Bleomycin-Induced Alterations in DNA Replication: Relationship to DNA Damage†

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ABSTRACT: Bleomycin (BLM), a well-known DNA scission agent, is assumed to inhibit intracellular DNA replication by damaging the DNA template (cis-acting mechanism), although other DNA damaging compounds can alter DNA replication through modulation of crucial replication factor(s) (trans-acting mechanism). The present study examines the relationship between DNA damage and inhibition of replication caused by BLM in the well-defined simian virus 40 (SV40) intracellular and cell-free in vitro systems. Treatment of SV40-infected BSC-1 cells for 2 h with BLM at 50 μg/mL, induced 0.3 break/viral genome. Under the same treatment conditions, analysis of replication intermediates on two-dimensional gels showed a decrease in both mass of SV40 replication intermediates and replication activity. The mass of SV40 intermediates was decreased to about 30%, whereas replication activity was reduced to less than 5%. These results suggest that BLM inhibits both initiation and elongation phases of SV40 replication. In a cell-free DNA replication system, extracts from BLM-treated cells (50 μg/mL) were able to support SV40 DNA replication by only 50%. In this study, non-drug-treated DNA template was used, implying that BLM can induce a trans-acting effect. Finally, the drug-induced effects on SV40 DNA replication in cell-free and intracellular viral systems were compared to the effects on genomic DNA replication in BSC-1 cells. Overall, the results support the concept that BLM-induced inhibition of DNA replication occurs by both trans- (inhibition of replication of nondamaged template) and cis-acting mechanisms (template damage).

Bleomycin (BLM),[1] a glycopeptide DNA-intercalating antibiotic isolated from cultures of *Streptomyces verticillus*, is active against several tumor types and is currently used in cancer therapy (for review see refs *1*−*3*). BLM is generally believed to act by disrupting the structure of chromosomal DNA similar to ionizing radiation (IR), although other cellular targets and different mechanisms of action against DNA have also been proposed (*4*−*9*). In addition, involvement of BLM-induced degradation of RNA (*10*, *11*) or DNA−RNA heteroduplexes (*12*) in its activity has been studied.

Bleomycin damages DNA in a reaction requiring Fe(II) and O_2 as cofactors (*13*, *14*). Strand breaks are generated through an oxidative cleavage of the (C-3′)−(C-4′) bond in deoxyribose, leaving a 3′-phosphoglycolate terminus, a base with an attached three-carbon sugar fragment (base-propenal), and a 5′-phosphate end group, or through the formation of an alkali-labile abasic site with concomitant formation of a free base (*15*, *16*). The drug induces double- and single-strand breaks in both isolated and intracellular DNA (*17*−*20*) and has a preference for cleavage of both linker regions of chromatin and transcribing genes (*21*, *22*). BLM-induced strand breaks show strong sequence preference, with d(GpC) and d(GpT) being the most susceptible to cleavage (*20*, *23*, *24*).

The cellular responses to DNA damage caused by DNA strand-scission agents include effects on DNA replication and repair and cell cycle progression (*25*, *26*). The biological consequences of BLM-induced DNA damage in cells are not completely understood, but DNA synthesis is thought to be a primary target. DNA damaging agents could inhibit DNA replication at the level of initiation or elongation. It has been shown that the enediyne antibiotic C-1027 (*27*), the cyclopropylpyrroloindole agents adozelesin (*28*, *29*) and bizelesin (*30*), ionizing radiation (*31*, *33*), and UV irradiation (*34*), at least at low concentrations, inhibit DNA replication at the level of initiation. Since the inhibition is observed at doses of drugs or radiation that produce only insignificant amounts of DNA damage, a trans-acting mechanism has been implicated. In such a model, inhibition of DNA replication occurs through DNA damage response pathways, rather than through direct arrest of replication forks on damaged template (cis-acting mechanism).

In this study, the relationship between DNA damage and inhibition of replication caused by bleomycin was evaluated in well-defined cell-free and intracellular simian virus 40

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[1] Abbreviations: BLM, bleomycin; IR, ionizing radiation; PFGE, pulsed-field gel electrophoresis; DSB, DNA double-strand break; SSB, DNA single-strand break; SV40, simian virus 40; RPA, replication protein A; TAg, large T antigen.

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(SV40) systems (*27*, *28*, *35*). SV40 DNA form conversion assay was used to determine damage induced by BLM in viral DNA, while two-dimensional neutral−neutral agarose gel electrophoresis of SV40 replication forms was employed to determine the ability of BLM to inhibit SV40 DNA replication in cells. SV40 DNA replication was assayed in cell-free system with extracts from drug-treated 293 cells (*30*,*35*), to evaluate whether the inhibition of DNA replication is due to cis or trans effects. Pulsed-field gel electrophoresis (PFGE) and [^{3}H]thymidine incorporation were deployed in whole cell system to assess drug-induced genomic DNA damage and replication inhibition, respectively.

