Clustered Damages and Total Lesions Induced in DNA by Ionizing Radiation: Oxidized Bases and Strand Breaks†

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ABSTRACT: Ionizing radiation induces both isolated DNA lesions and clustered damages—multiple closely spaced lesions (strand breaks, oxidized purines, oxidized pyrimidines, or abasic sites within a few helical turns). Such clusters are postulated to be difficult to repair and thus potentially lethal or mutagenic lesions. Using highly purified enzymes that cleave DNA at specific classes of damage and electrophoretic assays developed for quantifying isolated and clustered damages in high molecular length genomic DNAs, we determined the relative frequencies of total lesions and of clustered damages involving both strands, and the composition and origin of such clusters. The relative frequency of isolated vs clustered damages depends on the identity of the lesion, with ∼15−18% of oxidized purines, pyrimidines, or abasic sites in clusters recognized by Fpg, Nth, or Nfo proteins, respectively, but only about half that level of frank single strand breaks in double strand breaks. Oxidized base clusters and abasic site clusters constitute about 80% of complex damages, while double strand breaks comprise only ∼20% of the total. The data also show that each cluster results from a single radiation (track) event, and thus clusters will be formed at low as well as high radiation doses.

Clustered DNA damages—two or more oxidized bases, abasic sites, or strand breaks within a few helical turns—are hypothesized to be poorly repairable and thus critical lethal and/or mutagenic lesions induced by ionizing radiation *(10−12)*. Indeed, studies with oligonucleotides containing synthetic clustered damages on opposing strands and purified glycosylase/lyases indicate that both the identity of the component lesions and their relative spacing determine their repairability *(13−16)*. Although the yields of individual lesions and their repair have been studied extensively *(17, 18)*, little is known of the composition of bifilar complex damages or the relative frequency of clustered damages versus their component lesions.

Sensitive assays using a base excision enzyme to recognize and cleave DNA at specific lesion sites, followed by quantitative gel electrophoresis of the cleaved DNA and number-average length analysis have been developed for quantifying individual lesions and clustered damages in genomic DNA *(19−22)*. This approach has a lower limit of sensitivity of ∼1 lesion per megabase for a 40 kb DNA. Clustered damages and total lesions are measured by cleavage with a lesion class-specific glycosylase/lyase followed by electrophoresis of sample DNAs along with appropriate length standard DNAs on native gels for clusters and denaturing gels for total lesions, then quantitative electronic imaging of the ethidium-stained DNAs. From the molecular length vs migration distance of the length standards, a DNA dispersion curve is determined, the number-average lengths of the experimental samples are calculated, and from them the damage frequency is computed *(22)*.

We have determined the frequencies of component lesions (strand breaks, oxidized purines, and oxidized pyrimidines) vs those of the corresponding clustered damages (double strand breaks, oxidized purine clusters, and oxidized pyrimidine clusters) produced in DNA irradiated with ^{137}Cs γ-rays in a nonradioprotective solution. The frequency of abasic sites was calculated from the total break frequency determined in alkaline conditions (frank break plus alkali-labile sites) times the proportion of total breaks corresponding to alkali-labile sites determined by Brake under similar conditions. (The high pH of the denaturing gel results in cleavage at abasic sites even without enzymatic treatment.) Double strand breaks comprise only ∼20% of complex multilesion damages, while the other clustered damages constitute at least 80% of the total of such complex damages. Although oxidized purines and abasic sites are produced at similar levels to frank strand breaks, both oxidized purine and abasic sites as well as oxidized pyrimidines are found at least twice as frequently in damage clusters as are single strand breaks in double strand breaks. These data indicate that a significant fraction of the lesions induced by ionizing radiation is in clustered damage other than double strand breaks. Cellular repair of such clusters is virtually unknown.

EXPERIMENTAL PROCEDURES

DNA Irradiation and Enzyme Treatment. Bacteriophage T7 DNA, a linear double-stranded 40 kbp duplex, was prepared from CsCl-purified virus particles by successive extractions with redistilled phenol (foil-wrapped and stored at −20 °C under N_2), *sec*-butanol, and extensive dialysis vs 0.5 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8,

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followed by 50 mM NaCl, 0.1 mM EDTA, 10 mM Tris, pH 8. DNA was transferred to 20 mM potassium phosphate buffer, pH 7.4, using an Amicon Microcon (no. 42415, Millipore, Bedford, MA). DNA was irradiated at 50 μg/mL in plastic tubes on ice with ^{137}Cs γ-rays at dose rates from 0.16 to 1.6 Gy/min. Irradiations were carried out at the Brookhaven Controlled Environment Radiation Facility; irradiations and dosimetry were performed by the certified operator of the facility.

