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Bleomycin-induced DNA lesions at mutational hot spots: Implications for the mechanism of double-strand cleavage

(apurinic/apyrimidinic sites/oxidative mutagens/closely opposed lesions/endonuclease III)

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ABSTRACT **Using various end-labeled, defined-sequence DNA substrates, we examined bleomycin-induced damage at several G·C base pairs which correspond to mutational hot spots. The most frequent lesions detected were single-strand breaks and single apurinic/apyrimidinic (AP) sites at the C residue, suggesting that this was the primary site of damage. Strand breaks and AP sites also occurred, but less frequently, at a secondary damage site—i.e., the directly opposed G residue in the complementary strand. However, damage at the secondary site occurred only when a strand break was present at the primary site, and AP sites at the primary site were never accompanied by closely opposed damage in the complementary strand. Thus, formation of a strand break at the primary damage site was a necessary though not sufficient condition for attack at the secondary site. Similar patterns were seen at other sequences attacked by bleomycin, although primary and secondary sites were sometimes staggered by one nucleotide position rather than directly opposed. These and other results suggest a mechanism of double-strand cleavage in which bleomycin is reactivated during formation of the first strand break, and the reactivated drug subsequently attacks the complementary strand at a specific position which is not normally a site of bleomycin-induced cleavage. Regeneration of activated bleomycin could result from a reaction between Fe(III)·bleomycin and a 4′-peroxyl derivative of deoxyribose, both produced during formation of the strand break.**

The fact that bleomycin produces double-strand breaks with single-hit kinetics suggests that these lesions result from a single interaction between bleomycin and DNA (1, 2). However, the chemistry of activated bleomycin (3, 4) gives no suggestion of bifunctionality, and the mechanism of double-strand cleavage has remained uncertain. We recently showed that most bleomycin-induced double-strand breaks consist of a "primary" site, which follows the normal G-Y (Y =pyrimidine nucleoside) specificity characteristic of bleomycin-induced single-strand cleavage, and a secondary site (in the opposite strand), which is seldom a G-Y sequence and is usually a site where single-strand cleavage is rare (2). In addition to double-strand breaks, bleomycin induces formation of apurinic/apyrimidinic (AP) sites with closely opposed strand breaks, and these bivalent lesions have been implicated in bleomycin-induced mutagenesis in λ phage (5, 6). To clarify the mechanism by which bleomycin effects concomitant damage to both DNA strands, we have examined the detailed structure of bivalent lesions induced by bleomycin at specific sites in defined-sequence DNA substrates, particularly those corresponding to mutational hot spots.

MATERIALS AND METHODS

Materials. Recombinant plasmids were constructed by cloning either a synthetic 25-mer (pcI245, Fig. 1 *Upper*) or the 538-base-pair (bp) *Nsi* I fragment of λ DNA (pcI538) in the *Pst* I site of pUC19. Various restriction fragments were 3′-end-labeled with the Klenow fragment of DNA polymerase and an appropriate deoxynucleoside [α-^{32}P]triphosphate or were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, as described previously (2). Bleomycin A2 was a gift of W. T. Bradner (Bristol Laboratories) and Fe(III)·bleomycin A2 was prepared as described (5). *Escherichia coli* endonuclease III was a gift of R. P. Cunningham (State University of New York, Albany).

Bleomycin–DNA Reactions. Reaction mixtures, usually 0.1 ml, contained calf thymus DNA at 20 μg/ml, a small amount (<5 μg/ml) of a labeled DNA fragment, 1 μM Fe(III)·bleomycin, 40 mM 2-mercaptoethanol, 50 mM Hepes–KOH at pH 8, and 0.1 mM EDTA. After incubation for 30 min at 37°C, samples were passed over CM-25 Sephadex minicolumns (6) to remove bleomycin, split into two or three aliquots, and incubated for 1 hr at 37°C in the presence or absence of putrescine (20 mM) or endonuclease III (8 units/ml), in the same buffer but with 1 mM 2-mercaptoethanol. In some experiments, an aliquot was treated with hydrazine (20 mM) for 1 hr at 22°C (7).

