4,6-diamino-5-formamidopyrimidine (FapyAde), 6-azathymine, and 8-azaadenine were purchased from Sigma Chemical Co. 2-Amino-6,8-dihydroxypurine (8-hydroxyguanine) was from Chemical Dynamics Corp. Dialuric acid (5,6-dihydroxyuracil) was purchased from American Tokyo Kasei, Inc. Synthesis of other base products was described elsewhere (Dizdaroglu, 1985; Fuciarelli et al., 1989). Dialysis membranes with a molecular weight cutoff of 3500 were purchased from Fisher Scientific Co. Formic acid and calcium chloride were obtained from Mallinckrodt. Water passed through a purification system from Millipore was used for all purposes.

Cell Culture. The cells used for chromatin isolation were SP2/0-derived murine hybridomas. The cell line has been designated HyHEL-10 and produces IgG antibodies against hen egg white lysozyme (courtesy of Dr. S. J. Smith-Gill, National Cancer Institute, Bethesda, MD). Cells were grown in a 5% CO_2 atmosphere at 37 °C in 750-cm^2 Lifecell bags (Baxter Healthcare Corp.) in 1.8 L of a 1/1 (v/v) mixture of DMEM/F-12 medium supplemented with 4% fetal bovine serum (Sigma Chemical Co.). Cells were harvested at the mid- to late-exponential growth phase at a density of approximately 7×10^5 cells/mL. The cells were spun down in 250-mL bottles at 1700*g* for 10 min, washed once by resuspending them in 50 mL of PBS buffer containing 0.01% sodium azide, spun down again, and resuspended at 4 °C in 30 mL of fresh PBS buffer containing 0.01% sodium azide. Chromatin was isolated within 24 h.

Isolation of Chromatin. Chromatin was isolated according to a slightly modified procedure of Mee and Adelstein (1981). Approximately 4×10^8 cells in PBS buffer were centrifuged at 300*g*, and the PBS buffer was removed. The cell pellet was suspended in 35 mL of cold sucrose buffer (0.25 M sucrose, 3 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM DTT, 50 mM Tris-HCl at pH 7.4) and kept in an ice bath for 10 min. Cells were centrifuged at 300*g*, then lysed in 35 mL of cold sucrose buffer containing 1% Triton X-100, and kept in an ice bath for 10 min. The nuclei were centrifuged at 1000*g* for 15 min and then washed with 35 mL of cold sucrose buffer without Triton X-100 and recentrifuged. Sucrose buffer was removed, the nuclei were suspended in 30 mL of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM PMSF, kept in an ice bath for 15 min, and centrifuged at 1000*g*, and the buffer was removed. This procedure was repeated with 10, 5, and 1 mM Tris-HCl buffers (pH 7.4) containing 0.1 mM PMSF. Chromatin was obtained finally as a white gel in 1 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM PMSF. Subsequently, chromatin was dialyzed extensively against 1 mM phosphate buffer (pH 7.4). All operations were carried out at 4 °C. After dialysis, chromatin was homogenized briefly with a few strokes in a glass homogenizer.

Characterization of Chromatin. The DNA content of chromatin was determined both by measurement of the absorbance at 258 nm using a molar extinction coefficient of 6.6×10^3 M^{-1} cm^{-1} and by Burton's assay (Burton, 1968). The amounts of DNA in chromatin (0.26 mg/mL) measured by these two different assays were in good agreement. The protein content of chromatin was measured according to Smith et al. (1985). The ratio of the amount of protein to that of DNA was 2 (w/w). The RNA content of chromatin was measured according to Schneider (1956) and was found to be ≤5% of the amount of DNA. The chromatin exhibited the following spectral characteristics: $A_{258}/A_{280} = 1.58$; $A_{258}/A_{230} = 1.1$; $A_{258}/A_{320} = 8.5$; A(maximum)/A(minimum) = 1.26.

The protein components of chromatin were analyzed by gel electrophoresis essentially as described by Laemmli (1970) with a modification in the ratio of acrylamide to bis(acrylamide). Separating gel (18%) and stacking gel (4%) were prepared from a stock solution of 29.8% acrylamide and 0.2% bis(acrylamide). Electrophoresis was carried out in a 15 × 18 cm slab gel at 40 mA for 2 h. Gels were stained with Coomassie blue.

