Deletions at short direct repeats and base substitutions are characteristic mutations for bleomycin-induced double- and single-strand breaks, respectively, in a human shuttle vector system

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ABSTRACT

Using the radiomimetic drug, bleomycin, we have determined the mutagenic potential of DNA strand breaks in the shuttle vector pZ189 in human fibroblasts. The bleomycin treatment conditions used produce strand breaks with 3'-phosphoglycolate termini as >95% of the detectable dose-dependent lesions. Breaks with this end group represent 50% of the strand break damage produced by ionizing radiation. We report that such strand breaks are mutagenic lesions. The type of mutation produced is largely determined by the type of strand break on the plasmid (i.e. single versus double). Mutagenesis studies with purified DNA forms showed that nicked plasmids (i.e. those containing single-strand breaks) predominantly produce base substitutions, the majority of which are multiples, which presumably originate from error-prone polymerase activity at strand break sites. In contrast, repair of linear plasmids (i.e. those containing double-strand breaks) mainly results in deletions at short direct repeat sequences, indicating the involvement of illegitimate recombination. The data characterize the nature of mutations produced by single- and double-strand breaks in human cells, and suggests that deletions at direct repeats may be a 'signature' mutation for the processing of DNA double-strand breaks.

INTRODUCTION

Ionizing radiation causes a wide range of mutations in mammalian cells, including deletions, large chromosomal rearrangements, insertions, frame shifts and single and multiple base substitutions (1–3). It has been difficult, however, to link these mutational spectra with any specific DNA lesions, because of the large diversity of damage produced by ionizing radiation (4) and the relatively limited sequence specificity of the lesions. Also, the differential production of lesions and their different mutagenic potentials makes it difficult to assess the mutagenicity of all but the most abundant or most highly mutagenic lesions in phenotypic selection assays. These limitations have impeded evaluation of the mutagenicity of individual ionizing-radiation-induced DNA lesions to the extent that cyclobutane dimers and 6–4 photoproducts have been studied for ultraviolet radiation (5). Consequently, knowledge of the mutagenic consequences of specific ionizing-radiation-induced DNA lesions is quite limited.

Our laboratory has been studying the repair of DNA single-strand breaks that have 3'-phosphoglycolate (3'-PG) termini. These end groups represent 50% of the strand breaks produced by ionizing radiation (the remaining half contain 3'-phosphate) (6). 3'-phosphoglycolate can physically block DNA repair synthesis (7,8) and act as a competitive inhibitor for DNA polymerases (9,10). Recent studies indicate that 3'-PG termini may also be an important toxic lesion (11,12), but their mutagenic potential is unknown.

We have previously identified conditions of bleomycin treatment of DNA which produce strand breaks with 3'-PG termini as >95% of the detectable dose-dependent lesions (13). In work presented here, we have used these conditions to generate such strand breaks on the shuttle vector plasmid pZ189 to specifically study their mutagenic potential. We find that these strand-breaks are mutagenic and the mutagenesis spectrum predominantly contains base substitutions and a characteristic type of deletion at short direct repeat sequences.

We were able to determine that the base substitutions mostly arose from DNA single-strand breaks, probably due to error-prone DNA polymerase repair synthesis. In contrast, double-strand breaks were responsible for deletions at short direct repeat sequences, suggesting illegitimate recombination repair is involved in rejoining these breaks. Furthermore, we show that the mutagenesis spectrum of bleomycin-induced strand breaks differs from that produced by bleomycin-induced apurinic/apyrimidinic (AP) sites. By comparing our data to the literature on strand-break mutagenesis, we suggest that deletions at direct

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repeats may be a 'signature' mutation for misrepaired DNA double-strand breaks in mammalian cells.

MATERIALS AND METHODS

Materials

Simian virus 40 (SV40) transformed human fetal lung fibroblasts, WI38VA13, were purchased from American Type Culture Collection (Rockville, MD). *Escherichia coli* strain MBM7070 [*lacZ*(Am)*CA7020*], carrying the amber mutation in the β-galacto-sidase gene from CA7020 (14), was used as the indicator strain to identify mutants and was kindly provided by Dr Michael Seidman, as was the human shuttle vector, pZ189. Bleomycin was generously provided in the form of bleomycin sulfate (Blenoxane™) by Bristol-Myers Pharmaceutical (Evansville, IN).

Human cell culture

WI38VA13 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, (1×) non-essential amino acids (Life Technologies; Gaithersburg, MD), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cultures were tested for mycoplasma twice during the course of experiments and found to be negative.