EXPERIMENTAL PROCEDURES

Materials. Bleomycin (Bristol-Myers Squibb Co., Syracuse, NY) stock solution (2 mg/mL) was prepared in water and stored at −20 °C. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]dATP (3000 Ci/mmol) were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). [*methyl*-^{3}H]Thymidine (48 Ci/mmol) and [2-^{14}C]thymidine (55 mCi/mmol) were from Moravek Biochemicals, Inc. (Brea, CA). GeneScreen membranes were obtained from Dupont NEN (Boston, MA). The DECAprime II DNA labeling kit was from Ambion (Austin, TX). Cell culture materials were purchased from Life Technologies (Grand Island, NY). SV40 large T-antigen was purified from baculovirus-infected High-Five insect cells (Invitrogen, CA) according to published procedure (*35*), and SV40 origin-containing plasmid pSV011 was isolated as described elsewhere (*36*).

SV40 DNA Replication in BSC-1 Cells. BSC-1 cells (70−80% confluence) in 60-mm dishes were infected with SV40 virus (moi = 1−3) in MEM containing 2% calf serum. At 24 h postinfection, BLM solution was added to the medium for 2 h. During the last 30 min of incubation the newly synthesized DNA was labeled with [^{3}H]thymidine (10 μCi/mL). Following drug treatment, the samples were rinsed three times with PBS, and SV40 DNA was extracted ("Hirt extraction") and purified according to a previously described method (*27*, *28*). The purified SV40 was dissolved in TE (10 mM Tris, pH 7.6, and 1 mM EDTA) and analyzed either by neutral agarose gel electrophoresis or by neutral−neutral 2-D agarose gel electrophoresis.

For 2-D gels, aliquots (10 μL) of SV40 DNA were digested with an excess of *Bam*HI for 2 h at 37 °C and then electrophoresed on a 0.6% high-strength analytical agarose gel (Bio-Rad Laboratories, Hercules, CA) in 1× TAE and 0.1 μg/mL ethidium bromide for 24 h at 0.7 V/cm. Lanes from the first-dimension gel were cut and placed across the top of another gel (1% agarose and 0.5 μg/mL ethidium bromide in 1× TBE). The second-dimension electrophoresis was carried out in 1× TBE buffer containing 0.5 μg/mL ethidium bromide at 4 °C for 19 h at 4 V/cm. DNA was then transferred to GeneScreen and probed with ^{32}P-labeled SV40, and replication intermediates were visualized by exposure to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Alternatively, gels were processed by fluorography to visualize newly synthesized DNA.

Changes in replication intermediates (from Southern blots) and replication activity (from fluorography) were determined as described elsewhere (*27*, *28*). Briefly, for calculating of the amount of replication intermediates, the volumes of pixels

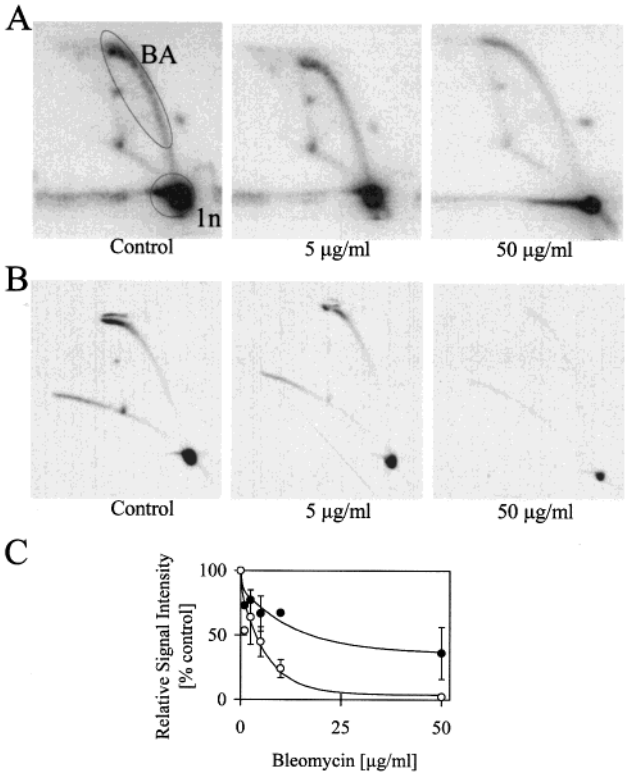

FIGURE 1: Bleomycin effects on SV40 DNA replication intermediates (panel A) and nascent SV40 DNA synthesis (panel B). SV40-infected BSC-1 cells were treated for 2 h with the indicated concentration of bleomycin (0, 5, or 50 μg/mL). SV40 replication intermediates were separated by two-dimensional agarose gel electrophoresis and detected by Southern blotting. Nascent SV40 DNA synthesis (replication activity) was visualized by fluorography of similar two-dimensional agarose gels. Panel C shows quantitation of the decrease in mass of SV40 DNA replication intermediates (bubble arc signal, ●) and in nascent SV40 DNA synthesis (replication activity, ○), based on densitometry analysis of the autoradiograms from three experiments. Points represent mean values ± SD. Electrophoresis conditions, blotting, hybridization, fluorography, and autoradiogram analysis methods are described under Experimental Procedures.

in ellipses inserted over the tip of the bubble arcs (*BA*, Figure 1A) and circles inserted placed over the 1*n* spots (1*n*, Figure 1A) were quantitated by use of the ImageQuant software (Molecular Dynamics) and the bubble arc signal (BA_{signal}) was calculated according to

$$BA_{signal} = \frac{BA_{sample} - bkg}{(1n_{sample} - bkg)/(1n_{control} - bkg)}$$

where $1n_{control}$ and $1n_{sample}$ refer to the amount of unreplicated SV40 DNA in the control and sample, respectively, and *bkg* refers to background value from the blot. Similarly to this, the BA_{sample} and *bkg* values were quantitated from fluorographic analysis of 2-D gels, and the replication activity (*RA*) was calculated according to

$$RA = \frac{BA_{sample} - bkg}{FIII_{sample}/FIII_{control}}$$

where $FIII_{sample}$ and $FIII_{control}$ are the intensities of linearized SV40 DNA in the drug-treated sample and the control, respectively, taken from the ethidium bromide-stained first-dimension gel.