Irradiations. The dialyzed suspension of chromatin was diluted with 1 mM phosphate buffer (pH 7.4) to a DNA concentration of 0.1 mg/mL. Aliquots of this solution were bubbled separately with argon, air, N_2O, and N_2O/O_2 (4/1) for 20 min prior to and then throughout the irradiations, which were done in a ^{60}Co γ-source at doses ranging from 20 to 200 Gy ($J \cdot kg^{-1}$). The dose rate of the ^{60}Co γ-source (124 Gy/min) was determined by Fricke dosimeter (Fricke & Hart, 1966). After irradiation, 1 nmol of 6-azathymine and 1 nmol of 8-azaadenine were added as internal standards to each of the chromatin samples containing 0.1 mg of DNA. Samples were then lyophilized.

Hydrolysis with Formic Acid and Trimethylsilylation. Lyophilized aliquots of chromatin samples were hydrolyzed with 0.5 mL of formic acid (88%) in evacuated and sealed tubes for 40 min at 150 °C. Samples were lyophilized and then trimethylsilylated in poly(tetrafluoroethylene)-capped hypovials (Pierce) with 0.12 mL of a BSTFA/acetonitrile (2/1 v/v) mixture by heating for 40 min at 130 °C. Unirradiated chromatin samples were treated in the same manner.

Gas Chromatography/Mass Spectrometry (GC/MS). Analysis of derivatized samples was performed by using a mass-selective detector interfaced to a gas chromatograph (both from Hewlett-Packard) equipped with an automatic sampler and a computer work station. The injection port, the

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

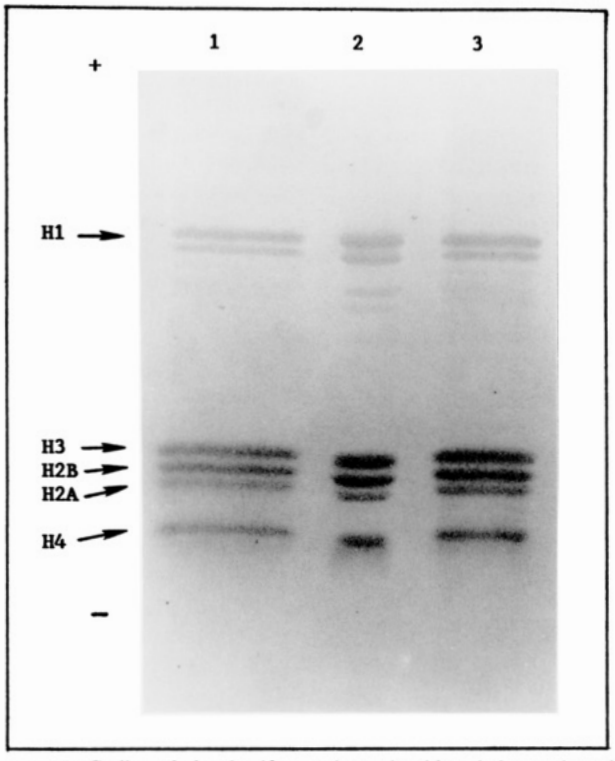

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of chromatin suspended in Tris-HCl buffer (lane 1), histones H1, H3, H2B, H2A, and H4 (lane 2), and chromatin dialyzed against and suspended in phosphate buffer (lane 3).

ion source and the interface were maintained at 250 °C. Separations were carried out by using a fused silica capillary column (12.5 m × 0.20 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness, 0.33 μm) (Hewlett-Packard). Helium was used as the carrier gas at an inlet pressure of 40 kPa. Selected-ion monitoring (SIM) was performed in the electron-ionization mode at 70 eV. An aliquot (0.4 μL of each derivatized sample was injected without any further treatment into the injection port of the gas chromatograph using the split mode of injection. The amount of DNA in chromatin samples injected onto the column for each analysis was approximately 0.4 μg.

Results

The objective of this investigation was to determine the modifications of DNA bases in isolated mammalian chromatin in aqueous suspension under various conditions of free-radical formation by ionizing radiation. The ratio of the amount of protein to that of DNA in isolated chromatin used here was 2 (w/w). The authenticity of the protein components of the isolated chromatin was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using commercially available histones as reference compounds. Figure 1 illustrates gel electrophoretic patterns of the proteins of isolated chromatin suspended in Tris-HCl buffer (lane 1), histones (lane 2), and the proteins of isolated chromatin after dialysis against phosphate buffer (lane 3). The electrophoretic patterns of histones in Figure 1 are quite similar to those published previously (Panyim & Chalkley, 1969; Böhm et al., 1973; Mee & Adelstein, 1981). Figure 1 clearly illustrates that histones H1, H3, H2B, H2A, and H4 were present in isolated chromatin suspended in Tris-HCl buffer as well as after dialysis against phosphate buffer. Figure 2 illustrates an absorption spectrum of isolated chromatin. This spectrum resembles absorption spectra characteristic of mammalian chromatin published previously (Bonner et al., 1968).