Analysis of DNA Cleavage. Nondenaturing and denaturing gel electrophoresis and elution of fragments from gels have been described (2). Except in experiments employing hydrazine, all eluted fragments were treated with 0.2 M butylamine for 10 min at 90°C to cleave any remaining AP sites and remove any residual sugar moieties from the 3′ termini (7); butylamine was removed by lyophilization. For removal of 3′-phosphates, samples (20 μl) were treated with T4 polynucleotide kinase at 500 units/ml (8) for 4 hr at 37°C in a buffer containing 0.3 M NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 M morpholinoethanesulfonate–NaOH at pH 5.5 and precipitated with ethanol.

RESULTS

To determine single and bivalent lesions induced by bleomycin at the base-pair 245 mutational hot spot (9), fragments of the recombinant plasmid pcI245 (Fig. 1 *Upper*), which were 5′-end-labeled at either the *Ava* I site or at the *Hin*dIII site in the pUC19 polylinker, were prepared and subjected to bleomycin treatment followed by nondenaturing gel electrophoresis (Fig. 2). Bleomycin treatment produced several discrete shorter double-stranded fragments, indicating site-specific double-strand cleavage. Three double-strand cleavage sites were examined in detail, as indicated by the numbered bands in Fig. 2 (band 1 corresponds to the base-pair 245 hot spot). Putrescine posttreatment, which will efficiently cleave bleomycin-induced AP sites (5), increased the intensity of all these bands, suggesting that AP sites (or other putrescine-susceptible lesions) with closely opposed strand breaks were

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Abbreviation: AP, apurinic/apyrimidinic.

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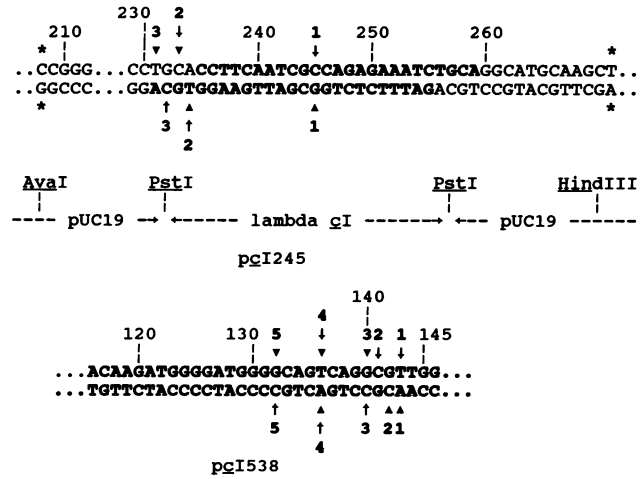

FIG. 1. (*Upper*) Bleomycin-induced damage in plasmid pcI245, which contains the base-pair 245 hot spot. Boldface numbers indicate sites of closely opposed damage in opposite strands and correspond to numbered bands on nondenaturing gels (Fig. 2). At all three numbered sites, both double-strand breaks and putrescine-susceptible sites (presumably AP sites) with closely opposed breaks were formed. For the AP sites with closely opposed breaks, arrows (↑) indicate the nucleotide where the break was formed (the primary site), while triangles (▲) indicate the nucleotide where the AP site occurred (the secondary site). At site 2, either lesion could occur in either strand. Asterisks (*) indicate positions of end-labeling; 5′-end-labeled fragments were used to distinguish direct breaks from cleaved AP sites, while 3′-end-labeled fragments were used to determine the pairing of sites of concurrent damage in top and bottom strands, as described previously (2). The synthetic insert is shown in boldface. Numbering of nucleotide positions is relative to the *c*I gene, even for sequences outside the insert. (*Lower*) Bleomycin-induced damage to bases 115–145 of the *c*I gene in the plasmid pcI538. Symbols are as in *Upper*, with lesions characterized as described for pcI245. Sites 1, 3, 4, and 5 were strong sites of closely opposed damage, while site 2 was a weak site. Sites 1 and 3, base pairs 143 and 140, are mutational hot spots. There was one exception to the usual selection rules for primary and secondary sites in this fragment: The T-G-A at base 136, bottom strand, was sometimes a primary site; a few other T-G-A sequences in other fragments were also weak primary sites. End-labeling was at base 100 (*Bst*NI site) or at base 172 (*Sal* I site in pUC19 polylinker).