Samples were brought to final concentrations of 70 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 50 ng/μL bovine serum albumin, and then treated with saturating levels of homogeneous enzymes as follows: Fpg protein[1] (specific activity 150 000 units/mg) *(1−5)*, Nth protein[1] (specific activity 33 300 units/mg) *(4−7)*, Nfo protein (the kind gift of B. Demple, Harvard), or T4 endonuclease V (the kind gift of S. Lloyd, University of Texas, Galveston), which recognizes 4,6-diamino-5-formamidopyrimidine residues *(9)*. The amount of each enzyme to be used was determined by titrating irradiated DNA with increasing enzyme concentrations, and determining that amount that gave maximal cleavage of specific sites (over any abasic site cleavage) as determined from alkaline gel electrophoresis. For the enzyme preparations in our reactions containing 500 ng of T7 DNA, this corresponded to 60 ng of Fpg protein and 120 ng of Nth protein. For Nfo protein, irradiated DNA was titrated with increasing amounts of enzyme, and the quantity of enzyme required to give maximal cleavage of abasic clusters (electrophoresis under neutral conditions) was determined, ~150 ng per 500 ng of T7 DNA. DNA was incubated without enzymes for determination of frank single or double strand breaks. After digestions were complete, enzymes were removed by addition of proteinase K and EDTA to 1.33 mg/mL and 0.1 M, respectively, and incubation at 37 °C overnight.

Damage Cluster Measurement. To the DNA samples was added a neutral stop mixture (0.125% bromphenol blue, 0.5% sodium lauryl sulfate in 50% glycerol) to ensure dissociation of any persistent DNA−protein complexes. Samples and double-stranded molecular length standards [bacteriophage T4 (169 kbp), bacteriophage λ (48.5 kbp), *Hin*dIII-digested λ (23.1, 9.4, 6.5, 4.4, 2.3, 2, and 0.56 kbp) and BioMarker Low (BioVentures, Inc., Murfreesboro, TN; 1, 0.7, 0.5, 25, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 kbp)] were electrophoresed in 0.4% agarose, in Tris−acetate buffer, pH 8, using static field electrophoresis (30 V, 6 °C, with buffer recirculation) for frequencies >~ 30 sites/Mbp (Mb,[2] megabase, one million bases), or unidirectional pulsed field electrophoresis [UPFE, 15 V/cm; 0.3 s pulse, 10 s interpulse period, 16 h, 10 °C, with buffer recirculation *(20)*] for lower frequencies. Gels were stained, destained, and imaged as described above. A DNA dispersion curve was determined using the double-stranded DNA length standards. From the profiles of irradiated and unirradiated (or 'enzyme-treated' 'untreated' DNA populations), the number-average length (L_n) of each DNA distribution was calculated by

$$L_n = \frac{\int f(x)\,\mathrm{d}x}{\int \frac{f(x)}{L(x)}\mathrm{d}x} \quad (1)$$

where $L(x)$ is the length of the DNA molecules that migrated to position x and $f(x)\,\mathrm{d}x$ is the intensity of ethidium fluorescence from DNA molecules at that position.

From these L_ns, ϕ_{DSB} (DSB frequency) and ϕ_C (oxidized purine or pyrimidine cluster frequency) were calculated by *(19)*

$$\phi_{DSB} = 1/L_n(+\mathrm{rad}) - 1/L_n(-\mathrm{rad}) \quad (2)$$

and

$$\phi_C = 1/L_n(+\mathrm{rad}, +\mathrm{enzyme}) - 1/L_n(+\mathrm{rad}, -\mathrm{enzyme}) \quad (3)$$

where $1/L_n(+\mathrm{rad})$, $1/L_n(-\mathrm{rad})$, $1/L_n(+\mathrm{rad}, +\mathrm{enzyme})$, and $1/L_n(+\mathrm{rad}, -\mathrm{enzyme})$ are the reciprocals of the L_ns of samples that were irradiated, unirradiated, irradiated and treated with enzyme, or irradiated and not treated with enzyme, respectively.