Damage to Intracellular SV40 DNA. To assess intracellular SV40 DNA damage, the "Hirt extracts" from infected BSC-1 cells treated with BLM were run on 1% agarose gels in 1× TAE for 16 h. The separated forms of SV40 DNA were stained with ethidium bromide (0.5 μg/mL), photographed under UV, and quantitated by densitometry. Frequencies of breaks in viral DNA expressed as single-strand (SSB) or double-strand breaks (DSB) per 10^6 bp were estimated as described previously (*37, 38*).

Cell-Free SV40 DNA Replication. Cell extracts were prepared as described elsewhere (*30*), with small modifications. Human 293 cells (embryonic kidney cells) were maintained in monolayer culture in D-MEM medium. BLM solution was added to a 50−80% confluent culture [(4−5) × 10^6 cells in 100-mm dish] for 2 h, and then cells were washed twice with cold PBS and scraped on ice. Harvested cells were washed twice in PBS and once in hypotonic buffer (20 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and 0.2 mM PMSF), spun, and incubated in the same buffer for 10 min on ice. Cells were disrupted by two cycles of rapid freezing in a dry ice/methanol bath and then thawed and left on ice for an additional 30 min. Cells debris was removed by centrifugation at 12000*g* for 15 min, and the soluble fraction was quick-frozen and stored at −80 °C. Protein concentrations were routinely determined by the Bradford method (Bio-Rad).

The conditions used for SV40 cell-free DNA replication were as described previously (*30, 35*). Briefly, extracts from BLM-treated or control cells (30 μg) in replication buffer (4 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM UTP, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 0.025 mM dATP, 7 mM $MgCl_2$, 0.024 unit of creatine phosphokinase, and 40 mM phosphocreatine) were supplemented with 30 ng of SV40 origin-containing plasmid pSV011, 600 ng of purified T antigen (TAg), and 1 μCi of [α-^{32}P]dATP. The mixtures (final volume 10 μL) were incubated at 37 °C for 1 h, and then the reaction was stopped by addition of 1% SDS, 10 mM EDTA, and 100 μg/mL proteinase K. Following a 30 min incubation at 37 °C and phenol−chloroform extraction, DNA was purified on a G50 column (Boehringer Mannheim, Indianapolis, IN) and electrophoresed on an 0.8% agarose gel in 1× TAE for 16 h at 1 V/cm. Gels were dried and DNA replication products were visualized and quantitated by exposure to a Phosphorimager screen.

Cell Culture and Cytotoxicity Assay. BSC-1 African green monkey kidney cells were cultured in MEM medium containing 10% calf serum as described previously (*39*). Human 293 cells (transformed embryonic kidney cells) were grown as a monolayer culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cytotoxicity of BLM was determined by colony formation assay as described previously (*37, 39*). Briefly, cells were grown in 60-mm dishes for 48 h [final density (1−1.5) × 10^6 cells/dish, 50−80% confluence] and then treated with drug for 2 h, harvested, and plated at various cell densities. After a 10 day incubation at 37 °C, the colonies were stained with methylene blue, and the relative plating efficiency was calculated.

Damage to Genomic DNA. The analysis of genomic DNA damage was performed by pulsed-field gel electrophoresis. BSC-1 or 293 cells were radiolabeled with [2-^{14}C]thymidine (0.0125 μCi/mL) for 24 h in 60-mm dishes (0.25 × 10^6 cells/dish) and then allowed to grow in fresh medium for 24 h. Cells at ∼50−80% confluence [(1−1.5) × 10^6 cells/dish] were treated with BLM for 2 h, harvested, and washed twice in PBS. Cells were resuspended in 0.66% low gelling temperature agarose at 1.4 × 10^7 cells/mL and digested with 2 mg/mL proteinase K in lysis buffer (1% Sarcosyl and 0.5 M EDTA, pH 8.0) for 2 h at 55 °C according to a method described elsewhere (*40*). After incubation, samples were spun down, and agarose plugs were formed at 4 °C and then stored in 0.5 mL of TE at 4 °C for at least 24 h to allow for diffusion of detergent and digested proteins. The plugs (containing ∼3 × 10^5 cells) were loaded into wells of 0.75% agarose gel. Gels were electrophoresed in 0.5× TBE for 90 h at 64 V with a switching time of 35 min, on a Chef DR II apparatus (Bio-Rad Laboratories Inc., Hercules, CA) (*40*). ^{14}C-Radiolabeled genomic DNA from the gels was transferred to a GeneScreen membrane and detected by phosporimaging. Damage to genomic DNA was calculated as a fraction of DNA from drug-treated cells that migrated from the well, compared to DNA from control cells, and converted to number of double-strand breaks per 10^6 bp as described previously (*38*).