Plasmid preparation and bleomycin treatment of pZ189

The plasmid pZ189 was grown in an *E.coli* host HB101 in Luria-Bertani (LB) medium and purified using QIAGEN (Chatsworth, CA) tip-100 purification columns following the manufacturer's recommended procedure. HB101 carries the *dam* gene (Dam methylase) which methylates the adenine residues in the sequence GATC on the plasmid. Bleomycin-treated pZ189 DNA was prepared as previously described (13). Briefly, a reaction mixture was prepared by incubating pZ189 at 145 μg/ml in 13 mM Tris–HCl (pH 8.0); 320 mM sucrose; 0.02% Triton X-100; 1.3 mM EDTA (pH 8.0); 8 mM β-mercaptoethanol; 266 μg/ml of heat-inactivated bovine serum albumin, with 0–1.0 μg/ml bleomycin and 100 μM ferrous ammonium sulfate. The reaction tubes were briefly spun with caps open at maximum speed in a microcentrifuge and incubated at 37°C for 20 min. The drug was removed by ethanol precipitation of the DNA at –70°C for 30 min and the DNA was collected by spinning at 16 000 *g* for 15 min. Following a 70% ethanol wash, the plasmid pZ189 was dissolved in 25 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and used for transfections.

Plasmid nicking assay

0.5 μg of bleomycin treated plasmid DNA was separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. The stained gel was photographed under UV light to reveal the fast migrating supercoiled (Form I) and slower migrating nicked (Form II) and linear (Form III) plasmid bands.

Transfection of DNA, plasmid recovery and mutation assay

Approximately 3 x 10⁵ logarithmically growing cells were plated in each of the five to ten 100 mm tissue culture dishes. After reaching more than 50% confluence (2–3 days), the cells were transfected with plasmid DNA by the DEAE-dextran method (15,16). Briefly, the cells were rinsed with 10 ml of TBS (25 mM
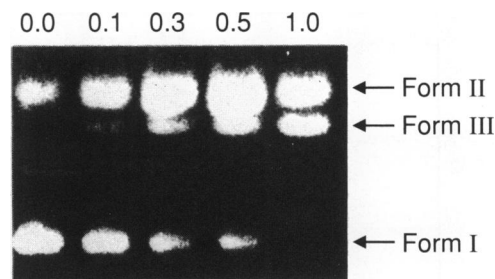

Figure 1. Bleomycin-induced strand-break cleavage of DNA. The plasmid pZ189 was treated with bleomycin and run on agarose gel after removal of the drug. The concentrations of bleomycin (μg/ml) are indicated above each lane. Strand-break cleavage is shown by the presence of form II (nicked plasmid) and form III (linear plasmid) DNA.

Tris–HCl, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and 400 ng of pZ189 (treated or untreated with bleomycin) was added to each plate in a solution of TBS containing DEAE-dextran at a concentration of 400 μg/ml (total volume 570 μl). The plates were then incubated at 37°C for 30 min. The DEAE-dextran was removed by rinsing with TBS and the cells immediately exposed to 10% dimethyl sulfoxide in TBS for 1 min. The cells were then rinsed twice with TBS and grown for 48 h in complete medium to allow plasmid replication.

pZ189 was recovered from WI38VA13 cells by an alkaline lysis method (17). The extracted plasmid DNA were treated with 5 U of *Dpn*I restriction endonuclease (New England Biolabs, Beverly, MA) to digest unreplicated plasmids. The enzyme was removed by ammonium acetate precipitation and DNA was precipitated with ethanol twice and electroporated into *E.coli* MBM7070 using a BioRad Laboratories (Melville, NY) Gene Pulser electoporator with 2.5 kV pulse from a 25 μF capacitor. The Gene Pulser was connected to a 400 ohms resistor in series, generating a time constant of 8.0–9.5 ms. The bacterium were prepared for electroporation as described by Ragsdale and coworkers (18) and typically gave transformation efficiencies up to 5 x 10⁸ transformants/μg of pZ189 DNA. The transformants were plated on LB agar plates containing 100 μg/ml of ampicillin, 40 μg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). Mutations in the *supF* target gene which inactivate the suppressor tRNA function give rise to clear or light blue colonies among mostly wild type blue colonies. Mutant colonies (clear and light blue) were restreaked at least twice for confirmation of the phenotype. The ratio of mutant colonies to total colonies gives the mutation frequency while the total number of colonies reflects plasmid survival. The plasmid DNA from mutant colonies were purified from 10 ml overnight cultures with QIAprep spin columns (QIAGEN; Chatsworth, CA). Sequencing of the suppressor tRNA gene in pZ189 was performed with the pBR322 *Eco*RI forward primer (New England Biolabs; Beverly, MA) using the dideoxynucleotide chain termination method (19).

