Site specificity of bleomycin cleavage in purified and intracellular simian virus 40 DNA

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ABSTRACT

The sites of bleomycin-induced cleavage of purified and intracellular simian virus 40 DNA were examined. Breaks in purified DNA were mapped to several discrete sites that were distributed throughout the viral genome, but were not associated with a common genetic element. Double-stranded breaks were made in positions of the first single-stranded nick, and regions of cuts were unaffected by changes in DNA superhelicity. Bleomycin cut intracellular chromosomes at the same sites that were cleaved in purified DNA. These results indicate that SV40 DNA contains DNA secondary structures that are highly preferred sites for BLM cleavage. These conformations appear to be unaffected by nucleoproteins bound to DNA.

INTRODUCTION

Bleomycin (BLM) is an antitumor antibiotic used alone and in combination with other agents in the treatment of several human cancers (1). The cytotoxicity of BLM is related to its ability to cut cellular DNA (2). BLM also introduces breaks into cell-free DNA, although ferrous ions must be added to the reaction for maximal cleavage activity (2-4). Extensive degradation of purified DNA results in the release of all four bases (5). Analysis of nucleotide sequence preference using DNA from diverse sources has revealed that BLM breaks DNA most frequently at 5'-GC-3' and 5'-GT-3', whereas several other dinucleotides are cleaved to a lesser extent (6-8). Not all of the preferred dinucleotides in a defined sequence of DNA are cut with equal frequency (9,10), and the strongest preference of the drug appears to be for alternating purine-pyrimidine sequences (10).

Although it has been suggested that perturbations in the double helix help determine BLM sequence specificity (10-12), the physical nature of DNA secondary structures recognized by BLM remains poorly understood. Furthermore, it is not yet clear whether BLM preferentially cuts in specific genetic elements. An approach which has been successful in determining the DNA conformations cut by micrococcal nuclease (13) and mung bean nuclease (14) has been to

identify highly preferred locations for enzymatic cleavage prior to examining the specific nucleotides cut in those regions. In contrast, most analyses on BLM nucleotide preference have been done without regard for the genomic location or the sensitivity of the examined regions to BLM relative to the entire DNA molecule. Studies using PM2 DNA have demonstrated that BLM does cut some regions of DNA more preferentially than others (11,12).

In addition to the influence of DNA secondary structure in directing BLM cleavage, drug action toward chromatin is subject to the effects of nucleoproteins. BLM cuts chromatin predominantly in the linker regions between nucleosomes (15) and has been shown to cut purified SV40 DNA more extensively than SV40 minichromosomes (16). In non-transcribed chromatin, the nucleosomes bound to DNA do not appear to perturb DNA structure in a way that alters BLM sequence preference (17). Chromatin associated with actively transcribed genes has been shown to be more sensitive to BLM than non-transcribed chromatin (18), although the molecular mechanism for this increased sensitivity is not known.

In this paper, we have examined whether DNA secondary structures exist at the sites of BLM-DNA interactions by mapping the locations of BLM-induced double- and single-stranded breaks in purified SV40 DNA and chromatin. SV40 has been employed as a model system for these studies because the circular genome of this DNA tumor virus is well characterized, and the locations of the five viral genes, origin of replication, and viral regulatory sequences are known (19,20). The structure of viral chromatin has been probed with a variety of nucleases, revealing a DNase I hypersensitive and nucleosome-free region adjacent to the origin of replication and encompassing the viral regulatory sequences (21-23). We have found that BLM cuts purified SV40 DNA with strong preference for several specific sites. The data suggest that in addition to the known dinucleotide specificity of BLM, long stretches of DNA contain structures which appear to be highly preferred sites for BLM-DNA interaction. Surprisingly, the cleavage sites in minichromosomes were strikingly similar to those mapped in purified DNA.

MATERIALS AND METHODS

Drugs and Reagents

Bleomycin (as the clinical mixture Blenoxane) was supplied by Bristol-Myers Company. Materials for cell culture were purchased from Grand Island Biological Company. Restriction enzymes were purchased from Boehringer

Mannheim. Agarose, ferrous ammonium sulfate, dithiothreitol, RNase, and proteinase K were purchased from Sigma Chemicals.