Incorporation of [^{3}H]Thymidine into Cellular DNA. Thymidine incorporation into cells was analyzed as described previously (*41*), with some modifications. Briefly, BSC-1 cells were plated onto 60-mm dishes (0.25 × 10^6 cells/dish) and prelabeled with [^{14}C]thymidine (0.0125 μCi/mL) for 24 h, followed by a 24 h chase in fresh medium. Cells at ∼50−80% confluence [(1−1.5) × 10^6 cells/dish] were treated with BLM for 2 h, and during the last 30 min [^{3}H]thymidine was added to a final concentration of 0.1 μCi/mL. After drug treatment, the cells were washed twice with cold PBS, scraped, and mixed with 0.5 M cold perchloric acid (PCA). Acid-insoluble radioactivity was measured with a liquid scintillation counter LS-3800 (Beckman). Inhibition of thymidine incorporation into cellular DNA was calculated as the ratio of [^{3}H] to [^{14}C] in drug-treated sample compared to nontreated control.

RESULTS

SV40 DNA Replication in Cells Treated with Bleomycin. SV40-infected BSC-1 cells were used to study bleomycin effects on DNA replication. SV40 DNA replicates in monkey cells by using the cellular replication machinery and only one viral protein, the large T antigen (TAg), therefore providing a good model for mammalian DNA replication. Additionally, in this system one can study DNA replication and damage in the context of the same well-defined SV40 genome. Two-dimensional neutral−neutral agarose gel electrophoresis of viral DNA extracted from drug-treated or nontreated cells can be used to determine the type and amount of SV40 DNA replication intermediates (*42*). In this technique, replication intermediates are isolated, digested with the appropriate restriction enzyme, and run on a 2-D agarose gel. In the first dimension the linearized SV40 DNA molecules are separated on the basis of size only, while in the second dimension the shape of DNA molecules influences their migration, allowing one to distinguish between replication bubbles (*BA*), replication forks, and nonreplicating molecules (1*n*). A more detailed description of the variant of the technique used in this laboratory is given in our earlier publications (*27, 28, 40*).

Simultaneously with 2-D gel analysis, the drug-induced damage to SV40 DNA in infected cells can be readily determined by topological forms conversion analysis on a 1-D agarose gel (*18*).

BSC-1 cells were infected with SV40 and then treated with bleomycin for 2 h (at 24 h postinfection). During the last 30 min of drug treatment, [^{3}H]thymidine was added to the medium. Viral DNA was then extracted, digested with *Bam*HI, and fractionated by 2-D gel electrophoresis as described under Experimental Procedures. Panels A and B of Figure 1 show representative blots and fluorograms of SV40 DNA replication from nontreated (control) and BLM-treated BSC-1 cells (5 and 50 μg/mL). The replication activity (*RA*) was determined by fluorographic detection of [^{3}H]thymidine, and the mass of SV40 DNA replicating intermediates was quantitated from the bubble arc signal (*BA*) as seen on the Southern blots. Data from two independent experiments are summarized in Figure 1C.

If drug inhibits SV40 DNA replication at the initiation phase, incorporation of [^{3}H]thymidine into SV40 replicating intermediates (replication activity, *RA*) should cease only when all of the already active replisomes complete their replication cycle (*43*). In this case, the number of replicating intermediates (mass of replicating intermediates measured as ^{32}P signal on Southern blots and expressed as the bubble arc signal, *BA*) should decline simultaneously with the reduction of replication activity. However, if drug inhibits DNA replication at the elongation phase (at replication forks), the mass of replicating intermediates will not decrease and the ^{32}P signal from Southern blot (*BA* signal) will be present, but replication forks will be inactive (no [^{3}H]thymidine signal from fluorography).

A progressive decrease in the mass of the bubble arc signal with increasing bleomycin concentration was observed (Figure 1A). The strong mass signal (*BA*) in the control sample was reduced by 35% at 5 μg/mL BLM and by 65% at 50 μg/mL BLM. While the 1*n* spot intensity also declined, possibly due to drug-induced DNA damage, this occurred to a lesser degree compared to the BA signal intensity. Under the same conditions, incorporation of [^{3}H]thymidine into replicating intermediates was reduced to a greater extent, by 55% at 5 μg/mL, and was almost undetectable at 50 μg/mL BLM (Figure 1B). The complete disappearance of replication activity in cells treated with 50 μg/mL BLM, in comparison to a partial reduction of the bubble arc signal, may indicate that the drug has effects on elongation and can arrest DNA replication fork movement. However, a "pure" elongation inhibitor [for example, aphidicolin, a specific inhibitor of replicative DNA polymerases (*27*, *28*)] would decrease only replication activity without affecting the bubble arc signal. Thus, presented results clearly indicate that bleomycin could inhibit DNA replication in both initiation and elongation phases.

Damage to Intracellular SV40 DNA. As bleomycin is a DNA strand-scission compound, inhibition of DNA replication could be the result of damage to the SV40 DNA molecules, which should be manifested by a decrease in the intensity of the 1*n* spot. Decrease in 1*n* spot intensity observed in samples from cell treated with 5 or 50 μg/mL BLM (Figure 1A) suggests that bleomycin-induced lesions in SV40 DNA could be responsible, at least partially, for replication inhibition.

