0026-895X/86/040358-06$02.00/0

MOLECULAR PHARMACOLOGY, 30:358-363

Measurement of Bleomycin, Neocarzinostatin, and Auromomycin Cleavage of Cell-Free and Intracellular Simian Virus 40 DNA and Chromatin

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Received April 29, 1986; Accepted July 8, 1986

SUMMARY

Simian virus 40 (SV40) was used as a model for eukaryotic chromatin to allow analysis of strand scission drug action on DNA of the same nucleotide sequence in different environments. Cleavage of purified DNA, cell-free chromatin, and intracellular chromatin by the antitumor drugs bleomycin, neocarzinostatin, and auromomycin was assayed. Comparison of total (single- and double-stranded) drug-induced cutting of purified SV40 DNA and isolated viral minichromosomes, as measured by the conversion of supercoiled DNA to the nicked circular form, revealed that a 2–3-fold higher concentration of all three drugs is required to cut minichromosomes as extensively as naked DNA. In addition, differences in drug activity on cell-free nuclear viral chromatin and intracellular SV40 chromatin were measured. Three times more auromomycin was needed to cut intracellular SV40 DNA as extensively as the viral DNA in isolated nuclei, whereas, surprisingly, the increases in bleomycin and neocarzinostatin concentrations were 200-fold and 1000-fold respectively. When the drugs were used to treat SV40-infected cells, no repair of strand scissions was detected. Formation of double-stranded breaks by bleomycin, neocarzinostatin, and automomycin, measured at equivalent levels of total cleavage, was also examined to provide information on the distribution of strand scissions in different environments. Nucleoproteins were found to cause a 3-fold higher level of neocarzinostatin-induced double-stranded breaks to be made on isolated minichromosomes than on purified DNA, but they had no effect on double-stranded break formation by either bleomycin or auromomycin. In contrast, auromomycin made twice as many double-stranded breaks on intracellular SV40 DNA as on minichromosomes in isolated nuclei. However, neither bleomycin- nor neocarzinostatin-induced double-stranded breaks on intracellular SV40 DNA were significantly different from those made on SV40 in isolated nuclei.

BLM is a glycopeptide antibiotic isolated from cultures of *Streptomyces verticillus* (1). NCS and AUR are also antibiotics isolated from strains of *Streptomyces* but, unlike BLM, they consist of a protein wrapped around the active component of these two drugs, a hydrophobic chromophore (2, 3). BLM is currently used in cancer therapy, both alone and in combination with other agents (4), whereas NCS has undergone limited clinical trials (5). All three drugs are active in several experimental tumor systems (4, 6, 7), and the cytotoxicity of these antitumor antibiotics is closely related to their ability to cut DNA (8–10).

When soluble chromatin or chromatin in isolated nuclei is treated with BLM, NCS, or AUR, the drugs cut linker regions between nucleosomes (11–13), and at high drug concentrations NCS also cuts core regions of the nucleosome (14). Actively transcribing genes in chromatin have also been reported to be more sensitive than bulk chromatin to cutting by BLM (15). Preference for specific nucleotide sequences of DNA is exhibited by all three drugs (16–18), but the sequences recognized by each drug differ.

Although AUR will cut DNA in the absence of activator, BLM, NCS, and AUR treatments in cell-free systems require the use of endogenous activators for maximal cleavage activity, and this activation may differ from the intracellular metabolism of these drugs (18–20). In addition, cellular permeability and repair may affect the amount of drug cleavage inside cells (21). Thus, a eukaryotic system in which drug-induced cleavage of DNA could be measured using the same nucleotide sequence in environments ranging from cell-free to intracellular would be advantageous for studying the action of antitumor strand scission drugs. SV40 provides such a system. SV40 is a DNA tumor virus with a circular genome of 5243 base pairs. During lytic infection, the viral genome associates with histones and other nuclear proteins to form circular molecules of nucleosomal chromatin, known as minichromosomes. Minichromosomes

This work was supported by the United States National Cancer Institute Grants CA-28495, CA-13038, and CA-24538. This work was done in partial fulfillment of the requirements of the degree of Doctor of Philosophy in the Department of Pharmacology, Roswell Park Division of the Graduate School of the State University of New York at Buffalo. J. E. G. was supported by a fellowship from New York State.

