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Novel Method For Quantifying Radiation-Induced Single-Strand-Break Yields In Plasmid DNA Highlights Tenfold Discrepancy

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Abstract

The widely used agarose gel electrophoresis method for assessing radiation-induced single-strand-break (SSB) yield in plasmid DNA involves measurement of the fraction of relaxed-circular (C) form that migrates independently from the intact supercoiled (SC) form. We rationalized that this method may underestimate the SSB yield since the position of the relaxed-circular form is not altered when the number of SSB per DNA molecule is >1 . To overcome this limitation, we have developed a novel method that directly probes and quantifies SSBs. Supercoiled ^3H -pUC19 plasmid samples were irradiated with γ -rays, alkali denatured, dephosphorylated, kinased with γ - ^{32}P -ATP, and the DNA-incorporated ^{32}P activities used to quantify the SSB yields per DNA molecule employing a standard curve generated using DNA molecules containing known number of SSBs. The same irradiated samples were analyzed by agarose gel and SSB yields were determined by conventional method. Comparison of the data demonstrated that the mean SSB yield per plasmid DNA molecule of $[21.2 \pm 0.59] \times 10^{-2} \text{ Gy}^{-1}$ as measured by direct probing, is ~ 10 -fold higher than that obtained from the conventional gel-based method. These findings imply that the SSB yields inferred from agarose-gels need re-evaluation, especially when they were utilized in the determination of radiation risk.

Keywords

γ -rays; single-strand breaks; plasmids; endonucleases; radiation

INTRODUCTION

Radiation induced damage to DNA is extremely complex and produces a spectrum of base lesions and products including a basic sites, base/sugar oxidation, cross links, and strand breaks as identified by physicochemical techniques [1–5]. Among these, single and double strand breaks in the DNA comprise the most well studied [6–8] radiation induced lesions due to their role in cytotoxicity [9], mutagenesis [10; 11], and other radiobiologic cellular effects [12].

Numerous studies demonstrated that the efficiency of single and double strand break formation in DNA greatly varies with the type of ionizing radiation, depending on whether it is low or high LET (linear energy transfer). Further, the ratio of DSB to SSB has been

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shown to be greater for densely ionizing (high LET) radiation such as α -particles, neutrons, and Auger electron, in comparison with sparsely ionizing (low LET) X-rays and γ -rays [13]. Ionizing radiation produces DNA strand breaks by two major mechanisms; either through direct ionization of the sugar-phosphate backbone in the DNA (direct mechanism) or by the reaction of hydroxyl radicals ($\bullet\text{OH}$), hydrogen atoms ($\bullet\text{H}$), and solvated electrons (e^-_{aq}) [14] with the DNA backbone (indirect mechanism). Studies using hydroxyl radical scavengers [15; 16] suggest that hydroxyl radical mediated indirect mechanisms is largely responsible for the induction of radiation induced strand breaks by low LET radiation; whereas the strand breaks resulting from high LET radiation arise from both direct and indirect mechanisms [17; 18].

There are many *in vitro* methods currently in practice to measure DNA strand breaks. Analytical methods such as sedimentation coefficient determination [4], contour length electron microscopy [1] and anion exchange HPLC [5] have been employed in the quantification of strand breaks. Low angle light scattering has also been employed to measure γ -ray induced SSB and DSB yield through molecular weight estimations [3]. These experiments demonstrated that for low LET radiation, double strand breaks mediated by direct effects and by single hit radical transfer mechanisms form a minor contribution emphasizing the importance of single strand breaks produced by radical mediated indirect effects in deciding the DSB yield. Consequently, it is generally accepted that in the absence of a hydroxyl radical scavenger, the majority of DSBs originate from two or more SSBs being proximal and clustered in a locus of a few base pairs [3]. Several lines of evidence suggest the involvement of two radicals (two hit mechanism) in the formation of a DSB [19–21]. On the other hand, it has been proposed that a significant fraction (45%) of DSB induced by fast neutrons (high LET) is caused by single hit and direct mechanisms [18]. Therefore, to determine the origin of DSBs, reliable quantification of single-strand breaks is necessary.