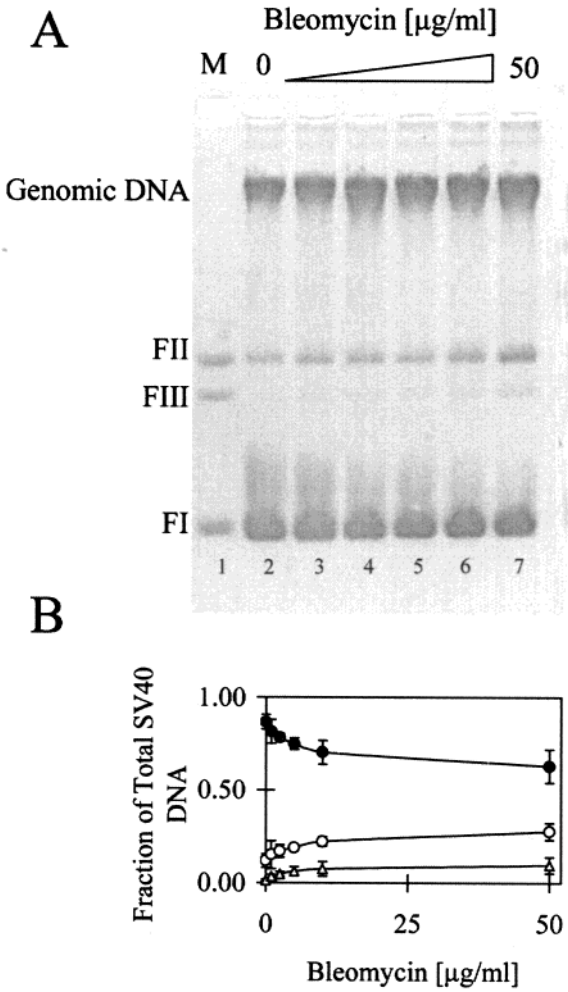

FIGURE 2: Bleomycin damage to intracellular SV40 DNA measured by forms conversion. Panel A is a representative agarose gel after electrophoresis of viral DNA from SV40-infected BSC-1 cells incubated with bleomycin at 0, 1, 2.5, 5, 10, and 50 μM (lanes 2−7, respectively) for 2 h. Positions of supercoiled (FI), nicked (FII), and linear (FIII) forms are indicated in lane 1. In panel B, forms conversion [FI (●), FII (○), and FIII (△)], are calculated on the basis of densitometric analysis of the gels. Relative amounts of the forms are expressed as a fraction of the total SV40 DNA. Data were collected from three experiments and are expressed as mean values ± SD.

Since bleomycin-induced SV40 DNA replication inhibition could be attributed to DNA damage, we assayed its ability to damage intracellular SV40 DNA. Strand breaks in SV40 DNA are easily measured by analyzing forms conversion of supercoiled (form I) molecule (*18*). Introducing one single-strand break converts supercoiled form I DNA to nicked circular form II, while a double-strand break (or two close single-strand breaks on opposite strands) is needed to linearize DNA to form III.

BSC-1 cells were infected with SV40 virus for 24 h and then treated with BLM for 2 h. Viral DNA was isolated and BLM-induced DNA damage was assessed by neutral agarose gel electrophoresis (Figure 2). At 50 μg/mL BLM, approximately 30% of viral DNA was converted from form I to form II or III, resulting in a total number of DNA breaks (DSB + SSB) of 61 per 10^6 bp, or 0.3 break per one viral genome. This could contribute to the loss of replication activity due to cis-acting effects, as 30% of the SV40 DNA molecules were damaged at the highest drug concentration