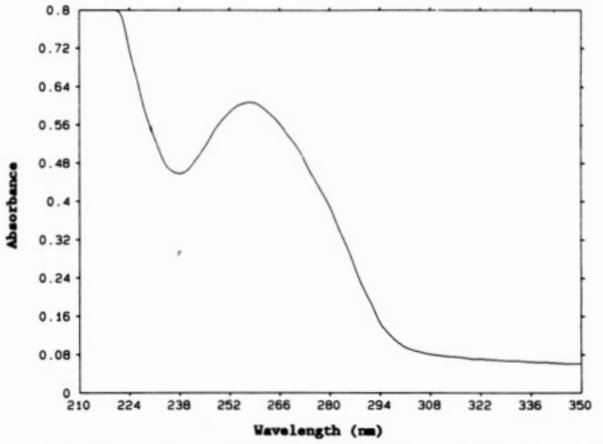

FIGURE 2: Absorption spectrum of isolated chromatin in phosphate buffer.

Table I: Yields ($\mu mol \cdot J^{-1}$) of Radical Species Generated by Ionizing Radiation in Aqueous Solution under Different Gaseous Conditions[a]

	argon	air	N_2O	N_2O/O_2
$^{\bullet}OH$	0.28	0.28	0.56	0.56
e_{aq}^-	0.27			
H atom	0.057		0.057	
O_2^-		0.33		0.057

[a] Adapted from von Sonntag (1987).

Isolated chromatin suspended in 1 mM phosphate buffer was exposed to ionizing radiation under four different gaseous conditions. When water is exposed to ionizing radiation, three radical species, hydroxyl radical ($^{\bullet}OH$), hydrated electron (e_{aq}^-), and hydrogen atom (H atom), are produced (von Sonntag, 1987). In the presence of oxygen, e_{aq}^- and H atom react with oxygen and are converted to the superoxide radical (O_2^-). When N_2O is present in solution, e_{aq}^- reacts with N_2O in a diffusion-controlled reaction to yield additional $^{\bullet}OH$ (von Sonntag, 1987). The yields of these species depending on the presence of various gases used in the present study are illustrated in Table I.

For identification of DNA base modifications in chromatin, trimethylsilylated hydrolysates of chromatin samples were analyzed by GC/MS-SIM. The use of this technique for chemical characterization and quantitation of free-radical-induced base modifications in DNA has been described previously (Dizdaroglu, 1985; Fuciarelli et al., 1989; Dizdaroglu & Gajewski, 1990). Figure 3 illustrates typical ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of chromatin exposed to ionizing radiation in the presence of N_2O/O_2. Peak identification is given in the figure legend. 5-Hydroxyuracil (5-OH-Ura) (peak 4 in Figure 3) and 5-hydroxycytosine (5-OH-Cyt) (peak 6) arise by acid-induced modification of Cyt glycol (Dizdaroglu et al., 1986). Similarly, 5,6-dihydroxyuracil (dialuric acid and/or isodialuric acid) (peak 8) is produced by acid-induced deamination of 5,6-dihydroxycytosine (5,6-diOH-Cyt). 5-Hydroxy-5-methylhydantoin (5-OH-5-MeHyd) (peak 2) and 5-hydroxyhydantoin (5-OH-Hyd) (peak 3) are believed to result from acid-induced modification of 5-methyl-5-hydroxybarbituric acid and 5,6-diOH-Cyt, respectively (Teoule & Cadet, 1978). The products identified and their yields are listed in Table II. Figure 4 illustrates representative dose–yield plots of some products. Three independent measurements were performed for each of the five dose points. Linear dose–yield relationships were obtained for all products identified at doses ranging from 20 to 200 Gy. With the exception of 5,6-diHThy, 5-hydroxy-6-hydrothymine (5-OH-6-HThy),