A popular method for quantifying single- and double-strand breaks in cell free systems is based on changes in the structure of supercoiled plasmid DNA. When irradiated, plasmid DNA molecules containing one or more SSB migrate independently on agarose gels, from the intact supercoiled form, enabling quantification [3; 17; 18; 22–26]. In plasmid DNA, both SSB and DSB produce break down products that can be separated and identified. This method has produced a wealth of information on strand breaks caused by ionizing (different types of external beam radiations as well as radionuclides such as ^{125}I and ^{123}I [17; 26–28]) and non-ionizing radiation. Recently, we realized that the enumeration of SSB through the formation of relaxed plasmid DNA has an underlying caveat given that molecules with one SSB and those with multiple SSBs (as long as SSBs are not close enough to make a DSB) will migrate together to the same position on the gel. To overcome this inherent technical limitation, we developed a new method to quantify SSB yield directly. This method relies on probing the single strand breaks in the irradiated plasmid DNA directly with ^{32}P and quantifying the number of SSB (per ^{32}P incorporation at the SSB sites of the plasmid DNA molecules).

Normally, single-strand breaks produced in plasmid DNA either by irradiation or by enzymatically are not accessible to DNA modifying enzymes such as calf intestinal phosphatase or polynucleotide kinase which act at the very ends of the DNA. Therefore, the success of the current method was dependent on developing a reliable method to expose the broken 5' ends at each SSB formed. This was achieved by using the alkali denaturation routinely used in Saenger's dideoxy sequencing strategy. Our studies demonstrate that γ -ray induced single strand break yields measured quantifying the fraction of nicked-circular form in gels is significantly lower than the actual SSB yield.

MATERIALS AND METHODS

Preparation of ^3HT -pUC19 Plasmid DNA

Stocks of *E. coli* DH5a bacterial cultures harboring pUC19 were grown in Luria broth for 16 h at 37°C in the presence of ampicillin (50 µg/ml) and ^3H -thymidine (^3H -TdR, 37 MBq). The plasmids (^3HT -pUC19) were isolated using the Qiagen Maxi preparation kit and dissolved in PBS (pH 7.4). The concentration was determined by measuring A_{260} . The plasmid DNA was stored at -20°C.

Preparation of ^3HT -pUC19 with a Single SSB

^3HT -pUC19 plasmid DNA with a single SSB was prepared as described previously [17; 26]. In brief, ^3HT -pUC19 plasmid DNA (20 µg) was digested with EcoRI (160 units in 8 µl, New England Biolabs, Beverly, MA) in the presence of ethidium bromide (0.4 mg/ml) and EcoRI buffer (1X, 80 µl), New England Biolabs, for 24 h at 37°C. The reaction mixture was extracted with phenol (pH >7.5) and chloroform:isoamyl alcohol (24:1, v/v), followed by five *n*-butanol extractions to remove ethidium bromide completely. The DNA was ethanol-precipitated and redissolved in 1X PBS. Since this circular DNA preparation was found to have traces of L DNA, the latter were digested with λ exonuclease and RecJ_F [17]. Agarose gel analysis indicated that no detectable linear DNA in the preparation. Finally, the λ exonuclease was inactivated thermally at 65°C for 10 min and the reaction mixture was extracted with phenol and chloroform:amyl alcohol (24:1, v/v) and the DNA was ethanol precipitated.

Preparation of ^3HT -pUC19 with >1 SSB

Nicking endonucleases Nb.BsrDI, Nb.BtsI, Nt.BstNBI and Nt.AlwI (New England Biolabs, Beverly, MA) were used to create ^3HT -pUC19 (20 µg) plasmid DNA molecules with known number of SSBs (2, 3, 4 and 10, respectively). In essence, ^3HT -pUC19 (20 µg) plasmid SC DNA samples were each digested with 100 units (10 units/µl) of one of the above mentioned nicking endonucleases in 100 µl volume under appropriate conditions. For Nb.BtsI digestion, DNA was incubated (37°C for 18 hrs) in 1X NEBuffer 4 (New England Biolabs, Inc.); for Nt. AlwI digestion (37°C for 18 hours), 1X NEBuffer 2 was used. Nt.BstNBI digestion (55°C, overnight) was carried out in NEbuffer 3 while Nb.BsrDI digestions was performed in NEbuffer 2 at 65°C for 18 hours. Following heat denaturation at 65°C for 15 minutes, the reaction mixtures were extracted with phenol (pH >7.5) and chloroform:isoamyl alcohol (24:1, v/v). The DNA was ethanol-precipitated and redissolved in 1X PBS. Agarose gel analysis indicated that all the digestions were complete and no SC DNA was left intact.

DNA-Gamma Irradiation

Supercoiled ^3HT -pUC19 plasmid DNA samples (9 µg in 100 µl of 1× PBS, pH 7.4) were placed on ice and irradiated (0–40 Gray) with ^{60}Co γ -rays (3.75 Gy/min).