Net Lesion Measurement. For quantification of net lesions [here including frank breaks and oxidized bases (for glycosylase-treated samples)], DNAs were denatured by addition of an alkaline stop mixture [equal volumes of dye mix (0.125% bromphenol blue, 50% glycerol) and 6 N NaOH] and incubation at 25 °C for 30 min. They were then electrophoresed along with molecular length standard DNAs (also denatured by alkaline stop mix) on an 0.4% alkaline agarose gel using UPFE for lesion frequencies <~30/Mb or static field electrophoresis buffer for frequencies >~30 sites/Mb. The gel was neutralized by two (45 min) treatments in 0.1 M Tris, pH 7.5, stained with ethidium bromide (1 μg/mg in H_2O), and destained, and a quantitative electronic image was obtained using ImageSystem, a cooled charge coupled device-based system *(23)*.

DNA profiles (fluorescence from ethidium bound to DNA) were obtained by integrating fluorescence intensity across all pixels at each migration distance down each lane. The length standards were used to construct a DNA dispersion curve (molecular length vs migration position). From the distribution of DNA in each experimental sample, the number-average molecular lengths (L_n) were calculated and the frequency of lesions computed from them by the equations *(19)*

[1] Substrates for glycosylases include: for *E. coli* Fpg protein, (FaPy = 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine) FaPyAdenine, FaPyGuanine, C8-oxoGuanine, C8-oxoAdenine, some abasic sites, and to a lesser extent, other modified purines and pyrimidines, including N−(2-Deoxy-β-D-erythro-pentofuranosyl)-N-3-[(2R)-hydroxyisobutyric acid]urea (αRT, formed from 5R-thymidine C5-hydrate) *(1−5)*; for *E. coli* Nth protein, pyrimidine residues damaged by ring saturation, fragmentation, or ring contraction (including 5,6-dihydrothymine, thymine glycol, urea, β-ureidoisobutyric acid, 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil, 5-hydroxy-2′-deoxyuridine, 5-hydroxy-5-methyl hydantoin, and αRT, DNA damaged at guanine sites, and some abasic sites *(4−7)*. 5-hydroxycytosine and 5-hydroxy-2′-deoxyuridine are substrates for Fpg protein and Nth protein, but neither is formed at significant levels during aerobic irradiations. Substrates for T4 Endonuclease V include cyclobutyl pyrimidine dimers, FaPyAdenine, and some abasic sites *(8,9)*.

[2] Abbreviations: DSB, double strand break; L_n, number-average molecular length; Mb, megabase, 1 million bases; OxyPur, oxidized purine; OxyPyr, oxidized pyrimidine; ϕ, frequency (of DNA lesions or clustered damages); SSB, single strand break; UPFE, unidirectional pulsed field electrophoresis.

$$\phi_{SSB} = 1/L_n(+\text{rad}) - 1/L_n(-\text{rad}) \quad (4)$$

and

$$\phi_{SITE} = 1/L_n(+\text{rad}, +\text{enzyme}) - 1/L_n(+\text{rad}, -\text{enzyme}) \quad (5)$$

where ϕ_{SSB} is the frequency of single strand breaks (SSB/Mb), ϕ_{SITE} is the frequency of lesions recognized by the enzyme, and $1/L_n(+\text{rad})$, $1/L_n(-\text{rad})$, $1/L_n(+\text{rad}, +\text{enzyme})$, and $1/L_n(+\text{rad}, -\text{enzyme})$ are the reciprocals of the L_n of samples that were irradiated, unirradiated, irradiated and treated with enzyme, or irradiated and not treated with enzyme, respectively.