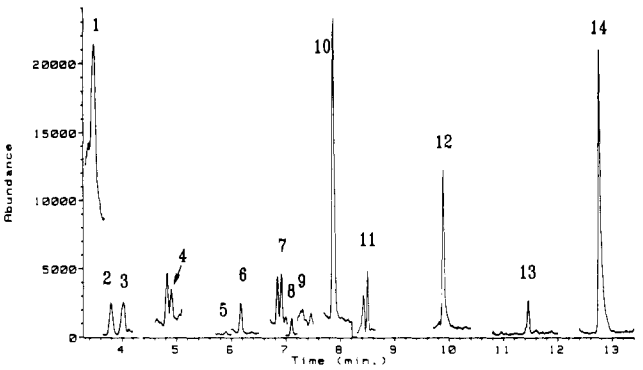

FIGURE 3: Ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of mammalian chromatin exposed to ionizing radiation in N_2O/O_2-saturated aqueous suspension. Radiation dose was 40 Gy. The column was programmed from 150 to 260 °C at a rate of 8 °C/min after 2 min at 150 °C. Amount of an aliquot of DNA in chromatin injected onto the column was approximately 0.4 μg. For other details see Experimental Procedures. Peaks (ion monitored): 1, 6-azathymine (*m/z* 256) (internal standard); 2, 5-OH-5-MeHyd (*m/z* 331); 3, 5-OH-Hyd (*m/z* 317); 4, 5-OH-Ura (*m/z* 329); 5, 5-OHMeUra (*m/z* 358); 6, 5-OH-Cyt (*m/z* 343); 7, *cis*-Thy glycol (*m/z* 259); 8, 5,6-dihydroxyuracil (*m/z* 417); 9, *trans*-Thy glycol (*m/z* 259); 10, 8-azaadenine (*m/z* 265) (internal standard); 11, FapyAde (*m/z* 354); 12, 8-OH-Ade (*m/z* 352); 13, FapyGua (*m/z* 442); 14, 8-OH-Gua (*m/z* 440) (all compounds as their trimethylsilyl derivatives).

and 5-OH-6-HCyt, all the compounds identified in irradiated chromatin were also found in unirradiated chromatin. The amounts measured in unirradiated chromatin are listed in Table II.

Discussion

The results obtained in the present study clearly indicate that DNA base products identified previously in model systems such as bases, nucleosides, and nucleotides, and in DNA [for reviews see Teoule and Cadet (1978) and von Sonntag (1987)], are also formed in mammalian chromatin in aqueous suspension upon exposure to ionizing radiation. The results further indicate that the modified DNA bases can be identified and quantitated in mammalian chromatin by our GC/MS-SIM methodology without any difficulty despite the presence of histones. Thus, the isolation of DNA from chromatin is unnecessary. This capability of analyzing chromatin directly is important for in vitro and in vivo studies of DNA damage in chromatin, because a significant amount of DNA may not be extracted from chromatin efficiently, due to the formation of DNA–protein cross-links in chromatin upon exposure to ionizing radiation or to any other free-radical-producing system (Smith, 1976; Yamamoto, 1976; Mee & Adelstein, 1979; Oleinick et al., 1987; Lesko et al., 1982). Moreover, the unextracted DNA may contain a significant portion of damaged bases.

The types of modified bases and their quantities depended on the radical environment and the gas present in the aqueous system (Table II). The higher yields of the products in the presence of N_2O/O_2 than in the presence of air indicate that O_2^- (see also Table I) had no effect on the production of the products observed, in agreement with previous observations (Aruoma et al., 1989). In the presence of oxygen (air and N_2O/O_2), 5,6-diHThy, 5-OH-6-HThy, and 5-OH-6-HCyt were not detected. 5,6-DiHThy arises from reactions of e_{aq}^- and H atom with the thymine moiety of DNA (Teoule & Cadet, 1978; von Sonntag, 1987). Thus this compound is not formed in the presence of oxygen because of the scavenging of e_{aq}^- and H atom by oxygen in diffusion-controlled reactions (von Sonntag, 1987). As expected, the yield of 5,6-diTHy in the presence of argon is higher than that in the presence of N_2O because of the diffusion-controlled reaction of e_{aq}^- with