Denature, Dephosphorylate, and Kinate (DDK) SSB Quantifying Method

Relaxed-circular ^3HT -pUC19 plasmid DNA containing a known number of nicks (i.e., SSBs) as well as γ -ray irradiated SC ^3HT -pUC19 plasmid DNA (5 µg) were denatured with 5 µl of 2 M NaOH/2 mM EDTA in 50 µl of 10 mM Tris pH 8.0 for 5 minutes at ambient temperature. DNA was ethanol precipitated by adding 17.5 µl of water, 17.5 µl of 3 M sodium acetate (pH 5.3), and 190 µl of ethanol and incubating the samples at -20°C overnight. The DNA pellets were redissolved in 10 mM Tris (pH 8.0) and dephosphorylated with 10 units of calf intestinal phosphatase in 120 µl of 1X NEBuffer 3 at 37°C for 45 min-1 hour. The reaction mixture was extracted with phenol/chloroform/isoamylalcohol and DNA

was ethanol precipitated. The DNA pellet was dissolved in 60 μ l of autoclaved water. DNA concentration was determined at A_{260} . Subsequently, 500 ng (equivalent to 0.283 pmoles of intact supercoiled pUC19 DNA or 2.82 pmoles of 5' OH ends for the standard with 10 SSBs per plasmid DNA molecule) of this DNA was kinased with 30 μ Ci (10 pmoles) of γ -[32 P-ATP] and 20 units of T4 polynucleotide kinase (New England Biolabs, Inc.) in 50 μ l of 1X PNK buffer (New England Biolabs, Inc) at 37°C for 1 hour. The unincorporated γ -[32 P-ATP] was removed by desalting the reaction mixture on MicroSpin™ G-25 columns (Amersham Biosciences) pre-equilibrated with water. Samples were diluted and 32 P content was counted in Beckman Coulter LS6000 IC scintillation counter.

Agarose Gel Analysis And Calculation Of Single-Strand Break Yield By Conventional Method

The γ irradiated 3 H-labeled DNA samples were analyzed on a 1% agarose gel as described previously [17; 26]. Essentially, 6- μ l of each irradiated DNA sample was mixed with agarose-gel-loading-dye mixture (3 μ l) and PBS (3 μ l) and loaded onto 1% agarose gel in 0.5X TAE buffer with ethidium bromide (0.5 μ g/ml). The gels were run at 200 volts (~7 volts/cm) for 1 h and then photographed on a transilluminator (long wavelength) attached to a CCD camera. The DNA bands — corresponding to SC, L, and C states — were excised and the gel pieces were dissolved in OPTI-FLOR scintillation fluid (10 ml). The DNA was quantified by assaying the associated tritium. The fraction of DNA in each of the three states (SC, L and N) were calculated from tritium counts.

The mean number of single strand breaks per DNA molecule (X_{SSB}) were calculated from the experimentally observed fractions of L DNA formed and SC DNA remaining after exposure to a given dose of γ -rays, using the following relationship which assumes Poisson distribution of DNA breaks [29]:

$$\bar{X}_{SSB} = \ln [(1 - F_L)/F_{SC}],$$

where F_{SC} is the fraction of SC DNA remaining and F_L is the fraction of L DNA formed as a result of exposure to γ -rays.

The rate of formation of SSB per DNA molecule per gray is obtained by plotting the average number of single (X_{SSB}) strand breaks as a function of radiation dose. Since a single SSB leads to the appearance of a circular DNA molecule, the slopes of these linear regressions is believed to reflect the rate of SSB formation and the reciprocal of the slopes represents D_0 , the dose required to form one SSB. The slope, when multiplied by the total number of plasmid DNA molecules/ml, gives the yield of SSB per gray:

$$Y_{SSB}/\text{Gray} = [\text{DNA}]/D_0,$$

where $[\text{DNA}] = 3.06 \times 10^{13}$ molecules/ml.

Calculation Of Single-Strand Break Yield By Direct Probing With 32 P

The mean single-strand-breaks per DNA molecule at each dose of γ irradiation was obtained from 32 P incorporation (cpm) and the slope of the standard curve (linear fit of the 32 P incorporation plotted as a function of number of enzymatically generated SSBs). This was done by substituting the slope of the standard line and the 32 P incorporation using the following equation:

$$\bar{X}_{SSB} \text{ (for each dose)} = {}^{32}\text{P (cpm)} / \text{Slope of Standard Curve}$$

The mean SSB/DNA molecule, in turn, was plotted as a function of dose and as described above, the slope of the linear regression reflects the SSB yield per DNA molecule per gray.