formed at the same sequence positions as the direct double-strand breaks. Consistent with this proposal, endonuclease III, a type I AP endonuclease, also increased the intensity of all but a few bands (discussed below). To distinguish which strand contained the putative AP site in these double closely opposed damages, 3′ termini of direct and putrescine-dependent double-strand breaks were examined. Cleavage of AP sites by putrescine leaves a 3′-phosphate terminus which can be converted to a 3′-hydroxyl by the 3′-phosphatase activity of T4 polynucleotide kinase. For short fragments, this 3′-hydroxyl terminus can be distinguished from a 3′-phosphoglycolate terminus (characteristic of a direct strand break) by differential mobilities of the fragments on sequencing gels (8).

When band 1 from the fragment labeled at the *Hin*dIII site and treated with bleomycin alone was eluted and run on a denaturing sequencing gel, a single band was seen corresponding to cleavage at the G at position 245 in the bottom strand (Fig. 3, site 1). The band ran just ahead of the Maxam–Gilbert marker, consistent with a 3′-phosphoglycolate terminus (10, 11). When band 1 was isolated from the sample treated with bleomycin plus putrescine, a similar but somewhat broadened band was seen on the denaturing gel. When this sample was treated with 3′-phosphatase, a second, slower-migrating, less-intense band appeared, suggesting conversion of a 3′-phosphate to a 3′-hydroxyl terminus (Fig.

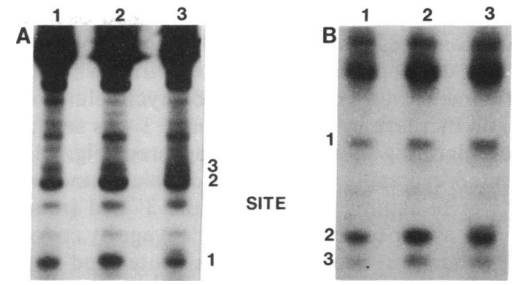

FIG. 2. Direct, putrescine-dependent, and endonuclease III-dependent site-specific double-strand cleavage by bleomycin in 5′-end-labeled fragments of pcI245, as determined by nondenaturing gel electrophoresis. An 80-bp fragment labeled at the *Hin*dIII site at base pair 271 (*A*), or a 159-bp fragment labeled at the *Ava*I site at base pair 208 (*B*) was treated with 1 μM bleomycin, passed through a CM-25 Sephadex column to remove bleomycin, and then given no treatment (lane 1) or treated with putrescine (lane 2) or with endonuclease III (lane 3). Numbered bands correspond to numbered sites of closely opposed damage shown in Fig. 1 *Upper*; since the two labeling sites lie on opposite sides of these cleavage sites, the order of fragments is reversed in *A* and *B*. Full-length DNA appears at the top of the gel in *A* but is not shown in *B*. Putrescine treatment increased the intensity of all bands. Densitometric scans indicated increases in band 1 of 28% ± 6% (SD, $n = 6$) for the fragment labeled at *Hin*dIII and 37% ± 19% ($n = 11$) for that labeled at *Ava* I. Increases in bands 2 and 3 were somewhat greater, approaching 2-fold. Endonuclease III produced increases in band intensities comparable to those seen with putrescine, except for band 1 in *A* and band 3 in *B* (see text). Analysis of eluted bands is shown in Figs. 3 and 4.

3, lane 4). This result suggests that bleomycin induced lesions consisting of an AP site at the G at position 245 plus a closely opposed strand break; treatment with putrescine would convert these lesions to double-strand breaks bearing a 3′-