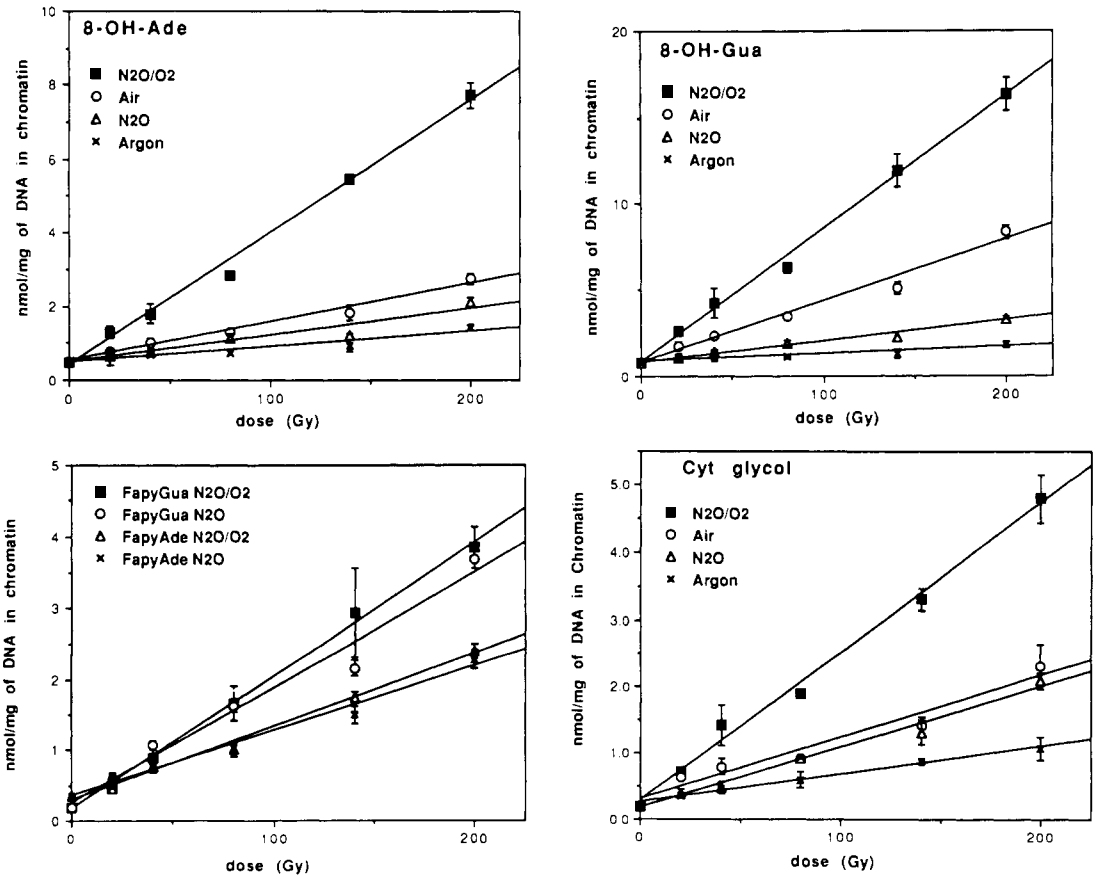

FIGURE 4: Dose–yield plots of some products. Error bars represent standard deviations of the means from three independent measurements.

Table II: Yields ($nmol \cdot J^{-1}$) of the DNA Base Products in Chromatin

product	argon	air	N_2O	N_2O/O_2	amount (μmol/mol of nucleotides) in control chromatin
5,6-diHThy	0.48 ± 0.04	nd[a]	0.26 ± 0.03	nd	nd
5-OH-5-MeHyd	*b*	0.21 ± 0.02	*b*	0.42 ± 0.02	64 ± 6.4
5-OH-Hyd	*b*	*b*	*b*	0.22 ± 0.02	73.6 ± 4.5
Cyt glycol	0.50 ± 0.025	1.10 ± 0.04	0.95 ± 0.03	2.30 ± 0.18	64 ± 19.2
5-OH-6HThy	0.30 ± 0.03	nd	0.30 ± 0.03	nd	nd
5-OH-6-HCyt	0.17 ± 0.02	nd	0.17 ± 0.02	nd	nd
5-OHMeUra	0.045 ± 0.005	0.02 ± 0.003	0.064 ± 0.008	0.052 ± 0.007	3.2 ± 1.28
Thy glycol	0.045 ± 0.004	0.18 ± 0.02	0.094 ± 0.01	0.40 ± 0.03	35.2 ± 2.56
5,6-diOH-Cyt	*b*	0.14 ± 0.007	*b*	0.33 ± 0.03	25.6 ± 3.2
FapyAde	0.82 ± 0.04	0.60 ± 0.04	0.96 ± 0.06	1.02 ± 0.08	48.0 ± 1.9
8-OH-Ade	0.45 ± 0.02	1.10 ± 0.08	0.80 ± 0.07	3.50 ± 0.30	89.6 ± 5.8
FapyGua	0.96 ± 0.03	1.11 ± 0.05	1.81 ± 0.18	1.81 ± 0.18	16.0 ± 5.4
8-OH-Gua	0.55 ± 0.03	3.85 ± 0.20	1.35 ± 0.12	8.05 ± 0.65	182 ± 4.2

[a] Not detected. [b] No increase in the amount above the background level was observed at doses ranging from 20 to 200 Gy.