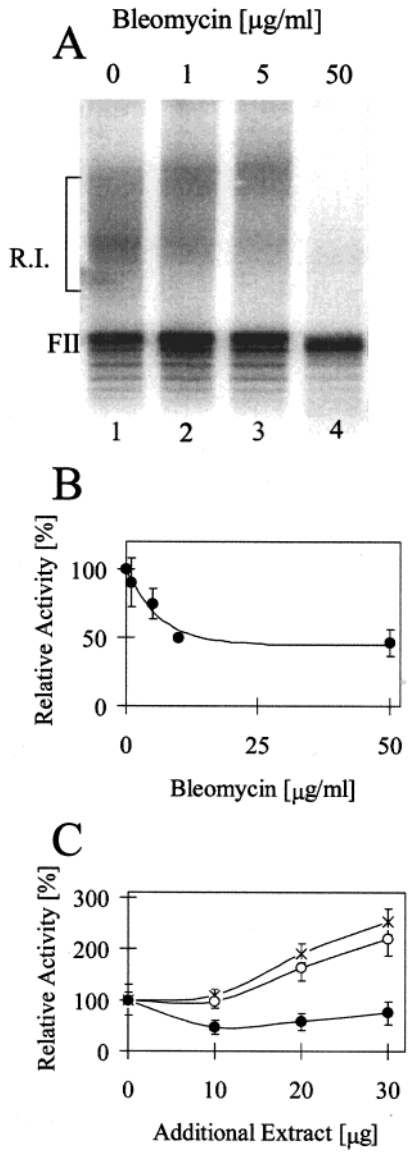

FIGURE 3: SV40 DNA replication in cell-free system with extracts from bleomycin-treated 293 cells. (A) Cells were treated with drug at the indicated concentrations (control, lane 1; 1 μg/mL, lane 2; 5 μg/mL, lane 3; and 50 μg/mL, lane 4) for 2 h. Reaction mixtures contained 30 μg of cell extracts, 30 ng of SV40 origin-containing DNA, and 0.6 μg of SV40 T-antigen and were incubated at 37 °C for 1 h. Replication products were isolated, resolved on 1% neutral agarose gels, visualized by autoradiography, and analyzed as described under Experimental Procedures. Positions of form II (FII) and DNA replication intermediates (RI) are indicated. (B) Quantitation of ^{32}P incorporation into newly replicated SV40 DNA (points represent data from five independent experiments ± SD). (C) Mixing extracts experiment. Changes in the SV40 DNA replication carried on by control cell extract were induced by addition of extracts from control (×) or bleomycin-treated cells (○, 5 μg/mL; ●, 50 μg/mL). Cell extract proteins (10, 20, or 30 μg) were added to 20 μg of control extract (100% replication).

tested. However, there is also a trans-acting component since the inhibition of DNA replication was nearly complete at the same drug concentration. If the inhibition of DNA replication arose only from the damage of DNA template, the number of damaged SV40 molecules would be expected to correlate with replication inhibition and reach a level of at least one break per viral genome at a concentration that completely inhibits SV40 DNA replication (in this study 50 μg/mL BLM). Therefore, it is reasonable to assume that both cis- and trans-acting mechanisms are the sources of DNA replication inhibition by BLM.

SV40 DNA Replication in Cell-Free in Vitro System. To more clearly delineate between BLM-induced cis and trans effects on DNA replication, an in vitro SV40 DNA replication assay was used, in which extracts from 293 cells treated with BLM were examined for DNA replication activity (*30*, *35*). As shown in Figure 3A, SV40 DNA replication activity in extracts from BLM-treated cells was suppressed compared to extracts from control, nontreated cells. After a 2 h treatment with BLM at 50 μg/mL, replication activity declined to 46% of the initial value (Figure 3A, lane 4). In these experiments, the plasmid template was never exposed to drug treatment, excluding the possibility that inhibition of DNA replication was caused by template damage (cis mechanism). Additionally, the possibility that traces of BLM could remain in extracts from the drug-treated cells and inhibit in vitro replication was tested by incubating the extracts with pSV011 DNA for 1 h at 37 °C and analyzing the DNA damage on agarose gel electrophoresis. Even at the highest drug concentration, no measurable DNA damage was observed (data not shown). Thus, it is safe to assume that the inhibition of DNA replication in a cell-free system by bleomycin is due to a trans-acting mechanism, not direct drug action on DNA template.

Trans inhibition of DNA replication could be mediated by a drug-induced inhibitor or by drug-induced depletion or inactivation of one (or more) crucial replication factor(s). Induction of a trans-acting inhibitor of DNA replication has been shown previously for ionizing radiation (*33*), camptothecin (*44*), and bizelesin (*40*), while adozelesin seems to inhibit DNA replication by inactivation of RPA, a protein complex indispensable for SV40 replication (*29*).

To differentiate between DNA replication inhibition by induction of inhibitor(s) or by inactivation of replication factor(s), "mixing" experiments were carried out in which extracts from control untreated cells (20 μg) were supplemented with increasing amount of extracts from control or drug-treated cells (Figure 3C). If BLM treatment induces a trans-acting DNA replication inhibitor(s), addition of extract from the drug-treated cells should reduce replication activity in control cell extract. Inactivation/depletion of the crucial replication factor(s) in extracts from BLM-treated cells would not influence replication activity of control cell extract, already having all necessary components.

Extracts mixtures were used to carry out in vitro SV40 DNA replication for 1 h at 37 °C as described above. The relative activity of control extract in the absence of additional extracts (from drug-treated or control cells) was adjusted to 100%. Addition of 10−30 μg of extracts from nontreated cells or cells incubated with 5 μg/mL BLM had a similar effect on DNA replication, resulting in an increase in incorporation of ^{32}P-labeled nucleotides. A decrease to a rate below control in radiolabeled nucleotide incorporation into DNA was observed only after addition of extracts derived from cells treated with the highest dose of BLM (50 μg/mL), suggesting that drug treatment induces a trans-acting inhibitor(s).