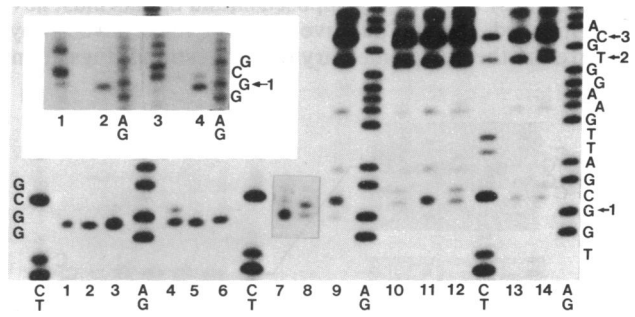

FIG. 3. Sequencing gel analysis of cleavage sites and termini of closely opposed damages in pcI245, with label at the 5′ terminus at the *Hin*dIII site (bottom strand in Fig. 1). Samples in even-numbered lanes were treated with 3′-phosphatase just before sequencing gel analysis. Lanes 1–6 show band 1 eluted from the gel shown in Fig. 2*A*, for samples treated with bleomycin only (lanes 1 and 2), bleomycin plus putrescine (lanes 3 and 4), or bleomycin plus endonuclease III (lanes 5 and 6). Lanes 7 and 8 show the eluted shoulder band from the endonuclease III-treated sample; because bands in lanes 7 and 8 were very faint, a longer exposure of the same gel has been superimposed. Lanes 9–14 show single-strand breaks and single AP sites in the remaining full-length fragment for samples treated with bleomycin only (lanes 9 and 10), bleomycin plus putrescine (lanes 11 and 12), or bleomycin plus endonuclease III (lanes 13 and 14). Numbered cleavage sites correspond to those shown in Fig. 1 *Upper*. (*Inset*) Similar experiment in which the same initial fragment was treated with bleomycin (lanes 1 and 2) or bleomycin plus hydrazine (lanes 3 and 4) and band 1 (lanes 2 and 4) or the remaining full-length fragment (lanes 1 and 3) was isolated from a nondenaturing gel and run on a sequencing gel (without 3′-phosphatase treatment). Splitting of bands into doublets in the hydrazine-treated samples is due to formation of a 3′-pyridazinylmethyl derivative upon cleavage of AP sites by hydrazine. CT and AG lanes are Maxam–Gilbert sequencing markers.

phosphate terminus where this AP site had been cleaved. Two additional results confirmed that the 3′-phosphate terminus was derived from an AP site. First, when the same initial fragment was treated with bleomycin plus endonuclease III, no increase in intensity of band 1 was seen; instead, a slower migrating shoulder band appeared (Fig. 2*A*, lane 3). This band presumably represents a double-strand break with an AP site remaining attached to the 3′ end (retarding its migration), as expected for AP site cleavage by endonuclease III (12). When the shoulder band was eluted and treated with butylamine to remove the cleaved AP site, subsequent treatment with 3′-phosphatase apparently converted most of it to 3′-hydroxyl (Fig. 3, lane 8). Second, when same initial substrate was treated with bleomycin plus hydrazine, material from band 1 generated two bands on the sequencing gel, one consistent with a phosphoglycolate terminus, and a less intense band migrating one nucleotide more slowly, characteristic of the pyridazinylmethyl derivative (7) produced when bleomycin-induced AP sites are cleaved by hydrazine (Fig. 3 *Inset*). In addition to confirming the presence of an AP site, this result suggests that the AP site has the unique 4′-ketone, 1′-aldehyde structure characteristic of bleomycin-induced AP sites (3, 4), since only this type of oxidized AP site can form a pyridazinylmethyl derivative.

In contrast, when a fragment 5′-end-labeled at the *Ava* I site was treated with bleomycin, bleomycin plus putrescine (Fig. 2), or bleomycin plus hydrazine (not shown), material from band 1 gave a single band on nondenaturing gels, either with or without 3′-phosphatase treatment (Fig. 4, site 1), suggesting that it contained only molecules with phosphoglycolate termini. The mobility of this band indicated cleavage at the C at base 245 (top strand in Fig. 1 *Upper*), directly opposite the cleavage in the bottom strand. Since putrescine (as well as endonuclease III) consistently increased the intensity of band 1, putrescine-susceptible lesions (presumably AP sites) with closely opposed strand breaks must have been induced, but the putative AP sites must have always occurred in the complementary (bottom) strand (Figs. 1 and 5).