Cells and Virus

SV40 (strain 776) was grown in the BSC-1 line of African Green Monkey kidney cells. Cells were infected with 5-15 pfu of SV40 virus per cell and maintained in Minimal Essential Media supplemented with 2% fetal bovine serum and 1.2 mM glutamine for either 24 hr or 40 hr. Cells were labeled with 5 μ Ci/ml of 50 mCi/mmol 14 C-thymidine (Moravek) either immediately or 24 hr after infection.

Isolation of SV40 DNA

SV40 DNA was isolated 40 hr after infection by the method of Hirt (24). The Hirt supernatant was further purified by RNase treatment (10 μ g/ml for 1 hr at 37°C) and phenol extraction. The DNA was precipitated with ethanol, and supercoiled DNA was isolated on CsCl/ethidium bromide gradients.

Drug Treatment

BLM treatment of either supercoiled or full-length linear purified SV40 DNA was done in a reaction mixture containing 1 μ g of DNA/10 mM Tris-HCl, pH 8.0/5 mM CaCl₂/2 mM dithiothreitol/1 nM ferrous ammonium sulfate at 37°C for 2 min. Reactions were stopped by adding EDTA to 20 mM and cooling in ice-water. Drug-treated DNA was precipitated with ethanol.

For treatment of intracellular minichromosomes, BSC-1 cells were grown in 35 mm tissue culture dishes and infected as described above. BLM treatments were performed either 24 hr or 40 hr after infection in 1 ml of media for 30 min at 37°C. The media was then aspirated, the cells were rinsed and then lysed with 0.5 ml of 10 mM Tris-HCl, pH 8.0/1% SDS/20 mM EDTA. BLM activity is inhibited by these lysis conditions, and drug-induced breakage could not be detected in cells treated with 20 μ M/ml of BLM and lysed immediately. Lysates were treated with 50 μ g/ml of RNase for 2 hr at 37°C, and then with 500 μ g/ml of proteinase K at 60°C for 4 hr. Following extraction with phenol and chloroform, the DNA was precipitated with ethanol. All DNA pellets were resuspended in 10 mM Tris-HCl, pH 7.2

To measure the amount of drug-induced strand scission, aliquots of treated samples were electrophoresed through 1% agarose gels run at 30V for 18 hr in 50 mM Tris-HCl, pH 8.3/20 mM sodium acetate/2 mM EDTA as the running buffer. The gels were stained in 0.5 μ g/ml of ethidium bromide, and illuminated with 300 nm ultraviolet light. Quantitation of DNA in different topological forms was done as described elsewhere (16).

Mapping of Double-stranded Cleavage Sites

Drug-treated DNA was digested with either Eco RI, Bam HI, or Bgl I according to manufacturer's instructions. These enzymes cut at a single location in the SV40 genome, and linearize intact and nicked SV40 DNA, while full length linear SV40 DNA is cut into two fragments. The DNA fragments were separated by electrophoresis on 1.5% agarose gels run in 50 mM Tris-HCl, pH 8.3/20 mM sodium acetate/2 mM EDTA at 30V for 20 hr. The DNA was transferred to nitrocellulose filters (25) and annealed to nick-translated plasmids containing one of the following fragments of SV40 DNA: the 751 bp Eco RI/Bam HI fragment (pJL1) or the 497 bp Bgl I/Taq I fragment (pKD1). pJL1 was hybridized to DNA cut with Eco RI or Bam HI, and pKD1 was annealed to DNA cut with Bgl I. Hybridizations were done in 4X SSC/2X Denhardt's solution/0.1% SDS/0.1% sodium pyrophosphate/1.5 mg of sheared denatured salmon sperm DNA for 18 hr at 65°C. (1X SSC is 0.15 M NaCl/0.015 M sodium citrates and 1X Denhardt's is 0.02% Ficoll/0.02% polyvinyl pyrrolidine/0.02% bovine serum albumin.) The filters were then air dried and exposed to Kodak XAR-5 film. The size of the fragments revealed on the autoradiograms corresponded to the number of bp from the unique restriction site at which BLM made a double-stranded break.