Mutation frequencies of supercoiled, nicked and linear plasmids

pZ189 treated with bleomycin at 1.0 μg/ml was separated by electrophoresis in a 1% agarose gel to resolve the various plasmid forms (Form I, supercoiled; Form II, nicked; Form III, linear). A
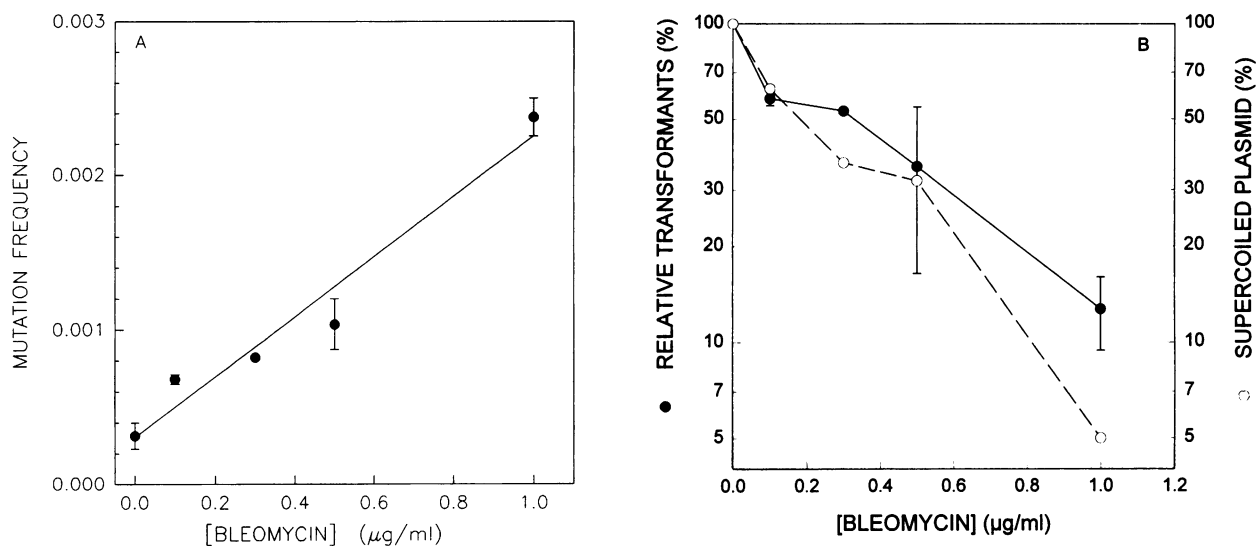
Figure 2. Mutation frequency of bleomycin-induced strand-breaks. (**A**) Mean mutation frequency in the *supF* gene of pZ189 is shown at indicated concentrations of bleomycin. Each value represents data from two or more experiments except at 0.3 μg/ml (one experiment). (**B**) Relative number of bacterial colonies observed after transformation of plasmids harvested from human fibroblasts, WI38VA13, transfected with bleomycin-treated pZ189 (●). Each value represents data from two experiments except at 0.3 μg/ml (one experiment). The percentage of supercoiled (Form I) DNA remaining following bleomycin treatment at indicated concentrations (○). The data is derived from agarose gel in Figure 1.

single lane was excised from the gel and stained with ethidium bromide to visualize the positions of the three bands. The gel slice was realigned with the gel and bands corresponding to super-coiled, nicked and linear plasmids were carefully excised and the DNA extracted from the agarose. The purity of the various forms was verified in a 1% agarose gel. The purified nicked plasmid was typically contaminated with a small amount of linear DNA, presumably due to further conversion during isolation.

2.75 μg of each DNA form was separately transfected into WI38VA13 using the DEAE-dextran method to determine their individual mutation frequencies as described above. The mutants were sequenced as described above.

RESULTS

Bleomycin treatment and evaluation of pZ189 DNA

Earlier work in our laboratory had identified bleomycin treatment conditions, for calf thymus DNA, under which 3′-PG containing strand breaks represented >95% of the dose-dependent lesions produced (13), without any significant induction of AP sites. The 3′-PG lesions produced under these conditions were exclusively linked to guanine nucleotides, consistent with the known 5′-G-Py-3′ sequence preference for DNA cleavage by bleomycin (20,21). We had further quantitated the lesion induction rate to be 110 fmol of 3′-PG/μg bleomycin for each μg of DNA treated. We used these same treatment conditions for the supercoiled pZ189 shuttle vector DNA in the present study.

The treated plasmid was assessed for bleomycin-induced nicking by agarose gel electrophoresis. Bleomycin produced a dose-dependent increase in nicked and linearized plasmids (Fig. 1). Nicked plasmids contain only single-strand breaks whereas linear plasmids have one double-strand break which is thought to arise from induction of two closely opposed single-strand breaks (21,22). The absence of bleomycin-induced AP sites in the DNA was further

confirmed by incubating the bleomycin-treated plasmid with recombinant human apurinic/apyrimidinic endonuclease 1 prior to electrophoresis. The enzyme failed to produce any further nicking (data not shown), confirming that the presence of AP sites in the treated plasmid was negligible. Likewise, hot alkali treatment did not produce any further strand breaks on the plasmid (23).

The quantity of 3′-PG in our treated samples was independently assessed by Dr Michael Weinfeld at the Cross Cancer Institute (Alberta, Canada) by a postlabelling assay (13). At a concentration of 1.0 μg/ml bleomycin, there were 380 fmol 3′-PG end groups/μg pZ189 which translates to 1.5–2 breaks per molecule, a value consistent with our nicking assay results.