N_2O. 5-OH-6-HThy and 5-OH-6-HCyt are produced by reactions of •OH with the Thy and Cyt moieties of DNA, respectively, followed by reduction of so-formed C-5 OH-adduct radicals (von Sonntag, 1987). Their formation is inhibited in the presence of oxygen due to the diffusion-controlled reaction of oxygen with the OH-adduct radicals of Thy and Cyt. An increase in the amount of 5,6-diOH-Cyt was observed only in the presence of oxygen, and its yield was 2-fold higher in the presence of N_2O/O_2 than in the presence of air. This is because 5,6-diOH-Cyt is produced by addition of •OH to the 5,6-double bond of Cyt followed by addition of oxygen to the OH-adduct radical and subsequent oxidation (Teoule & Cadet, 1978). An increase in the amount of 5-OH-Hyd due to irradiation was observed only in the presence of N_2O/O_2. This is presumably because 5-OH-Hyd is not a primary product and results from acid-induced modification of 5,6-diOH-Cyt. Other remaining products of pyrimidines Cyt glycol, Thy glycol, and 5-OH-MeUra, and all the products of purines, were observed under all four gaseous conditions. The yield of 5-OHMeUra, which has been identified previously in cellular DNA (Teebor et al., 1984), was the lowest among the yield of the observed products. The yields of Cyt glycol and Thy glycol were higher in the presence of oxygen than in its absence. This is expected because oxygen inhibits the formation of 5-OH-6-hydropyrimidines, which result from OH-adduct radicals of pyrimidines as do pyrimidine glycols. The yield of Cyt glycol was consistently higher that of Thy glycol under all four gaseous conditions. This is in contrast to the yields of these compounds observed with calf thymus DNA in aqueous solution (Fuciarelli et al., 1990).

The fact that the yield of 5-OH-6-HThy was higher than that of Thy glycol in the absence of oxygen might indicate the involvement of the C-5 OH-adduct radical of Thy in abstraction of an H atom from the sugar moiety leading to DNA strand breaks. In a previous work done with oligonucleotides of Thy, the yield of 5-OH-6-HThy had been found to be also higher than that of Thy glycol in contrast to the yields observed with dT and 5′-pdT (Karam et al., 1988). In view of the work done with poly(U) (Lemaire et al., 1984), this was thought to be the result of H abstraction by the C-5 OH-adduct radical of Thy from the sugar moiety of a neighboring or a distant nucleotide unit, leading to formation of strand breaks. The same mechanism might be operative in chromatin because of the higher yield of 5-OH-6-HThy than that of Thy glycol in the absence of oxygen. This is in contrast to the results observed with DNA in deoxygenated aqueous solution (Fuciarelli et al., 1990).