RESULTS AND DISCUSSION

Single-strand-break yields induced by γ rays in supercoiled plasmid DNA as determined by fractionating different topological forms on agarose gels

Supercoiled form of plasmid DNA has been an ideal model to quantify radiation induced single and double strand breaks in DNA as the products resulting from DNA strand breaks can be easily separated from the intact supercoiled DNA. In the current investigations, supercoiled (>99%) ${}^3\text{H}$ -pUC19 plasmid DNA was irradiated with 0, 5, 10, 15, 20, 25, 30, and 40 grays of γ rays. These doses were selected to insure a very low probability for two ionizations being sufficiently close to form a DSB. The results of such irradiated DNA samples on 1 % agarose gel (Figure 1A) show a gradual increase in the appearance of nicked (circular) DNA form with a concomitant disappearance of supercoiled form as the dose is increased demonstrating that single strand breaks are produced in the supercoiled DNA. Each band, corresponding to supercoiled and circular forms, was excised from the agarose gels and assayed for their ${}^3\text{H}$ content to determine the fraction of each of these forms of plasmid DNA. Since SSBs induced by external beam radiations are predominantly the results of hydroxyl radicals mediated indirect effects [15; 16], the mean SSB yield per DNA molecule at each dose was calculated on the assumption that the reactivity of hydroxyl radicals with DNA is random and hence the strand breaks follow a Poisson distribution [29]. The mean SSB per DNA molecule were then plotted as a function of dose (Figure 1B) and the data was fitted to a linear regression, the slope of which directly reports the SSB yield in *mean SSB per DNA molecule per Gray*. Using this conventional method, a mean SSB yield per DNA molecule per gray of $(2.31 \pm 0.07) \times 10^{-2}$ was obtained, with a D_0 of 43.29 ± 1.31 gray (Table 1). These values are in good agreement with the SSB yields reported by other workers, determined using the same approach [18]. However, since the position of the relaxed N form is not altered when the number of SSB in these DNA molecules is >1 and this method may thus underestimate the SSB yield, a novel method that directly probes 5' single stranded ends at SSBs and quantifies SSBs has been developed. The basis of this method relies on the direct labeling of the free dangling 5' hydroxyl groups by phosphorylation using polynucleotide kinase and γ - ${}^{32}\text{P}$ ATP. The efficiency of radiotracer incorporation at the DNA terminus would be directly proportional to the number of free 5' hydroxyl groups which in turn are generated at the sites of single strand breaks induced originally by the irradiation of the samples.

Single-strand-break yields induced by γ rays in supercoiled plasmid DNA as determined by direct labeling of 5' ends of single strand breaks

The endonucleases used to generate ${}^3\text{H}$ -pUC19 plasmid DNA molecules with varying number of SSBs are listed in Table 2, each with its recognition sequence. Restriction endonuclease EcoRI is known to cut both strands in the DNA between the bases G and A in the recognition sequence 5' GAATTC 3', leading to a DSB [30]. However, in the presence of ethidium bromide, EcoRI severs only one of the two DNA strands in its recognition sequence, resulting in relaxation of supercoiling and the production of a nicked-circular molecule [31]. To induce a single SSB in ${}^3\text{H}$ -pUC19 DNA, the plasmid was digested with EcoRI in the presence of ethidium bromide as described previously [17]. ${}^3\text{H}$ -pUC19 SC DNA was also digested with the nicking endonucleases Nb.BsrDI, Nb.BtsI, Nt. BstNBI, and

Nt. AlwI to generate a set of relaxed-circular plasmid DNA molecules with 2, 3, 4, and 10 SSBs, respectively (Table 2).

Each of the relaxed-circular ^3H -pUC19 DNA standards was alkali denatured to expose the sites of SSBs to phosphatase to generate 5' OH groups. This was followed by kination and the incorporated ^{32}P per given amount of DNA was quantified. The variation of ^{32}P incorporation as a function of number of SSBs per pUC19 DNA molecule is shown in figure 2A. As expected, a linear increase in ^{32}P incorporation with the number of SSBs per plasmid DNA molecule was observed among the standards containing known number of SSBs. The relationship between the 5' labeled ^{32}P and the number of SSBs was used to quantify the mean SSBs per DNA molecule in γ irradiated DNA samples containing unknown number of SSBs.