Mutation frequency of bleomycin-induced strand breaks

To investigate the mutagenic potential of the bleomycin-treated pZ189 in human cells, we transfected the plasmid into WI38VA13, a SV40-immortalized human fibroblast cell line, recovered the plasmid and quantified mutations in the *supF* reporter gene using a bacterial indicator strain. The plasmid pZ189 has been designed to score for point mutations and small deletions (<250 bases) in the *supF* marker gene. This gene is surrounded by sequences that are essential for replication and, thereby, selects against isolation of large deletions. We chose WI38VA13 cells as host cells, because they are derived from normal human cells, yet they express the SV40 large T antigen, which is required for replication of plasmids with the SV40 origin of replication. Mutations occurring during the processing of damaged plasmid in human cells are fixed on progeny plasmids following replication and were scored on the indicator bacteria. Non-replicated plasmids were eliminated prior to transformation into indicator strain by digestion with *Dpn*I, a restriction enzyme which cleaves only at GATC sequences carrying methylation at adenine residues from growth of plasmid in bacteria. This prevents the mutagenesis from taking place in the indicator strain.
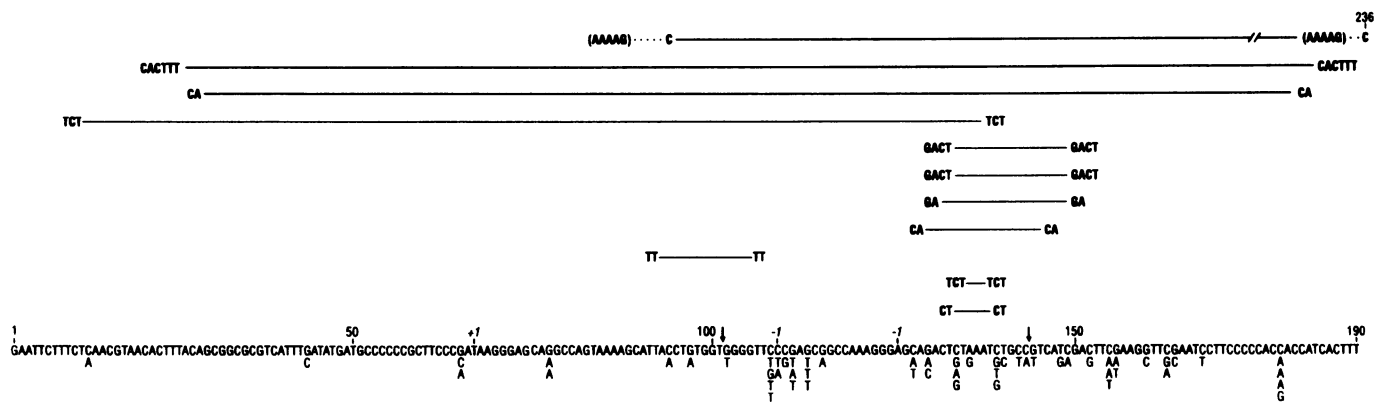
Figure 3. Bleomycin-induced strand-break mutagenesis spectrum in pZ189. The wild type sequence of *supF* gene beginning from the first nucleotide to 190 bases is shown. All base substitution mutations are indicated below the corresponding wild type bases. Mutations found in same base from distinct plasmids are indicated below each other. The deletions are shown above the gene as thickened lines. All deletions contain short direct repeat sequences at the termini, as depicted at start and end of each deletion. Only one copy of the direct repeat is retained in the deletion. The top most deletion extends beyond the indicated sequence to base 236, with recombination occurring just downstream of the direct repeat (AAAG), at the indicated C position. The arrows indicate the sites of large (>500 base pairs) insertions.

Bleomycin treatment produced a dose-dependent increase in mutants (Fig. 2A). Untreated DNA gave a background mutation frequency of 3.1×10^{-4}, which is comparable to backgrounds reported by other investigators for this vector (2,24,25). One microgram/ml of bleomycin, the highest concentration used, produced a mutation frequency of 2.4×10^{-3}, nearly a 10-fold increase above background. This concentration was subsequently used to produce mutants for the mutational spectrum analysis.

Survival of plasmid DNA dropped as a function of drug concentration (Fig. 2B). At a bleomycin concentration of 1 µg/ml, bacterial transformants dropped to 13%, indicating most of the molecules containing strand-breaks (single and double) did not survive. The survival of plasmid was found to be correlated closely with the percentage of remaining supercoiled (Form I) DNA in the transfected mixture (Fig. 2B). The majority of the colonies likely come from the survival of the supercoiled (i.e. undamaged) DNA (26). Only small fraction of plasmids with strand-breaks survive and result in mutations.

Mutagenesis spectrum of bleomycin-induced strand breaks in pZ189

The plasmid mutants generated from drug treatment at 1.0 µg/ml were sequenced to identify the molecular nature of mutations. Identical mutants arising from the same transfected plate were presumed to be siblings and scored only once. The mutational spectrum collected from five independent experiments is shown in Figure 3.

The mutational spectrum contains three classes of mutations; base substitutions, small deletions and insertions. The first class of mutations is represented by base substitution mutations (Table 1). Of the 44 mutants sequenced, 28 (63%) had one or more base substitutions. The proportion of plasmids with multiple point mutations (i.e. two or more base changes within single plasmid) was greater than plasmids with single base substitutions. Multiple point mutations accounted for 57% (16 of 28) of the total base substitutions whereas single base substitution constituted the remaining 43% (12 of 28). The distances between the multiple point mutations was sometimes large. In one mutant plasmid, four base substitutions were found where the distance between the first

and last mutation was separated by 152 bases. The base substitutions were a mixture of transversions (63%) and transitions (37%) and nearly all (96%) were located at G:C base pairs (Fig. 3). Several hot spots were seen within the mature (functional) tRNA region of the gene, which contains a high density of mutation detectable sites.