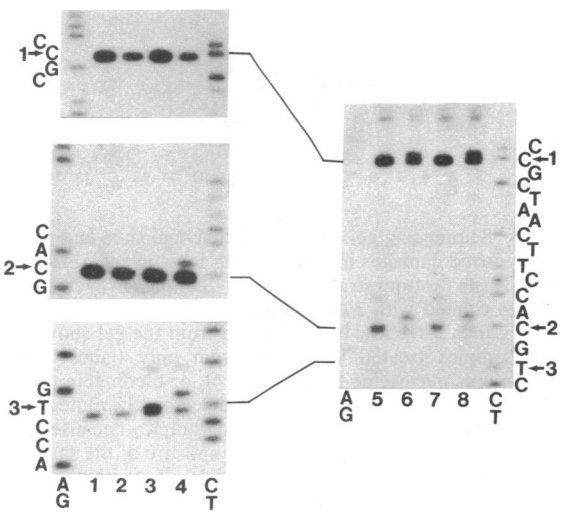

FIG. 4. Sequencing gel analysis of cleavage sites and termini of closely opposed damages in pcI245, with label at the 5′ terminus at the *Ava* I site (top strand in Fig. 1 *Upper*). Samples in even-numbered lanes were treated with 3′-phosphatase. Lanes 1–4 show bands 1, 2, and 3 eluted from the gel shown in Fig. 2*B*, for DNA treated with bleomycin alone (lanes 1 and 2) or bleomycin plus putrescine (lanes 3 and 4). Lanes 5–8 show the remaining full-length fragment for samples treated with bleomycin alone (lanes 5 and 6) or bleomycin plus putrescine (lanes 7 and 8) (lanes 5–8 were intentionally underexposed to show the doublet at site 1). Arrows correspond to cleavage sites indicated in Fig. 1 *Upper*.

DNA molecules containing only single-strand breaks or lone AP sites will still migrate on nondenaturing gels at the position of full-length undamaged DNA. Thus, these single lesions could be specifically analyzed by eluting full-length DNA and assaying for cleavage on sequencing gels. Analysis of the full-length DNA 5′-end-labeled at the *Ava* I site (Fig. 4, lanes 5–8) suggested that the C at base 245 (site 1) was a prominent site for both direct single-strand breaks and lone AP sites, as indicated by the splitting of the band at this position into a doublet with 3′-phosphatase treatment. In contrast, analysis of the full-length DNA 5′-end-labeled at the *Hin*dIII site (Fig. 3) revealed, for the sample treated with bleomycin alone, only a weak band at the G at base pair 245 (lane 9). This band was completely eliminated by 3′-phosphatase treatment (the resulting 3′-hydroxyl fragment would comigrate with the much stronger band at the C at position 244), implying that it consisted exclusively of 3′-phosphate-ended molecules. Thus, no *single-strand* breaks with 3′-phosphoglycolate ends were formed at site 1 in this strand. The small amount of 3′-phosphate-ended molecules probably reflected AP sites with directly opposed breaks, since the band was largely eliminated in the samples treated with putrescine or endonuclease III. Thus, virtually no *single* lesions were induced by bleomycin at position 245 in this strand.

In summary, four types of lesions appear to be produced by bleomycin at base pair 245: single-strand breaks at the C, lone AP sites at the C, direct double-strand breaks involving the C and directly opposed G, and AP sites at the G accompanied by strand breaks at the C (Fig. 5). Thus, at this and other positions (see below) of closely opposed damage, the primary (G-Y) site incurred extensive *single-strand* damage as well, while the secondary (non-G-Y) site incurred damage only in the context of closely opposed damage to the primary site.

For example, a similar distribution of lesions was seen at site 3, base pairs 231–232 (see Fig. 1 *Upper*). Analysis of band 3 derived from DNA 5′-end-labeled at the *Ava* I site suggested both phosphate and phosphoglycolate 3′ termini in the putrescine-treated sample (Fig. 4), implying the presence of AP sites at the T at base pair 231 (top strand), accompanied by closely opposed breaks. Experiments with DNA 5′-end-labeled at the *Hin*dIII site suggested that putrescine-dependent double-strand breaks at site 3 had exclusively phosphoglycolate ends in the bottom strand (not shown). The C residue in the bottom strand was a major site for single lesions (Fig. 3), while damage at the T residue in the top strand occurred almost exclusively in the context of double closely opposed lesions (Fig. 4). Thus, the C behaved as a primary site (as expected from its G-Y context), while the T behaved as a secondary site.