RESULTS

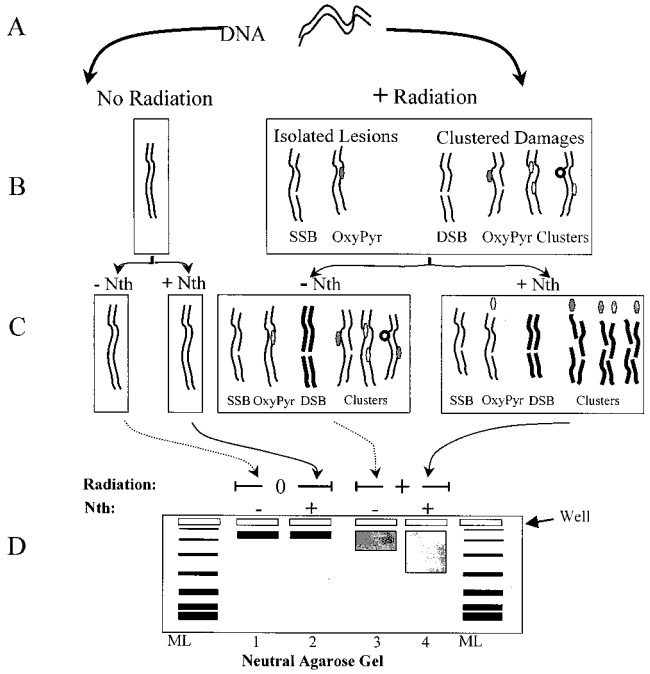
Clustered DNA damages—here defined as two or more oxidized bases, abasic sites, or single strand breaks on opposing strands within one or a few helical turns—can be identified by cleavage with an appropriate glycosylase/lyase and detection of enzyme-induced double strand cleavages by dispersal on neutral electrophoretic gels. Double strand breaks induced by radiation alone are detected by electrophoresis without enzyme treatment. Scheme 1 illustrates these principles for oxidized pyrimidine clusters. DNA (row A) is exposed to ionizing radiation or remains unirradiated (row B). Radiation induces both isolated lesions (single strand breaks and oxidized pyrimidines) and clustered damages (double strand breaks; oxypyrimidine clusters, composed of an oxidized pyrimidine and single strand break, two oxidized pyrimidines, or an oxidized pyrimidine vs an abasic site) (row B). Nth protein cleavage induces a de novo DSB at each cluster site (row C). [Abasic sites induced by ionizing radiation are ~10% regular abasic sites—a good substrate for Nth protein—and the rest 4′ and 2′ oxidized abasic sites *(24)*, reported to be poor substrates for Nth protein *(25)*; thus, Nth protein-recognized oxidized pyrimidine−abasic site clusters would be expected to be infrequent.]

Row D shows a schematic neutral agarose gel; Nth protein recognizes few if any clusters in unirradiated DNA (cf. lanes 1 and 2). Radiation-induced DSB reduce the size of the DNA molecules (lane 3), and Nth protein cleavage produces additional de novo DSB at oxidized pyrimidine clusters, resulting in smaller DNA molecules (lane 4). The frequency of DSB and other clustered damages is measured by quantitative electronic imaging, calculation of a DNA dispersion function (DNA migration vs molecular length) from the molecular length standards, and number-average length analysis of the sample DNAs (eqs 1−3). The total frequency of all oxypyrimidine lesions, whether isolated or clustered, is determined using denaturing separation media, as previously described *(19)*. Oxidized purine clusters and total oxidized purines are measured by Fpg protein treatment and separation on neutral or denaturing media; similarly, abasic site clusters and total abasic lesions are determined using Nfo protein.

Figure 1A shows an electronic image of a neutral agarose gel for quantifying damage clusters identified by Nfo protein, Nth protein, Fpg protein, or T4 endonuclease V [which cleaves at FaPyAdenine residues *(9)*, in addition to its well-known activity toward cyclobutyl pyrimidine dimers and abasic sites]. The sections of the gel (from left to right, A to E) show a dose response for induction of damage clusters by γ-rays: panel A contains unirradiated DNAs; panel B, DNA irradiated with 1 Gy of ^{137}Cs γ-rays; panel C, 2.5 Gy; panel D, 5 Gy; and panel E, 10 Gy. In each case, the samples were treated with (successive lanes, left to right) no enzyme, Nfo protein, Nth protein, Fpg protein, or T4 endonuclease V, respectively. Comparison of lanes 2−6 shows that there are few if any clusters in the unirradiated DNA. The frequency of all cluster classes increases as a function of dose. Radiation alone induces double strand breaks (cf. lanes 2, 7, 12, 17, and 22). Fpg-recognized oxidized purine clusters are induced at the highest frequency (lanes 10, 15, 20, and 25), and clusters involving FaPyAdenine are a measurable portion of such clusters (lanes 11, 16, 21, and 26).