Table 1. Mutations in the *supF* tRNA gene of pZ189 treated with bleomycin (1.0 µg/ml) and replicated in human fibroblasts, WI38VA13

	Number of mutants
Number of mutants analyzed	44 (100%)
Base substitutions	28 (63%)
Single	12
Multiple	16
Deletions	13 (30%)
Direct sequence repeats	11
Single base	2
Insertions	3 (7%)

Approximately, one-third (30%) of the total mutations were deletions (Table 1). Sequencing data revealed that in nearly all multiple base deletions (10 of 11), a short direct repeat sequence (2–6 bp) occurred precisely at each end point of the deleted region (Fig. 3). This is illustrated in Figure 4 with a deletion at a CACTTT repeat. The wild type *supF* sequence contains two CACTTT sequences spaced 165 bases apart, but only one copy of the sequence is retained in the deletion mutant. This structure of the deletion is consistent with an illegitimate recombination mechanism being involved in strand-break repair (27,28). The illegitimate recombination pathway utilizes short sequence homologies or microhomologies (eg. direct repeats), to generate recombinants (deletions). The deletion endpoints are ambiguous because of direct repeat sequences on each ends. The sizes of the deletions were variable, ranging from six bases to the entire *supF* gene. Two single-base deletions were also seen. Finally, a small
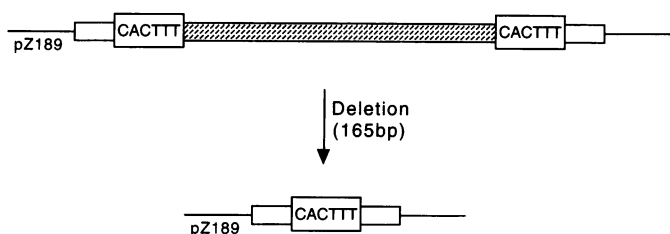
Figure 4. Deletion hot spots contain short direct repeat sequences. An example of an observed deletion is shown using CACTTT sequences. The two CACTTT direct repeats in *supF* (top rectangle) are boxed. The hatched rectangle represents the 165 bases deleted in the mutant below. Only one copy of the CACTTT sequence is retained in the deletion mutant. The exact endpoint of deletion is ambiguous due to direct repeats.

fraction (7%) of insertions (one single-base and two >500 bases) were observed in the spectrum (see Fig. 3).

Mutational analysis of supercoiled, nicked and linear plasmids

Since the bleomycin treatment contained a mixture of super-coiled, nicked and linear plasmids, it was not clear whether single strand-breaks and double-strand breaks were differentially contributing to the resulting spectrum. Some recent studies indicate that mammalian cells process single- and double-strand breaks through different pathways, producing different mutations. Seidman and coworkers (29) have shown that multiple point mutations (base substitutions) are produced with high frequency from enzyme-induced nicks in pZ189. These mutations are thought to result from error-prone DNA polymerase repair synthesis initiating from the site of the nick. This suggested that single-strand breaks, which represented 60% of the bleomycin-damaged plasmid, may have been responsible for the base substitution component seen in our mutational spectrum.

Also, recent studies on repair of restriction-enzyme-induced double-strand breaks in plasmids, using mammalian cells extracts (30), have shown that their ligation is frequently mediated by the illegitimate recombination pathway, resulting in deletions at short direct repeat sequences (31). Since 35% of our transfected mixture contained DNA double-strand breaks, the linear DNA could have specifically resulted in deletions around direct repeats. To test whether single- and double-strand breaks were differentially producing base substitutions and deletions respectively, supercoiled, nicked and linear forms were gel purified from plasmid treated with bleomycin at 1.0 µg/ml. The purified forms were separately transfected into WI38VA13 to determine their mutation frequency and the resulting mutants were sequenced.

Table 2 shows the mutation frequencies of the three forms of DNA. Supercoiled plasmid was weakly mutagenic with mutation frequency 3-fold above background (9.4×10^{-4}). Nicked plasmid, which contains only single-strand breaks, was 10-fold more mutagenic (2.7×10^{-3}) than background. Plasmids with double-strand breaks (i.e. linear) showed the highest mutation frequency (1.0×10^{-2}), nearly 4-fold increased over nicked and >32-fold increased over background. Based on the relative proportions of the three forms in the bleomycin-treated mixture (5% supercoiled, 60% nicked, 35% linear) and their individual mutation

frequencies, a mutation frequency of 5.17×10^{-3} is predicted for the mixture. This is similar to the observed experimental value of 2.4×10^{-3}. In fact, by remixing the supercoiled, nicked and linear plasmids after gel purification in the ratios seen at 1.0 µg/ml of bleomycin (see Fig. 1), a mutation frequency of 2.1×10^{-3} was obtained.