Site 2, base pairs 233–234, is a special case in that both the C at base pair 233 (top strand) and the T at base pair 234 (bottom strand) are potential primary sites in G-Y sequences, and analysis of full-length fragments confirmed that single lesions occurred relatively frequently at both these nucleotides (Figs. 3 and 4). Analysis of band 2 from nondenaturing gels suggested that, for AP sites with closely opposed strand breaks occurring at site 2, the AP site was sometimes in the top strand (Fig. 4) and sometimes in the bottom strand (not shown). This result suggests that both nucleotides could serve either as a primary site or as a secondary site.

Several additional sites of closely opposed damage were examined in a 30-bp region of the plasmid pcI538, spanning base pairs 115–145 of the λ cI gene (Fig. 1 *Lower*). Base pairs 140 and 143, which are mutational hot spots (9), were both prominent sites of directly opposed damage. At the C-G-C-C sequence (Fig. 1, site 4, bottom strand) at base 140, the same four types of lesions were detected as were found at the C-G-C-C at base 245 (see Fig. 5). Analogous types of lesions were seen at the C-G-T-T sequence at base 143 (Fig. 1, site

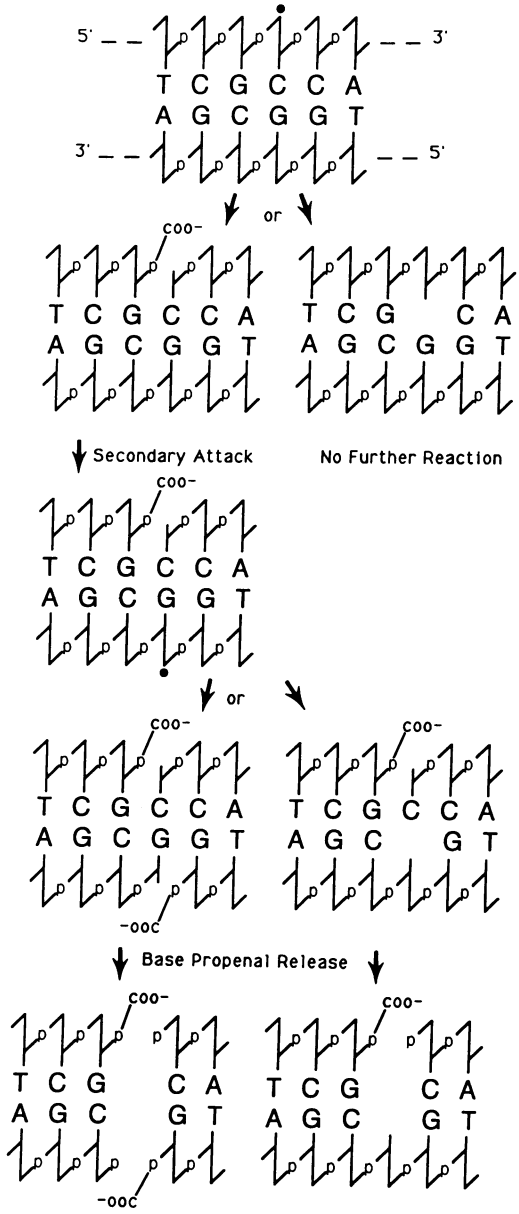

FIG. 5. Proposed mechanism for production of closely opposed damages by bleomycin. Activated bleomycin first abstracts hydrogen from C-4′ at the primary site (top strand). The C-4′ radical partitions into either an AP site or a strand break. If an AP site is formed, no further reaction occurs. If a strand break is formed, then, a significant fraction of the time, a secondary attack by bleomycin abstracts hydrogen from the directly opposed nucleotide in the complementary strand. This C-4′ radical likewise partitions into either a strand break or an AP site, and base propenal release produces either a double-strand break with 5′-phosphate and 3′-phosphoglycolate termini or a strand break at the primary site accompanied by an AP site at the secondary site. If the break occurs at the primary site but there is no secondary attack, the base propenal is likewise lost to leave a single-strand break with a 3′-phosphoglycolate terminus (not shown). The proposal that base propenal release occurs after single-strand or double-strand cleavage is based on kinetic studies (13). The fact that there are not closely opposed damages involving an AP site at the primary site (top strand) suggests that bleomycin can be reactivated during formation of a strand break but not during formation of an AP site.