Figure 1B shows an electronic image of a denaturing gel for measuring Fpg sites in T7 DNA exposed to 0−25 Gy of γ-rays. The samples (no irradiation, 1, 2.5, 5, 10, or 25 Gy of ^{137}Cs γ-rays) are in pairs in adjacent lanes, in each case without and with treatment by Fpg protein. [The T7 DNA preparation contained single strand breaks and/or alkali-labile sites not revealed on neutral gels (see Figure 1A, lane 2) that are evident on denaturing gels (Figure 1B, lane 2); they do not interfere with lesion quantitation, other than a loss of sensitivity resulting from the use of smaller initial molecules.]

Scheme 1: Quantifying DNA Damage Clusters and Total Lesions by Agarose Gel Electrophoresis[a]



[a]DNA (row a) is unirradiated or exposed to ionizing radiation. Radiation induces isolated lesions, e.g., a single strand break (SSB) and oxidized pyrimidine (OxyPyr), as well as clustered damages, shown here as a double strand break (DSB), and three classes of oxidized pyrimidine clusters (OxyPyr Clusters): an OxyPyr vs an SSB, two OxyPyrs, or an OxyPyr vs an abasic site (row b). Samples are treated with a lesion-recognizing glycosylase/lyase shown here for Nth protein, or incubated without enzyme, resulting in the molecules in row c. Molecules are analyzed at neutral pH to quantify damage clusters (as shown), or under denaturing conditions to measure total lesions (not shown). Molecules whose double strand molecular length is reduced by the radiation alone (−Nth) or radiation plus Nth protein digestion (+Nth) are shown in boldface. Row d shows a neutral pH electrophoretic gel with unirradiated DNA (without and with treatment with Nth protein), and irradiated DNA, without and with Nth protein, along with two molecular length marker lanes. The levels of other clustered and total lesions are measured similarly, with lesion recognition by other damage class-specific enzymes.