ABBREVIATIONS: BLM, bleomycin; NCS, neocarzinostatin; AUR, auromomycin; SV40, simian virus 40; EDTA, ethylenediaminetetraacetate.

with their full complement of DNA-associated proteins are present in nuclei isolated from infected cells and can also be separated from the host's genome (22). These isolated and nuclear minichromosomes, as well as purified SV40 DNA and virally infected cells, can be used for assaying drug action of DNA in different environments. Single- and double-strand scission in these assays is monitored easily because naked SV40 DNA is supercoiled.

Once nicked in a single strand, supercoiled DNA forms a relaxed closed circle. If a double-stranded break occurs, the DNA is linearized. These three topological forms, supercoiled DNA, relaxed circular DNA, and linear DNA, can be separated by agarose gel electrophoresis. Previously, our laboratory and others have used the supercoiled DNAs from PM2 phage (23), pBR322 plasmid (21), and SV40 virions (24–26) to study strand scissions caused by drugs and ionizing radiation.

This paper describes the damage caused by BLM, NCS, and AUR to naked SV40 DNA, isolated minichromosomes, nuclear minichromosomes (minichromosomes in nuclei isolated from virus-infected cells), and intracellular SV40 DNA. Comparison of the drug-induced cleavage of naked DNA and isolated minichromosomes provides information on the effect of DNA association with nucleoproteins on drug action. Nuclear minichromosomes and intracellular DNA are similar in chromatin structure, and drug cutting in these two assay systems is studied to help elucidate how factors such as cellular permeability, drug metabolism (activation and inactivation), repair of drug cleavage, and other cytoplasmic factors might influence BLM-, NCS-, or AUR-induced single- and double-strand scission.

Materials and Methods

Drugs and reagents. BLM (Blenoxane) and NCS were supplied by Bristol-Myers Co., Syracuse, NY. AUR was supplied in crude form by the Investigational Drug Branch of the National Cancer Institute and was further purified by the method of Woynarowski and Beerman (27). Materials for cell culture were purchased from Grand Island Biological Co., Grand Island, NY.

Growth of cells and virus. SV40 (strain 776) was grown in the BCS-1 line of African green monkey kidney cells. For these experiments, nearly confluent cells were infected with 5–10 plaque-forming units of SV40 per cell. After infection, the cells were maintained in minimal essential media supplemented with 2% fetal bovine serum and glutamine.

Isolation of SV40 DNA and minichromosomes. SV40 DNA was isolated from virus-infected BSC-1 cells 40 hr after infection, by the method of Hirt (28). The Hirt supernatant was further purified by RNase treatment (10 μg/ml, 1 hr at 37°) and phenol extraction. Following ethanol precipitation and pelleting of the Hirt supernatant, pure supercoiled DNA was isolated on CsCl/ethidium bromide gradients.

SV40 minichromosomes were isolated as described by Scott and Wigmore (29). Briefly, 40 hr after viral infection, BSC-1 cells were rinsed with phosphate-buffered saline and the cells were swollen for 20 min at 4° in 10 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, 10 mM NaCl. The swollen cells were scraped from the culture dishes and homogenized 10 strokes in a Dounce homogenizer using the A pestle. Nuclei were pelleted by centrifuging the lysed cells at 320 × *g* at 4° for 5 min. The nuclei were resuspended in 0.25% Triton X-100, 0.56 mM EDTA, 11 mM Tris, pH 7.8, and kept at room temperature for 15 min. One-half volume of 0.56 mM EDTA, 11 mM Tris, pH 7.8, 150 mM NaCl was then added and incubated for an additional 10 min at room temperature. The nuclear residue was pelleted at 320 × *g* for 5 min at 4° and the supernatant containing the minichromosomes was adjusted to 5% glycerol and either used immediately or frozen in single-use aliquots at −70°. The concentration of DNA in the minichromosomes was determined by comparing the fluorescence of viral DNA complexed with ethidium bromide with known standards after electrophoresis on 1% agarose gels.