Table 2. Mutation frequencies of different forms of pZ189 isolated from an agarose gel following bleomycin treatment at 1.0 µg/ml[a]

Forms of pZ189	Mutation frequency[b]
Supercoiled circular (Form I)	9.4×10^{-4}
Nicked-circular (Form II)	3.0×10^{-3}
	2.3×10^{-3}
Linear (Form III)	1.1×10^{-2}
	0.9×10^{-2}

[a]Control DNA, without bleomycin treatment, had a mutation frequency of 3.1×10^{-4}.

[b]Values represent independent determinations.

A total of 25 mutants were sequenced from the individual forms to correlate mutations in the spectrum with the type of strand-break damage. The results are shown in Table 3. All types of mutations obtained in the spectrum can be accounted for by the distribution of mutations observed from nicked and linear plasmids separately. Nicked plasmids mostly produce base substitutions (9/10), the majority (6/9) of which are multiples, agreeing with results reported by Seidman and coworkers on single-strand break mutagenesis (29). The single deletion may have arisen from a small amount of linear DNA which was generated during purification of nicked plasmids.

Table 3. Distribution of mutations produced from forms I, II and III after bleomycin treatment and passage through WI38VA13

Sequence alterations	Form of pZ189		
	I	II	III
Base substitutions	5	9	3
Deletions	0	1	5
Insertions	0	0	2
Number of mutants analyzed	5	10	10

Deletions and insertions were the major types of mutations observed from transfection with linear plasmids (Table 4). All deletions (5/10) occurred at short direct repeat sequences indicating repair by illegitimate recombination. The locations and sizes of deletions closely matched those seen in the spectrum (Fig. 3). These data strongly suggest that deletions in the spectrum come from rejoining of linear plasmids, which represented 35% of the transfected mixture. Some of the deletion mutants also contained base substitutions. Insertions (2/10) were only seen with linear plasmids. The insertions seen in the spectrum probably result from the processing of linear plasmids in the transfected mixture. Five mutants arising from supercoiled DNA were sequenced and all contained base substitution mutations.

Table 4. Sizes and sequences of deletions at direct repeats observed in mutant progenies of gel purified bloemycin-treated linear DNA

pZ189 sequence deleted[a]	Direct repeats[b]
9–131	TCT..........................TCT
119–134	AAA..........................AAA
153–160	TTCGAA.........TTCGAA
179–184	CAC..........................CAC
5501–214[c]	TCA..........................TCA

[a]The deletion site is indicated to begin from the first direct repeat nucleotide. Since one copy of direct repeat is retained, the exact site for deletion cannot be unambiguously established.

[b]The direct repeat sequences at the sites of deletions are shown. Only one repeat is retained in the deletions.

[c]The start of the deletion is four bases upstream of the first nucleotide in pZ189.

DISCUSSION

Using the radiomimetic drug, bleomycin, we have looked at the mutagenic potential of strand-breaks under conditions which do not produce any detectable AP sites. Our results demonstrate that strand-breaks are mutagenic lesions. Double strand-breaks are nearly 4-fold more mutagenic (1.0×10^{-2}) than single strand breaks (2.7×10^{-3}). These values are similar to the mutation frequency of $\sim 1.0 \times 10^{-2}$ for AP sites reported by Sarasin and coworkers (32) using a similar shuttle vector containing a single site-specific lesion per molecule. Under our bleomycin-treatment conditions, nicked plasmids contain one to two single-strand break lesions per molecule and linear plasmids represent one double-strand break lesion, indicating that strand-breaks and AP lesions have comparable mutation frequencies in mammalian cells.

The mutagenesis spectrum is an outcome of WI38VA13 cells' ability to use different pathways for repair of single- versus double-strand breaks. Base substitutions are the most predominant mutation in the spectrum. Most of these arise from nicked plasmids, which constitute 60% of the mixture, since transfection with nicked plasmid separately resulted in nearly all (9/10) base substitutions (Table 3). Our results support the previously proposed hypothesis by Seidman and coworkers (29) that the mutagenicity of single-strand breaks arises from error-prone DNA polymerase repair synthesis at nicks or breaks in DNA. According to the proposed mechanism, the probability of introducing base substitutions would solely depend on the fidelity of the DNA polymerase. The involvement of DNA polymerase in the mutagenesis process is further strengthened by our finding that almost all (96%) point mutations occurred at G:C base pairs, which have been shown to be sites for preferential misincorporation by eukaryotic polymerases (33). Previous studies have shown that β-polymerase is the least accurate enzyme in mammalian cells (33) suggesting that it may be involved in strand-break repair (29).