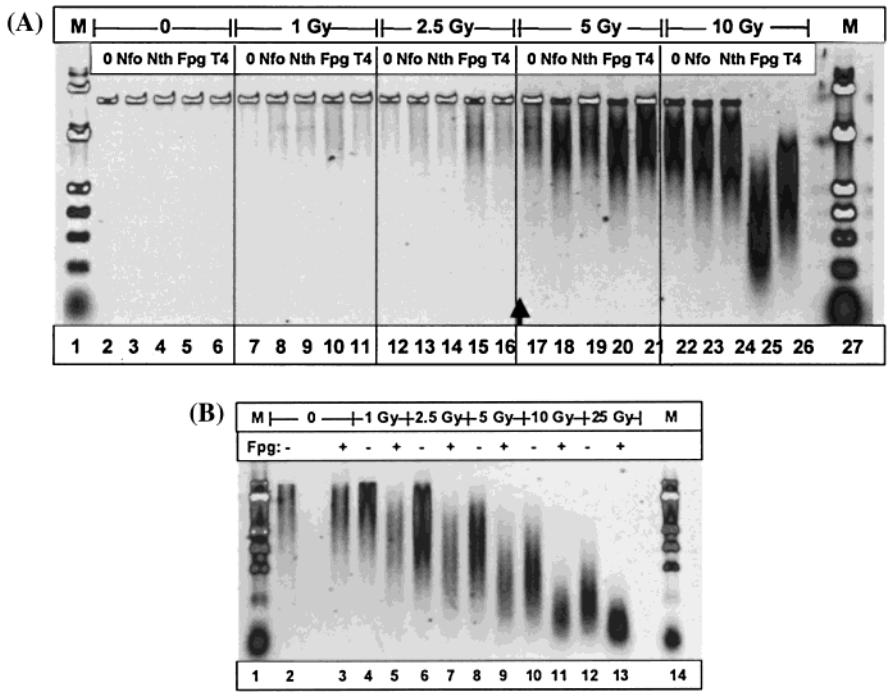


FIGURE 1: Electronic images of electrophoretic gels for analyzing DNA damage clusters and net lesions. (A) Damage cluster quantitation using a neutral agarose gel containing bacteriophage T7 DNA exposed to (from left to right, panels A to E) A, 0; B, 1 Gy; C, 2.5 Gy; D, 5, Gy, and E, 10 Gy of ^{137}Cs γ-rays, along with double stranded molecular length standard DNAs (T4, λ, *Hin*dIII-digested λ, and BioMarker Low DNAs). At each dose, DNAs were treated with no enzyme, Nfo protein, Nth protein, Fpg protein, or T4 endonuclease V. Two images were obtained for the left and right sides of the gel; for this figure, these images were merged, and the arrow between lanes 16 and 17 shows the intersection of the two images. (B) Fpg protein sites measured on an alkaline agarose gel containing pairs of T7 DNAs exposed to 0 (lanes 2 and 3), 1 Gy (lanes 4 and 5), 2.5 Gy (lanes 6 and 7), 5 Gy (lanes 8 and 9), 10 Gy (lanes 10 and 11), or 25 Gy (lanes 12 and 13) of ^{137}Cs γ-rays. Odd-numbered sample lanes were treated with Fpg protein, while even-numbered sample lanes were not. Lanes 1 and 14 contain single-stranded molecular length standard DNAs (λ, *Hin*dIII-digested λ and BioMarker Low).

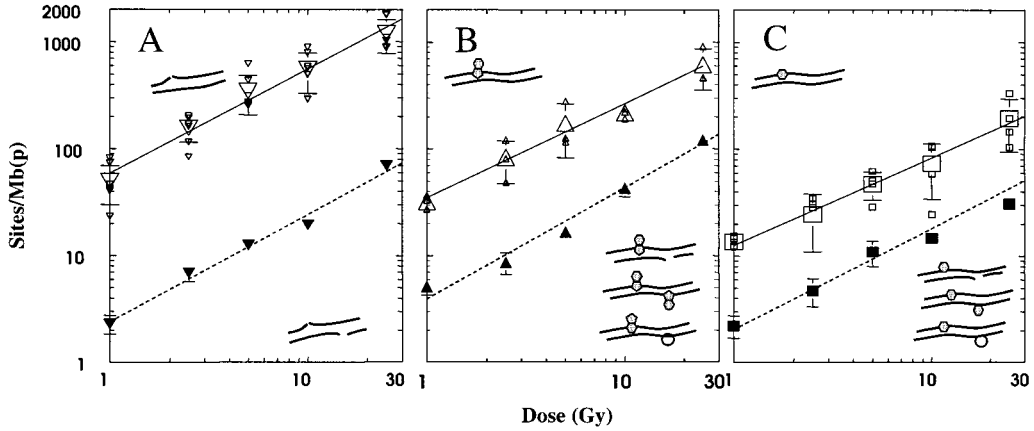


FIGURE 2: Frequencies of damage clusters and net lesions induced by ionizing radiation in T7 DNA in phosphate buffer; pertinent lesions (○, abasic site) and clusters are depicted schematically. (A) Strand breaks (including those in SSB or DSB (▽, —) and DSB (▼, - - -). (B) Net oxidized purines (△, —) and net oxidized purine clusters (▲, - - -). (C) Net oxidized pyrimidines (□, —) and net oxidized pyrimidine clusters (■, - - -). For all panels, the upper line represents net lesions (whether isolated or in clusters); small symbols show individual measurements, the large open symbols, the averages, and the error bars, the standard errors of the mean. The lower line shows damage cluster averages and standard errors [data from *(22)*]. In some cases, the error bars are smaller than the data point.

Radiation induces strand breaks, which reduce the size of the DNA molecules, even without enzyme digestion; Fpg protein treatment of irradiated DNAs clearly indicates the presence of sites, principally oxidized purines and abasic sites, recognized by this enzyme (see lanes 5, 7, 9, 11, and 13). The number-average lengths of the experimental DNAs are calculated (eq 1), and the frequencies of Fpg protein sites are computed using eq 5.

Figure 2 shows the quantitative data for the frequencies of strand breaks (single strand and double strand breaks), oxidized purines and oxidized purine clusters, as well as oxidized pyrimidines and oxidized pyrimidine clusters obtained from gels such as those in Figure 1. The upper line in panel A shows the induction of single strand breaks (some of which participate in double strand breaks) and double strand breaks (lower line) by γ-radiation. The slope of the line for induction of DSB is a straight line with a slope of 1.