3, top strand) and at the T-G-C-C at base 132 (Fig. 1, site 1, bottom strand), with primary-type damage occurring at the (underlined) pyrimidine and secondary-type damage at the directly opposed purine. One additional C-G-C-C hot spot, occurring at base pair 188 of *c*I, was examined in another recombinant plasmid, with results identical to those obtained for base pairs 140 and 245. Characterization of these and other positions of closely opposed damage in all three plasmids confirmed that, with few exceptions, the same four types of lesions were produced: single-strand breaks at the primary site, lone AP sites at the primary site, double-strand breaks involving the primary and secondary sites, and AP sites at the secondary site accompanied by strand breaks at the primary site (Fig. 5). Most importantly, for AP sites with closely opposed breaks, the putative AP site invariably occurred at the secondary site. Thus, the model of double-strand cleavage developed below, based on lesions seen at C-G-C-C sequences, should apply generally to nearly all bleomycin-induced double-strand breaks. As described previously (2), the secondary site is virtually always either directly opposite the primary site or opposite the base one position downstream; the choice between these two positions follows a hierarchy of sequence-dependent selection rules.

Table 1. Bleomycin-induced mutations and classification of mutable sites in the *c*I gene

Classification of sites	Sites available	Sites hit	Mutants	Mutants per site
Primary and secondary				
Directly opposed*	15	4	17	1.13
Staggered†	6	1	1	0.16
Primary only‡	13	4	5	0.38
Secondary only§	15	3	5	0.33
Neither	71	0	0	(0.00)

*Analogous to base pair 245, site 1 in pcI245 (see Fig. 1 *Upper*).
†Analogous to base pair 234, site 2 in pcI245.
‡Analogous to base pair 232, site 3 in pcI245.
§Analogous to base pair 231, site 3 in pcI245.

Table 1 classifies all base pairs in positions 1–250 of *c*I at which base substitutions have been shown to produce a clear-plaque phenotype (14), in terms of whether they would be expected to be primary or secondary sites of bleomycin-induced damage, according to the sequence selection rules discussed above and in ref. 2. Most mutations occurred at sequence positions where AP sites and strand breaks would be directly opposed, a classification which includes the hot spots at base pairs 140, 143, 188, and 245 (9). Some of the mutations occurred at positions which could only be secondary sites, including all mutations occurring at non-G-Y sequences. Some mutations also occurred at G-Y sequences which could only be primary sites. Remarkably, not a single mutation was detected at any of the remaining 71 positions in *c*I which would not be potential primary or secondary sites of bleomycin attack.

DISCUSSION

Mechanism of Double-Strand Cleavage. Analysis of the lesions induced at primary and secondary cleavage sites comprising bleomycin-induced double-strand breaks (Fig. 5) reveals several important features. First, the chemistry of the lesions at both sites is highly characteristic of specific attack by activated bleomycin at C-4′. Second, presence of a *strand break* at the primary site is a necessary condition for attack at the secondary site. Third, the secondary site exhibits an altered specificity, virtually always occurring at one of two nucleotide positions opposite the primary break, and usually violating the characteristic G-Y specificity of the drug. All these results can be explained by hypothesizing that (*i*) attack at the primary site occurs first and (*ii*) attack at the secondary site, and its altered specificity, are consequences of the formation of a strand break at the primary site. The simplest model incorporating these features is one in which bleomycin is sometimes reactivated during formation of the strand break

at the primary site, allowing the drug to attack the secondary site in the complementary strand without ever dissociating from DNA. In addition to explaining the types of lesions induced at primary and secondary sites, both separately and in concert (Fig. 5), this model accounts for the single-hit kinetics of double-strand cleavage (1), since only one binding event between DNA and bleomycin is required.