Deletions at direct repeat sequences constituted one-fourth of the bleomycin-induced mutation spectrum and were demonstrated to come from linear plasmids. Short direct repeats (2–6 bp) spaced as far apart as 165 bases in the parent molecule, were able to recombine to eliminate one copy of the repeat and form the deletion. Our data suggest that 50% of mutants (5/10) from linear plasmids arise from such an illegitimate recombination repair pathway. Several groups have recently shown illegitimate recom-

bination to be involved in the repair of a variety of double-strand breaks. Sikpi and coworkers have similarly found ~50% of the deletions in human cells from gamma-irradiated linear pZ189 are at or near direct repeats (26). These types of deletions have also been demonstrated to occur in chromosomal DNA after induction of double-strand breaks *in vivo* with restriction endonucleases (27,34). Taken together, the above data suggest that deletions at direct repeats resulting from illegitimate recombination are 'signature' mutations for DNA double-strand breaks. Deletions at direct repeats have been observed in cellular DNA of mammalian cells following exposure to X-rays (3). This suggests that DNA double-strand breaks are contributing to radiation-induced mutagenicity *in vivo*.

A number of studies with ionizing radiation have implicated double-strand breaks to be toxic lesions in mammalian cells (35,36). This is supported by recent findings that mammalian cell lines sensitive to ionizing radiation contain defects in repair of double-strand breaks (37,38). However, our data demonstrates that ionizing-radiation-induced double-strand breaks, which should have the same end groups as bleomycin-induced double-strand breaks, can be repaired but their repair often leads to mutagenesis. This class of mutations might be important to carcinogenesis. For example, a common mutation found in retinoblastoma (*Rb*) involves a deletion at direct repeats in the *Rb* gene (39).

Thacker and coworkers (30) have proposed a strand-exposure and repair (SER) model to explain the sequence-repeat dependence for joining double strand breaks. According to the SER model, the double-strand break occurs between the direct repeat sequences. Exonucleases can act on the 3'-ends, exposing single strands of DNA at direct repeats. The sequences at the direct repeats are then free to base pair with each other leaving extending tails which must be removed (see Fig. 7 in ref. 30). The recombinant molecule is then filled by polymerase and ligated to produce the deletion mutant with only one copy of the repeat. Although we have not experimentally mapped the 5'-G-Py-3' bleomycin hot spots in the *supF* gene where single- and double-strand breaks occur, all deletions contained several potential bleomycin hot spots within the deletion target.

Povirk and coworkers have recently determined the mutagenic spectrum of bleomycin in pZ189 and the *aprt* gene of chinese hamster ovary D422 cells under conditions which produced ~50% AP sites and 50% strand breaks (25,40). Their observed mutation frequency (3.5×10^{-3}) in pZ189 is similar to our data on strand-break mutagenesis. Our bleomycin conditions predominantly produced strand breaks, without any AP sites and our spectrum is different in several aspects.

First, Povirk and coworker's spectra (pZ189 and the *aprt* gene) contained 1 bp deletions at potential bleomycin hot spots, which the authors correlate with the presence of AP sites at closely opposed strand breaks. These single-base deletions represent 'signature' mutations of their substrate, which contained significant AP damage. In contrast, there were no detectable AP sites on our damaged pZ189 and we did not detect a single mutant plasmid with a one-base deletion at any potential bleomycin hot spot. Also, other investigators have demonstrated that the presence of AP lesions in DNA results in the preferential incorporation of adenine residues during replication (32,41). We did not observe any preferential adenine incorporation in our spectrum, which is consistent with our assessment that the bleomycin-treated substrate did not contain AP sites.

Secondly, base substitutions represented only a minor component of Povirk and coworkers' spectrum in pZ189 and most of their mutants were deletions. However, base substitutions represented the majority of mutations in our spectrum and were shown to arise from nicked plasmids. The base substitutions observed by Povirk and coworker's were proposed to arise from removal of bleomycin-induced AP sites, whereas, base substitutions seen in our spectrum likely came from error-prone DNA polymerase repair synthesis at bleomycin-induced strand break sites. Finally, Povirk and coworkers also observed deletions at short direct repeat sequences in both pZ189 and the *aprt* gene of D422 cells. We have now demonstrated that these may have arisen from repair of double-strand breaks.

Our data demonstrates the mutagenicity of bleomycin-induced single- and double-strand breaks, which mimic ionizing radiation-induced breaks in structure and chemistry. Furthermore, the mutation frequencies of single- and double-strand breaks are similar to AP lesions. Based on the mutation characteristics for these breaks we suggest that deletions at direct repeats may be a 'signature' mutation for double-strand breaks in mammalian cells.