The upper line in panel B shows the data for Fpg protein-recognized sites (oxidized purines), and the lower line shows that for Fpg protein-identified clusters (oxidized purine clusters). In calculating the frequencies of oxidized purines, the frequency of strand breaks was subtracted (see eq 5), and, similarly, the level of DSB was subtracted in computing the levels of oxidized purine clusters. Although the yield of

Table 1: Lesions and Clustered DNA Damage Induced by ^{137}Cs γ-rays in T7 DNA in Phosphate Buffer

lesion type	lesions (sites Mb^{-1} Gy^{-1})	clusters (sites Mbp^{-1} Gy^{-1})[a]	lesions in cluster (%)
breaks[b]	57.1	2.4	4.2
frank breaks[c]	32.	2.4	8.4
oxidized pyrimidine (Nth protein sites)	9.6	1.4	14.6
oxidized purine (Fpg protein sites)	29.2	5.2	17.8
abasic site (Nfo protein sites)	25.1[c]	3.6	14.3

[a] Data from *(22)*; OxyPyr (OxyPur) clusters may include two oxidized pyrimidines (purines), one oxidized pyrimidine (purine) vs a single strand break, or an oxidized pyrimidine (purine) vs an abasic site. [b] Frank + alkali-labile. [c] Calculated from the ratio obtained by Brake *(26)*.

frank strand breaks is approximately equal to that of oxidized purines (see Table 1), the yield of oxidized purine clusters is greater than that of DSB (cf. panels A and B.) About 18% of the oxidized purines are in clusters, about twice the frequency of frank strand breaks in DSB. The slope of the induction line for oxidized purine clusters is 1 on this log−log plot.

Panel C shows the data for Nth protein-identified oxidized pyrimidines and oxidized pyrimidine clusters. Both the oxidized pyrimidine sites and clusters are induced at lower frequencies than break sites and DSB or oxidized purines and oxidized purine clusters. About 15% of the oxidized pyrimidines are in such clusters, and the slope of the induction line in a log−log plot for oxidized pyrimidine clusters is $\cong$1.

DISCUSSION

Table 1 shows the yields of lesions and of clustered damages. For T7 DNA in dilute solution in phosphate buffer, 1 Gy of γ-rays induces about 32 frank breaks per million bases, 29 oxidized purines/Mb, slightly fewer (25/Mb) abasic sites, and ~10 oxidized pyrimidines/Mb. The results indicate that ~15% (14−18%) of oxidized pyrimidines, oxidized purines, and abasic sites are in clustered damages recognized by Nth, Fpg, and Nfo proteins, respectively, whereas only ~8.5% of the frank single strand breaks are in double strand breaks. Of course, some single strand breaks could participate in damage clusters other than DSBs (see Scheme 1, row B).

In these experiments, T7 DNA was irradiated in phosphate buffer, a nonradioprotective solution. The level of radioprotecting scavengers can affect the damage yields by as much as a factor of ~500 times that of highly protective conditions *(26, 27)*. The yields of lesions we obtain agree well with those obtained by others under these conditions. Brake found that among all breaks, 56% were frank strand breaks and 44% were alkali-labile sites *(26)*. For DNA irradiated in phosphate buffer, he obtained a yield of frank breaks of 30 Mb^{-1} Gy^{-1}, and Chen and J. Sutherland ~36 frank breaks Mb^{-1} Gy^{-1}. Thus, using Brake's proportions, our frequency of 57 total breaks corresponds to 32 frank breaks Mb^{-1} Gy^{-1}, and 25.1 abasic sites Mb^{-1} Gy^{-1}.

Lesions forming a damage cluster may occur on one or both DNA strands. Clusters detected by the approach described here are necessarily bifilar, or they would not be converted to double strand breaks by glycosylase/lyase treatment. The effect of the properties of the radiation producing double strand breaks, a type of clustered damage, has been studied extensively. Much less is known of the effect of radiation quality on the production of non-DSB clusters; however, Prise et al. reported that the production of clusters identified by endonuclease III (Nth protein) cleavage depended on LET *(28)*.