The formation of 8-hydroxypurines was affected substantially by the presence of oxygen in the system. Much higher yields of both 8-OH-Ade and 8-OH-Gua were observed with oxygen than without oxygen in the system. By contrast, the yields of formamidopyrimidines were not affected by addition of oxygen to the system and were even higher than those of 8-hydroxypurines in the absence of oxygen. The addition of N_2O to the system increased the yields of both formamidopyrimidines and 8-hydroxypurines, indicating the role of •OH in their formation. These two types of compounds result from addition of •OH to the C-8 of purines followed by respective one-electron reduction and oxidation of the C-8 OH-adduct radicals [for a review see Steenken (1989)]. The OH-adduct radicals of purines demonstrate a "redox ambivalence", meaning that different mesomeric structures of the same OH-adduct radical can be oxidized or reduced depending on their reaction partner (Steenken, 1989). Apparently, the presence of oxygen in the system strongly favors the oxidation of the C-8 OH-adduct radicals of purines as the ratios of the yields of 8-hydroxypurines to those of formamidopyrimidines measured suggest: 8-OH-Gua/FapyGua = 0.57 in argon vs 3.47 in air, and 0.75 in N_2O vs 4.45 in N_2O/O_2; 8-OH-Ade/FapyAde = 0.56 in argon vs 1.83 in air, and 0.83 in N_2O vs 3.43 in N_2O/O_2. In the case of Ade, the higher yield of 8-OH-Ade than that of FapyAde in the presence of oxygen might be explained by the addition of oxygen in a diffusion-controlled reaction to the C-8 OH-adduct radical of Ade followed by $HO_2^{\bullet}$ elimination (loss of O_2^- followed by deprotonation) as was described by Vieira and Steenken (1987). Whether this mechanism holds for the formation of 8-OH-Gua as well is not known. It is likely that oxygen also reacts with the C-8 OH adduct of Gua either by addition followed by O_2^- elimination as in the case of Ade or by electron transfer to produce O_2^- (Steenken, 1989). Both mechanisms would lead to the formation of the C-8 OH-adduct cation, which would yield 8-OH-Gua upon deprotonation. On the other hand, no satisfactory explanation can be given for the formation of formamidopyrimidines in the presence of oxygen. The fact that the ring-opening reaction of the C-8 OH-adduct radical of adenine, which precedes the formation of FapyAde, is suppressed by oxidants such as oxygen (Vieira & Steenken, 1987) is in agreement with the findings here. This might also hold for the C-8 OH-adduct radical of guanine, because the values of the ratio 8-OH-Gua/FapyGua had an analogous trend to those of the ratio 8-OH-Ade/FapyAde. Nevertheless, the formation of formamidopyrimidines is not suppressed, and these compounds have appreciable yields in the presence of oxygen, compared with the yields of the other products (Table II). This is in contrast to pyrimidines because 5-OH-6-HThy and 5-OH-6-HCyt, which result similarly from reduction of C-5 OH-adduct radicals of Thy and Cyt, respectively, were

not formed in the presence of oxygen. The one-electron reduction of the C-8 OH-adduct radicals of purines without the ring-opening reactions should also be taken into consideration for the formation of formamidopyrimidines in the absence of oxygen. This reaction leads to the formation of 7-hydro-8-hydroxypurines, which as hemiorthoamides are sensitive to hydrolysis and yield formamidopyrimidines (Steenken, 1989). Under our experimental conditions, 7-hydro-8-hydroxypurines should not be observed, because the acidic hydrolysis is an essential part of the sample preparation after irradiation.

The overall yields of the modified bases in chromatin irradiated in aqueous suspension are much lower than the yields of the same products determined in DNA irradiated in aqueous solution under similar conditions (Fuciarelli et al., 1990). This is expected because of the presence of histones in chromatin. Besides the simple scavenging of free radicals by the histones, the nucleosome structure might also play an important role in the production of the products. Because chromatin is in suspension rather than in solution, only those radicals formed in the immediate vicinity of DNA in chromatin might cause formation of the products. Furthermore, the DNA bases might participate in the formation of DNA–protein cross-links in mammalian chromatin as was described previously (Oleinick et al., 1987; Gajewski et al., 1988; Dizdaroglu et al., 1989; Dizdaroglu & Gajewski, 1989), resulting in the decreased yields of the modified bases. For these reasons, chromatin should be considered as a different system from DNA alone.

A number of products measured in irradiated chromatin were also found to be present in unirradiated chromatin (Table II). This is in agreement with the previous observations using DNA from various sources (Dizdaroglu & Bergtold, 1986; Kasai et al., 1986; Richter et al., 1988; Dizdaroglu et al., 1987). The presence of the products in mammalian chromatin may represent intrinsic damage to DNA due to cellular metabolic processes. On the other hand, isolation procedure and treatment of chromatin may also contribute in part to the formation of these products. However, this should be less likely with the mild isolation procedure used in the present study for chromatin than with that used for DNA in the aforementioned papers.

In conclusion, modified bases identified in previous studies on DNA and its constituents in aqueous solution are also formed in mammalian chromatin in aqueous suspension upon exposure to ionizing radiation. The methodology incorporating the GC/MS-SIM technique is well suited for identification and quantitation of the DNA base products in chromatin without any need for isolation of DNA first or removal of proteins. All the products reported here can be analyzed in a single sample by using as little as 0.4 μg of DNA in chromatin. The types of the products and their quantities markedly depend on the radical environment and the presence of oxygen in the system. Superoxide radical has no effect on product formation. Generally, the presence of oxygen increases the yields of the products with the exception of formamidopyrimidines. Although formamidopyrimidines and 8-hydroxypurines result from respective one-electron reduction and oxidation of the C-8 OH-adduct radicals of purines, the presence of oxygen has a marked effect on the yields of 8-hydroxypurines whereas the yields of formamidopyrimidines are not affected by the presence of oxygen. Further, the yields of the products measured are much lower than those observed with DNA alone. This is most likely due to the nucleosome structure, scavenging of radicals by histones, and involvement of DNA bases in the formation of DNA–protein cross-links in chromatin.