Bleomycin-Induced Inhibition of Cellular DNA Synthesis. To determine how bleomycin inhibits cellular, genomic DNA replication at the drug concentrations causing inhibition and damage to intracellular, viral SV40 DNA, drug-treated BSC-1

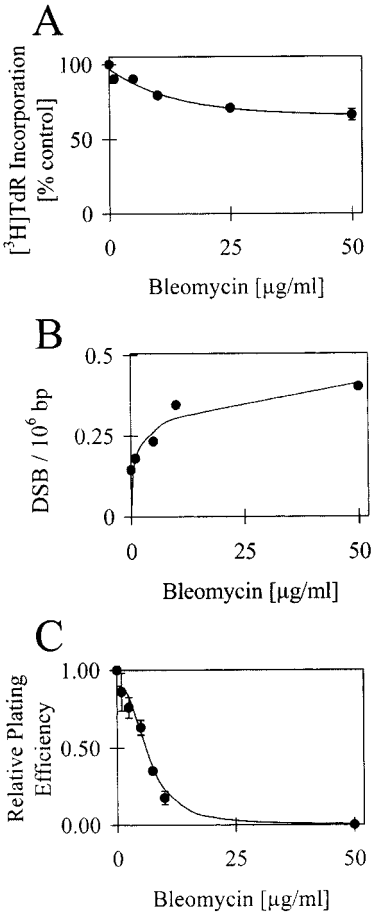

FIGURE 4: Bleomycin-induced effects in BSC-1 cells. (A) Inhibition of [^{3}H]thymidine incorporation into DNA of BSC-1 cells treated with bleomycin. For cellular DNA synthesis inhibition, bleomycin-treated cells were pulsed with tritium-labeled precursor for the last 30 min of incubation, and [^{3}H] incorporation into acid-insoluble material was quantitated by liquid scintillation counting. (B) Damage to genomic DNA after a 2 h incubation with drug. High molecular weight DNA from cells treated with bleomycin (0−50 μg/mL) for 2 h was resolved by pulsed-field gel electrophoresis and analyzed on a Southern blot. The radioactivity released from the wells was recalculated to double-strand breaks as described under Experimental Procedures. Data are from two experiments and are expressed as mean values ± SD. (C) For colony-forming inhibition, cells were drug-treated for 2 h at the indicated concentrations, replated, and grown for 10 days, followed by colony counting and relative plating efficiency calculation. Points represent mean values from at least two experiments ± SD.

cells were examined for genomic DNA synthesis measured by [^{3}H]thymidine incorporation as described under Experimental Procedures. Figure 4A shows the decrease in [^{3}H]-thymidine incorporation into BSC-1 cells after a 2 h incubation with 0−50 μg/mL bleomycin. The highest studied BLM concentration (50 μg/mL) was sufficient to inhibit bulk DNA synthesis by only 30%, although it almost completely inhibited SV40 DNA replication, as shown above.

Damage to BSC-1 Genomic DNA. Given that bleomycin is a DNA scission agent, the damage induced by BLM in intracellular, genomic DNA was studied. Figure 4B, shows the number of double-strand breaks in genomic DNA from BSC-1 cells treated with 0−50 μg/mL BLM for 2 h. About 0.2 double-strand break (DSB) per 10^6 bp was detected for BSC-1 cells treated with 5 μg/mL drug. The highest BLM concentrations (50 μg/mL) during a 2 h incubation produced 0.4 DSB per 10^6 bp.

BSC-1 Cell Growth Inhibition. To compare DNA damage and DNA replication inhibition induced by BLM with cytotoxic activity of this drug, we examined plating efficiency of drug-treated BSC-1 cells. Figure 4C demonstrates the decrease in relative plating efficiency of cells after a 2 h treatment with 0−50 μg/mL BLM and a 10 day postincubation. The plating efficiency of BSC-1 cells was inhibited by 50% (EC_{50}) after treatment with 6.6 ± 0.55 μg/mL BLM.

DISCUSSION

Although it has previously been shown that bleomycin can induce DNA damage and inhibit DNA replication (*2*, *45*, *46*), a relationship between them has not been described in detail. DNA replication can be inhibited at the phase of initiation or at elongation; additionally, the DNA damaging agents can inhibit replication in cis (i.e., directly damaging the DNA template) or in trans (i.e., by inducing a regulation signal and arresting replication on nondamaged template). In the present work we attempted to characterize the mechanisms by which BLM inhibits DNA replication and its relationship to drug-induced DNA damage.

To study BLM-induced inhibition of DNA replication, we have utilized 2-D gel techniques, which have the advantage that effects on initiation and elongation can be distinguished, using the well-defined SV40 DNA replication system in monkey cells. Using this approach, we have shown previously that cell treatment with several DNA damaging agents [e.g., adozelesin (*28*), C-1027 (*27*), and bizelesin (*30*)] inhibits initiation of DNA replication. Bleomycin also inhibits SV40 DNA synthesis at the stage of initiation, as indicated by the fading of the bubble arc signal on 2-D gels with increasing drug concentrations. However, under treatment conditions where decreases in the bubble arc were detected, replication activity also decreased, and it almost completely disappeared at 50 μg/mL BLM. This indicates that BLM arrests DNA replication at both stages, initiation and elongation. "Pure" initiation inhibition (for example, by C-1027) would result in a simultaneous decrease in both intensity of the bubble arc and replication activity (*27*), whereas "pure" elongation inhibition (for example, by aphidicolin) would only reduce replication activity, while leaving a strong bubble arc signal (*27*, *28*, *42*).