Mapping of Single-stranded Cleavage Sites

Single-stranded breaks were mapped by a modification of the method described for locating double-stranded breaks. Following restriction enzyme digestion, the treated DNA was precipitated with ethanol, resuspended in 5 μ l of 10 mM Tris-HCl, pH 7.2, and incubated with 20 μ l of 30 μ M NaHPO₄/0.36% SDS/1 M deionized glyoxyl/50% DMSO at 50°C for 1 hr to denature the DNA. The DNA was then electrophoresed through 1.5% agarose gels at 30V for 20 hr using 10 mM sodium phosphate, pH 6.0 as the running buffer. DNA transfer and hybridizations were done as described above.

RESULTS

Specificity of BLM-induced double-stranded breaks in purified SV40 DNA

Purified SV40 DNA was treated with concentrations of BLM that converted from 10% to 80% of supercoiled molecules to nicked circular and full-length linear forms. In order to determine whether the first BLM-induced double-stranded break was located in a specific site, the drug-treated DNA was digested with Eco RI, which cut the full-length linear molecules into 2 fragments and linearized the uncut and nicked circular forms. The resulting fragments were separated by agarose gel electrophoresis. DNA was transferred to nitrocellulose filters and hybridized to ³²P-labeled fragments of SV40, as

described in Material and Methods (see Fig. 1). As shown in Fig. 1a, Eco RI digestion of untreated SV40 DNA formed only the 5243 bp linear SV40 molecule (Fig. 1a, lane 1). However, treatment of SV40 DNA with BLM prior to Eco RI digestion caused specific sub-bands to appear below full-length SV40. Multiple sub-bands were detected at even the lowest drug dose, which converted less than 5% of SV40 DNA molecules to the linear form (Fig. 1a, lane 2). These results indicate that the first double-stranded break made by BLM can be located at several preferred sites. Increasing the BLM concentration caused greater linearization of SV40 molecules and resulted in an overall enhancement of sub-band intensity (Fig. 1a, lanes 3 and 4). Some sites appear to be cut more frequently than others, as shown by the varying intensity of the sub-bands made at any of the BLM doses used.

For a more definitive analysis of BLM-induced breakage throughout the SV40 genome, drug-treated SV40 DNA was digested with one of three restriction enzymes: namely Eco RI, Bam HI, or Bgl I. Digestion with each of the three restriction enzymes revealed multiple distinct sub-bands, and demonstrated that preferred sites for the first BLM-induced double-stranded break are distributed over the entire SV40 genome (Fig. 1b). The locations of these cleavage sites were mapped as described in Materials and Methods and are shown in Fig. 1c. BLM did not recognize only one type of genetic element. For example, two sites in the viral regulatory region (nucleotides 120 and 280), a site in the T-antigen intron (nucleotide 4830), a site at the 3'-end of the T-antigen (nucleotide 2660) and multiple sites in the coding regions of the viral genes were cut. (The locations of all cleavages are accurate within $\pm$ 50 bp.)

Specificity of BLM-induced single-stranded breaks in purified SV40 DNA

BLM-induced double-stranded breaks are thought to result predominantly from two opposing single-stranded nicks (2), and therefore reflect multiple cleavage events. To examine the site of the first BLM-DNA interaction, purified SV40 DNA was cut with doses of BLM which resulted in an average of one or fewer single-stranded breaks per molecule. The drug-treated DNA was digested with Bam HI, denatured by glyoxalization, and analyzed for sub-band formation as described in Materials and Methods. As shown in Fig. 2a, distinct sub-bands were seen in lanes containing BLM-treated DNA (Fig. 2a, lanes 2 and 3), whereas only the full-length linear band was seen after Bam HI digestion of untreated SV40 DNA (Fig. 2a, lane 1). Like double-stranded breaks, BLM produces single-stranded breakage at specific sites in the SV40 genome. The intensity of the sub-bands increased as higher doses of BLM con-