A precise mechanism for bleomycin reactivation is difficult to propose, since it is necessarily dependent on the uncertain events (3, 4) occurring between formation of "activated bleomycin" (bleomycin ferric peroxide) and abstraction from C-4'. In particular, the actual abstracting species is unknown, although high-valence bleomycin–iron–oxo species (4) and species formally equivalent to a sequestered hydroxyl radical (15) have been proposed.

One possible reactivation mechanism is an addition reaction between Fe(III)·bleomycin, which is the final bleomycin product formed after strand cleavage, and a 4'-peroxide derivative of deoxyribose, which is a nearly obligatory intermediate in the strand cleavage reaction (16). It has been demonstrated that organic peroxides can combine with and reactivate Fe(III)·bleomycin (15). The one uncertainty in this mechanism is whether the putative deoxyribose–peroxo–iron–bleomycin conjugate would still decay to form the observed 3'-phosphoglycolate terminus at the primary site. Products formed from organic peroxides used to activate bleomycin have been identified (15), but it is difficult to extrapolate these results to the rather different structure of the putative peroxide intermediate at C-4'.

An alternative reactivation mechanism is a reaction between the putative 4'-peroxyl radical intermediate at the primary site (16) and the immediate bleomycin–iron–oxo product of the primary abstraction, that is, a simple recapture of the abstracted hydrogen by the peroxyl radical, directly regenerating the abstracting bleomycin species. This mechanism would be analogous to the "propagation" step in lipid peroxidation (17), but like the first mechanism, its likelihood depends on the exact nature of the bleomycin species present immediately before and after abstraction. It should be noted that both of the proposed models require the formation of 4-peroxyl intermediates, which are generated only when a strand break is produced; thus, either model could explain the apparent failure of bleomycin to be reactivated when AP sites are formed at the primary site.

Recently, Keller and Oppenheimer (18) synthesized short DNA duplexes containing a one-base gap with 3'- and 5'-phosphate termini (a structure very similar to a bleomycin-induced strand break) and showed that bleomycin preferentially attacked sites opposite the gap. In general terms, these results agree with our proposal that the presence of a break at the primary site restricts the placement of the secondary break to a few closely opposed nucleotide positions. However, the detailed specificity of secondary cleavage seen with these synthetic substrates is not always consistent with that seen with bleomycin alone (e.g., breaks with 3'-overhangs are sometimes seen with the synthetic substrates). Furthermore, kinetic studies (13) show that bleomycin-induced double-strand cleavage occurs much faster than base-propenal release, implying that secondary attack must occur before the gap and the 5'-phosphate terminus are formed. Thus, the synthetic substrates appear to be useful though imperfect models for intermediates in double-strand cleavage.

Role of Bivalent Lesions in Mutagenesis. Studies with repackaged λ phage suggest that although AP sites with closely opposed strand breaks constitute a minor component of bleomycin-induced DNA lesions, they may play a major role in mutagenesis (6). This proposal is confirmed by sequence specificity data (Table 1), which reveal that some bleomycin-induced base substitutions occur at sequence positions which can only be secondary sites of bleomycin attack. Furthermore, at sequence positions where potential primary and secondary sites are directly opposed, most (11/15) of the substitutions were transversions (9), suggesting that they resulted from bypass of an AP site at the purine (secondary site) rather than the pyrimidine (primary site) at these sequence positions. Since AP sites at these purines occur only in the context of a directly opposed strand break, this result suggests that such bivalent lesions rather than lone AP sites were responsible for most of these mutations. It appears that directly opposed lesions may be more mutagenic than staggered lesions.

Bivalent Lesions and Cytotoxicity. Although the evidence is not conclusive, double-strand breaks are believed to be the primary cause of bleomycin-induced cytotoxicity (1). The detailed mechanisms for repair of double-strand breaks are not known, but they could depend critically on the structure of the termini. The cleaved bleomycin-induced AP site represents a unique terminus which preliminary studies suggest is relatively resistant to enzymes that normally remove 3' blocks (e.g., *E. coli* endonuclease IV and exonuclease III). Thus, double-strand breaks with this terminus may be particularly difficult to repair and therefore highly cytotoxic.

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