Theoretically, a cluster could result from multiple independent events, each producing one lesion constituting a cluster, or from a single radiation hit (with its accompanying track). For example, among strand breaks, frank breaks could occur as isolated single strand breaks (each resulting from a single event), or as closely spaced single strand breaks on opposite phosphodiester backbones, constituting a double strand break. The data in Figure 2 provide insight into this question. The fact that the plots of the log of DSB frequency vs the log of the dose are straight lines implies that the break frequency is proportional to some power of dose, described by

$$\phi = \alpha D^x \tag{6}$$

where ϕ is the frequency of DSBs induced either by radiation alone (eq 2) or by radiation followed by glycosylase/lyase treatment (eq 3), D is the radiation dose, α is a proportionality constant, and x is an exponent indicating the degree of the power law relationship. Experimentally, x is determined from the slope of the log−log plot from the equation:

$$\log \phi = x\,(\log \alpha + \log D) \tag{7}$$

The experimental results show that x is close to unity for all the complex damages (DSB, 1.00; abasic clusters, 1.05; Fpg sites/oxidized purine clusters, 1.05; Nth protein sites/oxidized pyrimidine clusters, initial slope 0.94). This indicates that the frequency of such clusters is directly proportional to dose, i.e., $\phi = \alpha D$. Thus, each cluster results from a single radiation track. Prise et al. found that the clusters detected in supercoiled DNA by Nth protein treatment did not result from independent events *(28)*.

Further, from the frequencies of the component lesions in a cluster, we can calculate the probability that one cluster resulted from multiple independent events—the oxidized purine frequency times the number of sites at which that lesion could occur as part of a cluster. [For this approximation, we ignore the lack of glycosylase cleavage at very closely opposed sites *(13, 15, 16)*.] For a cluster composed of 2 oxidized purines with a maximum separation of ±20 bases, at 1 Gy, the probability is $40 \times (29/10^6)$, or 1×10^{-3}. For oxidized purine clusters containing a strand break, the probability is also 1×10^{-3}, and thus the total probability of both types of oxidized purine clusters is then $\sim 2 \times 10^{-3}$, suggesting that few of the clustered damages we measure result from independent events.

Other investigators have presented evidence for cluster induction by radiation: S1 induced de novo DSB in λ DNA irradiated with ^{60}Co γ-rays *(29)*; *M. luteus* γ endonuclease produced DSB at some S1 sites in ^{60}Co γ-ray-irradiated λ DNA *(30)*. Further, Lam and Reynolds reported that UV produced clustered pyrimidine dimers *(31−33)*.

Ionizing radiation-induced complex damages have been modeled using Monte Carlo track structure simulations *(34)*, and termed DSB (a double strand break), DSB^+ [a DSB with additional single strand break(s) within 10 base pairs], and

DSB^{++} (a DSB with another DSB within 10 base pairs). One can also envision complex damages containing both closely opposed strand breaks as well as oxidized bases on one or both strands. All these configurations would appear as frank double strand breaks in our assay. Since frank, radiation-induced DSBs comprise only ~20% of the total of complex damages, at least 80% of the clustered damages induced under our conditions in this DNA are oxidized basic or abasic site clusters.

These figures may underestimate the frequencies of clustered damages. First, several glycosylase/lyases cleave DNA poorly at very closely opposed clusters in specific polarity *(13−16)*. Second, if the constituent lesions are too far apart to yield a de novo double strand break upon enzyme cleavage, the cluster would not be counted even if it were processed by the cell as a cluster. Third, glycosylase/lyase cleavage of multiple clusters very close together could yield two macromolecules plus many very small fragments that could migrate off the gel and thus not be detected. Fourth, multiple damages on one strand (without any on the opposing strand) would not be detected by these methods. Thus, the estimate above of 80% of complex damages as non-DSB clusters may understate their potential importance in the biological effects of ionizing radiation.

Since both Fpg protein and Nth protein recognize abasic sites, clusters could also contain these lesions. However, the abasic sites produced by ionizing radiation are principally oxidized abasic sites (with only about 10% regular abasic sites) *(24)*. Since neither protein apparently has significant activity toward oxidized abasic sites *(25)*, this suggests that few clusters we measure contain radiation-induced abasic sites.

Clustered damages are induced by ionizing radiation in human cells: Fe^{+26} (1 GeV/amu) particles produce approximately equal levels of oxidized pyrimidine clusters and DSB *(22)*. Further, preliminary data indicate that γ-rays and X-rays also induce damage clusters in cells (Sutherland and Bennett, unpublished). Cellular repair paths for clusters are not known, but on the basis of the model oligonucleotide studies *(14, 15, 35)*, the composition of the spacing of lesions within a cluster would be expected to determine the mode of repair. Increased DSB levels in Chinese hamster ovary cells after ionizing radiation exposure *(36, 37)* could result from simultaneous scissions at opposing lesions in a cluster.

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