Figure 1. Sites of BLM-induced double-stranded breaks in purified SV40 DNA. Supercoiled SV40 DNA was treated with BLM, resulting in the formation of nicked circular and full-length linear DNA. The treated DNA was then digested with a restriction enzyme, electrophoresed through agarose gels, blotted to nitrocellulose filters, and hybridized to a radioactively-labeled plasmid containing a fragment of the SV40 genome, which revealed only one band from each sub-band pair. (A) Eco RI was used to digest SV40 DNA that had been treated with BLM. The drug concentrations and percentages of full-length linear

molecules formed were as follows: lane 1) no drug, 0% linear; lane 2) 2.5 ng/ml, 5% linear; lane 3) 5 ng/ml, 10% linear; lane 4) 10 ng/ml, 13% linear; lane 5) untreated SV40 DNA cut with several restriction enzymes to give fragments of known size. (B) Eco RI (RI), Bam HI (Bam) or Bgl I were used to digest SV40 DNA treated with 5 ng/ml of BLM. Lane C contains SV40 DNA incubated without BLM and digested with Eco RI. In both (A) and (B), * designates the general location of the viral regulatory region. (C) Genomic map of SV40 with sites of BLM cleavage indicated by triangles. The sites are accurate to ± 50 bp. T and t represent the large and small T-antigens, respectively, VP1, VP2, and VP3 are the viral capsid proteins.

verted a greater percentage of supercoiled molecules to nicked circles.

To examine if the first single-stranded cut made by BLM determines the site of double-stranded breakage, the sub-bands made from single- and double-stranded cleavage were compared. These results, shown in Fig. 2b, reveal that the preferred sites for the first double-stranded break made by BLM mirror the locations of the first drug-DNA interaction.

Role of supercoiling in determining the locations of BLM-induced cleavage in SV40 DNA

Negative supercoiling has been shown to cause the formation of several types of altered DNA secondary structures (26). Since the nucleotide sequence preference of BLM has been reported to be affected by changes in DNA superhelicity (27), some of the sites in SV40 DNA cut by BLM could reflect regions in which torsional stress had caused helical perturbations. To examine this possibility, BLM was used to make an average of one or fewer single-stranded nicks in either supercoiled SV40 DNA or SV40 DNA that had been linearized with Bam HI. Following Bam HI digestion of the treated supercoiled DNA, the formation of sub-bands was analyzed under denaturing conditions as described in Materials and Methods. The similarity between the sub-band patterns (Fig. 3) indicate that BLM cuts supercoiled and relaxed DNA at the same sites.

Comparison of BLM-induced breaks in intracellular SV40 minichromosomes with cleavage in purified DNA

BLM-induced cutting of chromatin differs from purified DNA in that nucleoproteins protect DNA from drug attack and concentrate breaks in linker regions between nucleosomes (15,16). Furthermore, transcriptionally active and inactive chromatin have different sensitivities to BLM, with active genes being more susceptible to drug attack (18). To determine if organizing DNA into chromatin could alter BLM site preference, cleavage sites in intracellular SV40 DNA were examined. Cells were treated with drug for 30 min at two times after infection: 24 hrs, when most of the viral chromatin was not encapsidated (Fig. 4a, lanes 2 and 3), and 40 hrs, after encapsidation had begun and

Figure 2. Site specificity of BLM-induced single-stranded breaks in purified SV40 DNA. Supercoiled SV40 DNA was treated with BLM to convert a small percentage (less than 15%) of supercoiled molecules to nicked circles, resulting in an average of less than 1 break per strand. The DNA was then digested with Bam HI, denatured with glyoxal, and processed as described in the legend to Fig. 1. (A) The BLM concentrations were as follows: lane 1) no drug; lane 2) 0.5 ng/ml; lane 3) 1 ng/ml. The location of the viral regulatory region is shown (*). (B) Direct comparison of the site specificity of BLM-induced single-stranded (lane 2) and double-stranded (lane 3) breakage. Lanes 1 and 4 contain DNA incubated without drug. A partial map of the SV40 genome is shown to indicate the location of breaks. The early (T-antigens) and late (capsid proteins) gene sequences are labeled, and the solid region of the map corresponds to the 72 bp repeat sequences.

the majority of the minichromosomes were inactive (19) (Fig. 4a, lane 4). No cleavage of SV40 DNA was detected in cells treated with BLM and lysed immediately, indicating that breakage was induced in intact cells (data not shown). BLM-treatment of minichromosomes at both times produced a similar pattern, composed of multiple distinct sub-bands. Thus, BLM site specificity did not appear to be affected by encapsidation. Single-stranded breaks made by BLM in intracellular minichromosomes were also examined, and, as was observed with purified DNA, were located at the sites of double-stranded breaks (Fig. 4b).