ACKNOWLEDGEMENTS

REFERENCES

1 Sankaranarayanan,K. (1991) *Mutat. Res.*, **258**, 75–97.
2 Waters,L.C., Sikpi,M.O., Preston,R.J., Mitra,S. and Jaberaboansari,A. (1991) *Radiat. Res.*, **127**, 190–201.
3 Morris,T. and Thacker,J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1392–1396.
4 Von Sonntag,C. (1987) *The Chemical Basis of Radiation Biology*. Taylor & Francis, New York, pp. 79–115.
5 Mullenders,L.H.F., Hazekamp-van Dokkum,A.-M., Kalle,W.H.J., Vrieling,H., Zdzienicka,M.Z. and Van Zeeland,A.A. (1993) *Mutat. Res. Genet. Toxicol. Testing*, **299**, 271–276.
6 Henner,W.D., Rodriguez,L.O., Hecht,S.M. and Haseltine,W.A. (1983) *J. Biol. Chem.*, **258**, 711–713.
7 Niwa,O. and Moses,R.E. (1981) *Biochemistry*, **20**, 238–244.
8 Kow,Y.W., Faundez,G., Melamede,R.J. and Wallace,S.S. (1991) *Radiat. Res.*, **126**, 357–366.
9 DiCioccio,R. and Sahai Srivastava,B.I. (1976) *Cancer Res.*, **36**, 1664–1668.
10 Muller,W.E., Maidhof,A., Arendes,J., Geurtsen,W., Zahn,R.K. and Schmidseder,R. (1979) *Cancer Res.*, **39**, 3768–3773.
11 Weinfeld,M. and Soderlind,K.-J.M. (1991) *Biochemistry*, **30**, 1091–1097.
12 Decuyper,J., Piette,J., Merville,M.-P. and Van de Vorst,A. (1986) *Biochem. Pharmacol.*, **35**, 1345–1350.
13 Winters,T.A., Weinfeld,M. and Jorgensen,T.J. (1992) *Nucleic Acids Res.*, **20**, 2573–2580.
14 Keyse,S.M., Amaudruz,F. and Tyrrell,R.M. (1988) *Mol. Cell Biol.*, **8**, 5425–5431.
15 McCutchan,J.H. and Pagano,J.S. (1968) *J. Natl. Cancer Inst.*, **41**, 351–357.
16 Selden,R.F. (1994) In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds.) *Current Protocols in Molecular Biology*. Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. New York, pp. 9.2.1–9.2.6.
17 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18 Dower,W.J., Miller,J.F. and Ragsdale,C.W. (1988) *Nucleic Acids Res.*, **16**, 6127–6147.
19 Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4767–4771.
20 Giloni,L., Takeshita,M., Johnson,F., Iden,C. and Grollman,A.P. (1981) *J. Biol. Chem.*, **256**, 8608–8615.
21 Povirk,L.F., Han,Y.-H. and Steighner,R.J. (1989) *Biochemistry*, **28**, 5808–5814.
22 Steighner,R.J. and Povirk,L.F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8350–8354.
23 Drinkwater,N.R., Miller,E.C. and Miller,J.A. (1980) *Biochemistry*, **19**, 5087–5092.
24 Bredberg,A., Kraemer,K.H. and Seidman,M.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8273–8277.
25 Bennett,R.A.O., Swerdlow,P.S. and Povirk,L.F. (1993) *Biochemistry*, **32**, 3188–3195.
26 Sikpi,M.O., Dry,S.M., Freedman,M.L. and Lurie,A.G. (1992) *Int. J. Radiat. Biol.*, **62**, 555–562.
27 Phillips,J.W. and Morgan,W.F. (1994) *Mol. Cell Biol.*, **14**, 5794–5803.
28 Roth,D.B., Porter,T.N. and Wilson,J.H. (1985) *Mol. Cell. Biol.*, **5**, 2599–2607.
29 Seidman,M.M., Bredberg,A., Seetharam,S. and Kraemer,K.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4944–4948.
30 Ganesh,A., North,P. and Thacker,J. (1993) *Mutat. Res. Genet. Toxicol. Testing*, **299**, 251–259.
31 Roth,D.B. and Wilson,J.H. (1986) *Mol. Cell. Biol.*, **6**, 4295–4304.
32 Gentil,A., Cabral-Neto,J.B., Mariage-Samson,R., Margot,A., Imbach,J.L., Rayner,B. and Sarasin,A. (1992) *J. Mol. Biol.*, **227**, 981–984.
33 Kunkel,T.A. and Sue Alexander,P. (1986) *J. Biol. Chem.*, **261**, 160–166.
34 Rouet,P., Smih,F. and Jasin,M. (1994) *Mol. Cell. Biol.*, **14**, 8096–8106.
35 Whitaker,S.J., Ung,Y.C. and McMillan,T.J. (1995) *Int. J. Radiat. Biol.*, **67**, 7–18.
36 Schwartz,J.L., Mustafi,R., Beckett,M.A., Czyzewski,E.A., Farhangi,E., Grdina,D.J., Rotmensch,J. and Weichselbaum,R.R. (1991) *Int. J. Radiat. Biol.*, **59**, 1341–1352.
37 Kirchgessner,C.U., Patil,C.K., Evans,J.W., Cuomo,C.A., Fried,L.M., Carter,T., Oettinger,M.A. and Martin Brown,J. (1995) *Science*, **267**, 1178–1183.
38 Lees-Miller,S.P., Godbout,R., Chan,D.W., Weinfeld,M., Day,R.S.,III, Barron,G.M. and Allalunis-Turner,J. (1995) *Science*, **267**, 1183–1185.
39 Canning,S. and Dryja,T.P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5044–5048.
40 Povirk,L.F., Bennett,R.A.O., Wang,P., Swerdlow,P.S. and Austin,M.J.F. (1994) *J. Mol. Biol.*, **243**, 216–226.
41 Kunkel,T.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1494–1498.