Although SV40 DNA replication inhibition by bleomycin was observed at doses similar to those inhibiting genomic DNA replication, SV40 DNA synthesis in infected BSC-1 cells was inhibited to a greater extent than was genomic DNA in uninfected BSC-1 cells. This could be explained by the observation that BLM induced more damage in SV40 DNA in infected cells than in uninfected BSC-1 genomic DNA. When cells were treated with BLM at 50 μg/mL, DSB were 30-fold more frequent in SV40 than in genomic DNA, and the replication activity was almost totally diminished. The relationship between DNA damage level and replication inhibition in these two systems emphasizes the role of a cis factor in BLM action on DNA replication. Alternatively, increased damage to viral DNA compared to DNA in uninfected cells may be due to changes in permeability of the cell membranes (*47*), thus increasing diffusion of the drug into the transformed cells. BLM transfer into cells is known to be a limiting step in its biological action: approximately 0.1% of the BLM concentration in medium is present in cells (*2*).

In earlier work (*19*) we studied the relationship between DNA damage and cellular responses induced by bleomycin at therapeutic doses using 935.1 tumor-bearing mice as a model system. The episomal element contained in the 935.1 cells was used to evaluate the DNA damage, while the tumor weight was measured for drug therapeutic efficacy. Our data showed that therapeutic effects occur at bleomycin doses that induce detectable single- and double-strand breaks to the episomal DNA. This supports the role of the cis effect(s) in bleomycin biological activity, although additional involvement of trans-acting effect(s) could not be ruled out.

Data presented here demonstrate the presence of trans inhibition of SV40 DNA replication in BLM-treated cells. At the dose of 50 μg/mL BLM, which completely stopped replication activity and lowered the bubble arc signal by ~64%, only one of every three SV40 molecules was damaged. Because not all viral molecules were damaged, the signal to arrest replication activity had to be transmitted from other (damaged) SV40 molecules or from damaged genomic DNA.

To study trans regulation of DNA replication, we used SV40 in a cell-free in vitro DNA replication assay (*30, 35*). The observed inhibition of DNA replication in extracts from cells treated with BLM cannot be attributed to damaged DNA template since the plasmid DNA was never exposed to BLM. Therefore, participation of a cis mechanism of DNA replication inhibition could be excluded in these experiments. The reduced replication activity in extracts prepared from drug-treated cells suggests the presence of trans inhibitory factor(s) that downregulate DNA replication. Mixing experiments suggested the presence of a dominant inhibitory activity (or activities) in the extract of BLM-treated cells that efficiently stalls replication in reactions assembled with extract from nontreated cells. Taken together, these results demonstrate that bleomycin induces a trans-acting DNA synthesis inhibitor, as previously shown for other DNA-interacting agents [for example, ionizing radiation (*33, 48*), UV radiation (*49*), camptothecin (*44*), or bizelesin (*30*)].

The nature of the trans-acting DNA replication inhibitor is presently unknown, although a DNA-dependent protein kinase (DNA-PK) has been suggested as a good candidate (*50*). Studies of the replication kinetics suggest that the damage-induced inhibitory activity affects the initiation steps of DNA replication and acts by modifying TAg, the key initiation protein of SV40 DNA replication (*33, 50*). TAg is not a cellular protein, and its functions in SV40 DNA replication are probably carried out by multiple proteins during genomic DNA replication, which may be a nuclear target of DNA-PK. Our recent work (*29, 30*) as well as results from others laboratories (*50, 51*) suggests that a modulation or reduction in RPA activity may be involved. Although RPA is a good substrate for DNA-PK-dependent phosphorylation in both cellular and cell-free studies (*52, 53*), the DNA-PK-mediated RPA phosphorylation appears to not have a significant impact on the DNA replication-supporting activity of this protein complex (*54*).

Treatment of BSC-1 cells with various doses of bleomycin for 2 h resulted in damage to genomic DNA, yielding 0.4 double-strand break per 10^6 DNA base pairs at the highest drug dose used (50 μg/mL). The same drug concentration reduced the incorporation of [^{3}H]thymidine into cellular DNA to ~70% of control. At this level of DNA damage, one DSB is introduced per approximately 2.5×10^6 bp, which equals one DSB per 2−3 replication clusters as defined by Hand (*55*). Because an average replication cluster consists of about 20 independent replicons, this suggests that BLM-induced damage to a single replicon could inhibit DNA replication, probably at the stage of initiation, in other replicons.

The dose−response curve for bleomycin-induced inhibition of the thymidine incorporation into DNA curve revealed a biphasic character. Similarly to ionizing radiation (as reviewed in ref *56*), the curve has an initial, sensitive component, followed by a more flat, resistant component. The sensitive component could be attributed to drug action on initiating replicons and the resistant component to action on replicons already in the process of replication (elongation). Others have shown that, due to this relative resistance of the DNA elongation step to damage-induced inhibition, IR is unable to inhibit the rate of [^{3}H]thymidine incorporation by more than ~55% even at the highest doses (*32*).

In summary, BLM induces DNA damage and inhibits DNA replication in intracellular, genomic, or viral DNA. The consequence of drug treatment is a decrease in DNA replication that occurs at the level of both initiation and elongation of DNA synthesis. Although DNA replication inhibition could be related to the amount of damage induced by the drug, suggesting a cis inhibition mechanism, SV40 DNA replication studies in a cell-free system demonstrate the presence of a trans-acting factor that acts as an inhibitor of DNA replication. Future studies will examine in detail which DNA damage-sensing pathway(s) is(are) activated and which replication factors are affected in bleomycin-treated mammalian cells.

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