Figure 3. Comparison of the cleavage pattern produced by BLM-treatment of supercoiled and linear SV40 DNA. BLM (1 ng/ml) was used to treat 1 μ g of either supercoiled SV40 DNA (lane 2) or Bam HI-linearized SV40 DNA (lane 3). This drug concentration caused no more than 1 nick in 15% to 20% of the molecules. Following drug treatment, the supercoiled DNA was digested with Bam HI, and all samples were denatured with glyoxal and processed as described in the legend to Fig. 1. Lane 1 and 4 contain supercoiled or linearized SV40 DNA, respectively, incubated without drug. The location of the viral regulatory region is shown (*).

In Fig. 5, the cleavage sites in intracellular minichromosomes (lane 2) and purified SV40 DNA (lane 3) are directly compared. Even though intracellular minichromosomes are associated with proteins, the sites cut in intracellular minichromosomes and purified DNA are strikingly similar.

Figure 4. Specificity of BLM-induced single- and double-stranded breaks in intracellular SV40 minichromosomes. (A) Double-stranded breakage was examined by treating SV40-infected cells with BLM either 24 hr (lanes 1-3) or 40 hr (lane 4) after infection. Cellular and viral DNA were isolated from the cells, digested with Bam HI, and processed as described in the legend to Fig. 1. The concentrations of BLM that were used are as follows: lane 1) no drug; lane 2) 20 $\mu\text{g/ml}$; lanes 3 and 4) 100 $\mu\text{g/ml}$. (B) Single-stranded breakage was examined by treating SV40 infected cells with 20 $\mu\text{g/ml}$ of BLM (lane 2) 40 hr after infection. DNA was isolated from the cells, digested with Bam HI, denatured with glyoxyl, and processed as described in the legend to Fig. 1. Lane 1 contains Bam HI digested DNA from untreated cells. The location of the viral regulatory region is shown (*).

DISCUSSION

Studies of BLM sequence preference have consistently demonstrated that all 5'-GC-3' and 5'-GT-3' in a DNA segment are susceptible to BLM although they are not cut with equal frequencies (9,10). The drug has been reported to attack the preferred dinucleotides most frequently when they are located within a sequence of four or more alternating purine-pyrimidines (10). Discrete positions of BLM double-stranded cleavage have also been observed surrounding the Hpa II restriction site in PM2 DNA (11,12). However, breakage in PM2 DNA was examined using ethidium bromide-stained agarose gels, which would limit the detection of DNA fragments generated to those of higher

Figure 5. Comparison of BLM site specificity in purified SV40 DNA and intracellular minichromosomes. BLM-treated intracellular minichromosomes (lane 2) or purified SV40 DNA (lane 3) were digested with Bam HI and processed as described in the legend to Fig. 1. Lane 1 and 4 contain untreated intracellular minichromosomes and purified DNA, respectively. A partial map of the SV40 genome is shown to indicate the locations of breaks. The early and late genes are labeled, and the solid region marks the location of the 72 base pair repeats.

molecular weights. The studies in this paper employ the more sensitive technique of Southern blot analysis to demonstrate that BLM introduces both single- and double-stranded breaks in purified SV40 DNA in several distinct locations distributed throughout the viral genome. In both SV40 and PM2, the number of highly preferred breakage locations was far fewer than could be accounted for by the prevalence of the reported dinucleotide sequence specificity of BLM.

The conformation of DNA located in highly preferred cleavage locations is not yet clearly defined, despite extensive examination of the nucleotides cut by BLM in short (50-150 bp) segments of DNA (7-10). An advantage to using a

larger DNA molecule with a known nucleotide sequence (28,29) for our drug studies was that it was possible to examine base composition of regions in which breaks were both frequently and infrequently observed. Surprisingly, segments of the SV40 genome within 100 bp of breakage positions were not rich in either AT or GC bases. The 100 bp regions were also not distinguished by an exceptionally high content of the preferred dinucleotide sequences, nor were they particularly rich in alternating purine-pyrimidine sequences. Possibly, the most frequent positions for BLM-DNA interaction are determined by helical conformations that are induced at least in part by distant nucleotides.