Registry No. 5,6-DiHThy, 696-04-8; 5-OHMeUra, 4433-40-3; FapyAde, 5122-36-1; FapyGua, 51093-31-3; 8-OH-Ade, 21149-26-8; 8-OH-Gua, 5614-64-2; 5-OH-5-MeHyd, 10045-58-6; 5-OH-Hyd, 29410-13-7; 5-OH-6-HThy, 1123-21-3; 5-OH-6-HCyt, 123472-58-2; 5,6-diOH-Cyt, 3914-34-9; $OH^{\bullet}$, 3352-57-6; O_2^{-}, 11062-77-4; O_2, 7782-44-7; N_2O, 10024-97-2; cytosine glycol, 13484-98-5; thymine glycol, 2943-56-8.

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RNA Folding during Transcription by *Escherichia coli* RNA Polymerase Analyzed by RNA Self-Cleavage†

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ABSTRACT: We have used a self-cleaving RNA molecule related to a subsequence of plant viroids (a "hammerhead") to study the length-dependent folding of RNA produced during transcription by *Escherichia coli* RNA polymerase. Transcript elongation is arrested at defined positions using chain-terminating ribonucleoside triphosphate analogues (3′-deoxyNTP's or 3′-*O*-methylNTP's). When the transcript can form the "hammerhead" structure it self-cleaves to give a truncated product. The experiment yields an RNA sequencing ladder which terminates at the length at which cleavage becomes possible; the sequencing ladder is compared to those generated by using a noncleaving transcript or by using [α-thio]ATP in place of ATP. We have shown that 15-18 nucleotides (nt) of RNA past the cleavage point must be synthesized before the transcript can self-cleave within a ternary complex, whereas RNA freed from the complex by heating can cleave with only 3 or more nt present beyond the cleavage point. There are sequence-dependent as well as length-dependent effects. The results suggest that 12 ± 1 nt are sequestered within the ternary complex and are consistent with the presence of a DNA-RNA hybrid within the transcription bubble, as proposed by others. The results indicate that the "hammerhead" structure does not disrupt the hybrid. It appears that the RNA beyond the hybrid is not restrained by interactions with the enzyme, since the last stem of the self-cleaving structure forms as soon as the RNA composing it emerges from the DNA-RNA hybrid. Self-cleaving of the transcript offers a simple structural probe for studying less well-characterized transcription complexes. The relevance of the results to models for transcription termination is discussed.

Transcription is the most important level of control of gene expression. The process of transcription can be divided into four stages (Chamberlin, 1974): promoter binding, initiation of transcription, elongation of the transcript, and termination. Mechanistic details of all of these processes are becoming available, and it is clear that cellular regulatory mechanisms act at all four stages. Our work concerns the structure and dynamics of the ternary polymerase·DNA·RNA complex during elongation.

The formation of structure in the nascent RNA chain is an essential step in pausing, antitermination, and termination of transcription by *Escherichia coli* RNA polymerase. These processes are believed to require the formation of an RNA hairpin near the 3′ end of the transcript. The prevailing model for "simple", ρ factor independent transcription termination and for pausing requires that an RNA hairpin disrupt the RNA-DNA hybrid helix within the transcription bubble (Farnham & Platt, 1982; von Hippel et al., 1984; Yager & von Hippel, 1987, 1990). RNA structure formation during transcription is required for attenuation of transcription in the *E. coli trp* operon (Landick & Yanofsky, 1987). The action of several transcription regulation factors such as NusA, proteins N and Q, and ρ is affected by RNA structure (Brennan et al., 1987; Faus et al., 1988; Landick & Yanofsky, 1987; Yang & Roberts, 1989). These proteins have been shown to affect pausing and termination of transcription, but in some cases, it is not clear whether they interact with the nascent transcript, the polymerase itself, or both. Specific folding patterns in the nascent RNA have been proposed to direct splice site selection (Eperon et al., 1988), to mediate Tat protein activation of HIV-1 gene expression (Berkhout et al., 1989), and to affect RNA-RNA interactions responsible for the control of plasmid ColE1 replication (Polisky et al.,

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