To enable meaningful comparison, aliquots from the same γ irradiated supercoiled DNA samples that were analyzed by agarose gel (Figure 1A) were also alkali denatured and dephosphorylated and the resulting 5' OH groups were labeled with γ -[^{32}P -ATP] in the presence of polynucleotide kinase. The DNA samples with the known number of SSBs were processed and labeled in parallel to generate a standard line. For each dose, the mean SSB/DNA molecule was calculated by dividing the counts associated with ^{32}P (cpm) by the slope $(1.003 \pm 0.038) \times 10^7$ of the standard line ^{32}P cpm vs. number of single strand breaks (figure 2A). When the mean SSB/DNA molecule was plotted as a function of dose and the data fitted to a linear regression, an SSB yield of $(21.24 \pm 0.59) \times 10^{-2}$ (mean SSB/DNA molecule/Gray) with a D_0 of 4.71 ± 0.13 (gray/SSB) was obtained (Table 1). These results demonstrate clearly that for the same irradiated supercoiled DNA samples, the γ -ray induced single strand break yield obtained by our newly developed "Denature, Dephosphorylate, and Kinate" (DDK) method is ~10 fold higher than that obtained by conventional method using gel electrophoresis. Thus, radiation induced SSB yields reported previously in studies using the conventional gel electrophoresis method should be interpreted with great caution. We therefore propose that the SSB yields determined using gel-based methods should be re-evaluated using the DDK method described herein, particularly when such results are used to assess radiation associated health risks. Finally, it would be of further interest to see if such discrepancies exist when DNA is exposed to non-ionizing and high LET ionizing radiations.

The conventional agarose gel electrophoresis method, owing to its experimental simplicity and ease of application compared to our new method reported herein, could be sufficient for comparing the SSBs induced by two or more agents *relative to each to other*. However, our new method has the ability to determine the absolute number of single strand breaks more accurately and as shown herein, the SSB yield as determined by gel electrophoresis is ~1/10th the factual yield. One of the greatest advantage of our new method lies in its ability to probe the sites of SSBs, unlike other methods currently in use which monitor a physical property of the DNA (e.g. electrophoretic gel mobility of supercoiled DNA) that changes upon the introduction of single strand break. In this context, using agarose gel electrophoresis method, it is impossible to determine SSB yield in linear and relaxed-circular forms of plasmid DNA and eukaryotic chromosomal DNA (linear) that has been irradiated or treated with DNA damaging agents, as the introduction of SSB(s) in these topological forms of DNA do not alter their electrophoretic mobility. However, our method's ability to probe directly the sites of SSBs enables its use for these topological forms of DNA too. Thus, our new method is very versatile and robust and it will enable those interested in the accurate determination of SSB yields in various topological form of DNA (the conventional agarose gel electrophoresis method is limited only to supercoiled form).

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Figure 1.

Determination of SSB yield induced by γ -rays by conventional gel electrophoresis method. (A) Analysis of the γ -irradiated ^3H -pUC19 plasmid DNA samples on 1% agarose gel stained with ethidium bromide showing the increases in the fluorescence intensities of the bands corresponding to relaxed-circular DNA resulting from SSB formation and the simultaneous decreases in the fluorescence intensities of the SC DNA bands. (B) SSB yield per DNA molecule for γ -irradiated SC plasmid DNA, calculated from the appearance of N form. Linear regression (forced through zero) and R^2 was obtained using Origin.

Figure 2.

(A) Linear regression obtained by measuring ^{32}P incorporation into nicked plasmid DNA samples containing known number of SSB (generated using the endonucleases listed in Table 2). (B) SSB per DNA molecule as determined from the ^{32}P incorporation in the irradiated samples as function of dose. The slope of the linear regression provides the SSB yield. Linear regression (forced through zero) and R^2 was obtained using Origin.

Table 1

Mean γ ray induced SSB yield determined from conventional (gel) and the direct method (^{32}P incorporation)

Method	D_0 (Gray/SSB)	Mean SSB/DNA Molecule/Gray
Gel Electrophoresis	43.29 ± 1.31	0.0007
Direct Method (^{32}P labeling)	4.71 ± 0.13	0.0059

List of nicking endonucleases used to generate known number of single-strand-breaks in pUC19

Table 2

Enzyme	Recognition sequence	Number of sites in pUC19
EcoRI (Ethidium Bromide)	5' G▼AATTC 3'	1
Nb.BsrDI	5' N▼CATTGC 3'	2
Nb.BtsI	5' N▼CACTGC 3'	3
Nt.BstNBI	5' GAGTCNNNN▼ 3'	4
Nt.AfwI	5' GGATCNNNN▼ 3'	10