There was no apparent common biological function associated with the sites in SV40 DNA recognized by BLM. The drug demonstrated preference of several types of genetic elements in SV40 DNA, including the viral enhancer, coding regions and T-antigen intron. Since the SV40 genome is nearly saturated with sequences that encode a protein, these studies do not address whether BLM cleavage sites are located preferentially in coding or non-coding regions. Our findings are consistent with the data obtained using PM2 DNA, where BLM-induced breaks were found at several sites in the phage genome (11).

While we have examined the site of the first double-stranded break made in SV40 DNA, the 1:6 ratio of double-stranded breaks to single-stranded breaks in this system (based on determining the Poisson distribution of breaks, data not shown) indicates that the first double-stranded break already represents multiple single-stranded nicks. When the BLM concentration was lowered to introduce a maximum of 1 single-stranded break per molecule, the position of this first nick did not differ significantly from the sites of double-stranded breakage. Initial BLM-DNA interactions may be located at only a few distinct sites that are the preferred locations for subsequent drug-induced cleavage. Previous studies have indicated that BLM-induced cuts on different strands are independent of each other except in palindromic sites such as 5'-GC-3' or 5'-AT-3' (10). Thus, for single- and double-stranded breaks to be located in the same sites, it is likely that cuts were made at such palindromic sequences or at opposing dinucleotides that were separated by no more than 4 bp. Such nucleotide arrangements could represent some of the sequences which form structures preferentially cut by BLM.

Some of the sites cut in supercoiled SV40 DNA by BLM could have been susceptible because of conformations caused by torsional stress, which has the potential of disrupting base-pairing and inducing formation of DNA secondary structures such as Z-DNA and cruciforms (30). However, we have found that BLM

cuts supercoiled and linearized SV40 DNA in the same regions. Mirabelli *et al.* (27), using high resolution polyacrylamide sequencing gels to examine each nucleotide in a short (50 bp) sequence, have reported that the sequence preference of BLM on supercoiled and relaxed DNA differed. It is possible that while torsional stress might alter which nucleotide in a cleavage region is attacked by BLM, supercoiling has little effect on the location of breaks within a large DNA molecule.

Although it seems clear that BLM-DNA interaction is directed by DNA secondary structure, less is known about the effects of nucleosomes and other DNA-binding proteins on BLM-induced cleavage. Previously we reported that nucleoproteins in SV40 minichromosomes shield the DNA from BLM attack, and that further reduction in drug cutting was caused by cellular permeability and intracellular drug metabolism (16). It was of interest to determine if BLM site preference on SV40 minichromosomes in intact cells differed from that of purified DNA. Surprisingly, not only was BLM site preference unaffected by the level of viral encapsidation, but BLM cut purified SV40 DNA and intracellular SV40 minichromosomes at similar sites. These data support the results of Murray and Martin, who reported that BLM cut a non-transcribed sequence at the same sites in intact cells as in purified DNA (17). Furthermore, our data are not inconsistent with Murray and Martin's interpretation that nucleosomes are randomly phased.

Kuo (18) has shown that BLM cuts actively transcribed globin and ovalbumin genes at a more rapid rate than non-transcribed chromatin. In our study, we have found that BLM preferentially cuts at the same positions in chromatin and purified DNA, suggesting that the increased rate of cutting in active chromatin does not result from the introduction of new cleavage sites. It is likely that the enhanced sensitivity of active chromatin to BLM is a consequence of the transcribed gene sequences being less protected by nucleosomes.

A major difference in the cutting of purified and intracellular DNA by BLM appears to be the drug concentrations required to achieve equivalent cleavage (16). Apparently, nucleoproteins, cell permeability, and drug metabolism can affect drug activity by reducing the quantity of drug-induced breaks, but not by altering the site specificity of BLM cleavage. This conclusion is consistent with our earlier observations that the frequency of double-stranded break formation in purified and intracellular SV40 DNA was similar (16). It appears that much of what is learned about drug-DNA interactions using large segments of purified DNA is pertinent to more complex systems.

The studies in this paper have identified regions in SV40 DNA that are

preferentially cut by BLM. The structure of these cleavage positions can now be examined further by analyzing the nucleotide sequences cut by BLM. In addition, the effect of distant nucleotides on BLM site specificity can be examined by changing the location of SV40 DNA segments that contain preferred breakage locations. Studies of this type should increase our understanding of the molecular action of DNA reactive agents.

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