Isolation of nuclei from SV40-infected BSC-1 cells. SV40-infected BSC-1 cells were removed from the culture dishes by trypsinization 40 hr after infection. The cells were then washed free of any medium and swollen for 10 min on ice in 20 mM Tris, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3% Nonidet P-40. Nuclei were pelleted from the swollen cells by centrifugation at 320 × *g* for 5 min at 4°C. The nuclei were then resuspended in 10 mM Tris, pH 8, 5 mM CaCl, repelleted, and resuspended in the same buffer. The amount of DNA in the nuclei preparation was estimated by measuring the absorbance at 260 nm in 0.1 N NaOH (A_{260} of 1 equals 35 μg/ml).

Drug treatment of SV40 DNA, isolated SV40 minichromosomes, and nuclear minichromosomes. Drug reactions were done in a reaction mixture containing 10 mM Tris, pH 8.0, 5 mM $CaCl_2$, and 2 mM dithiothreitol. Ferrous ammonium sulfate (1.0 nM final concentration) was added to the BLM reactions. Routinely, 0.1 μg of DNA was treated with reactions using purified DNA or isolated minichromosomes, whereas 10 μg of nuclei were treated. All reactions were done under reduced lighting at 37° for varying times: 20 min (NCS reactions and AUR treatment of nuclei), 45 min (AUR treatment of DNA and minichromosomes), or 5 min (all BLM reactions). Reactions were stopped by adding EDTA to 20 mM and cooling to 0°. Following drug treatment, isolated minichromosomes were incubated with 0.5% sodium dodecyl sulfate for 30 min at 37°, and nuclei were lysed with 0.5% Sarcosyl and incubated with 50 μg/ml of proteinase k (Boehringer Mannheim) at 37° for 18 hr. All of the drug-treated DNA and isolated minichromosomes and 5 μl of the drug-treated lysed nuclei were used for analysis.

Drug treatment of intracellular SV40 DNA. BSC-1 cells were grown in 35-mm dishes and infected as described. Drug treatments of infected cells were performed 40 hr after infection. NCS treatments were done in 0.5 ml of minimal essential medium-2% fetal bovine serum, and the AUR and BLM reactions were done in a volume of 1 ml. The drugs were allowed to interact with the cells for 30 min. The medium containing drug was removed by aspiration and the cells were lysed with 0.5 ml of 10 mM Tris, pH 8, 1% sodium dodecyl sulfate, and 10 mM EDTA. The lysates were treated with 50 μg/ml of proteinase K at 37° for 18 hr. Aliquots (5 μl each) were used for analysis.

Agarose gel electrophoresis. DNA samples were routinely loaded onto 1% agarose gels and electrophoresed at 30 V for 18 hr using 50 mM Tris, pH 8.3, 20 mM sodium acetate, 2 mM EDTA as the running buffer. The gels were stained in 0.5 μg/ml of ethidium bromide and photographed. The photographic negatives were scanned using a Helena Quick Scan densitometer, and the relative amounts of DNA were determined by cutting out and weighing the peaks from the scans. The value for the amount of supercoiled DNA was adjusted to account for the decreased amount of ethidium bromide binding (approximately 70%) to supercoiled DNA relative to relaxed DNA (30).

Results

Degradation of supercoiled SV40 DNA by BLM. Naked SV40 DNA, isolated minichromosomes, nuclear minichromosomes, and intracellular SV40 DNA were treated with BLM. The results of these cutting assays are shown in Fig. 1. In all four of these systems, the amount of supercoiled material decreases as the drug concentration is increased, and for all but the cell-free DNA, this decrease clearly appears to be exponential.

The drug concentration required to reduce the fraction of supercoiled molecules by 50% was determined for each of the four cutting systems. These concentrations are highlighted by the *arrows* on Fig. 1. When the cleavage of purified DNA and

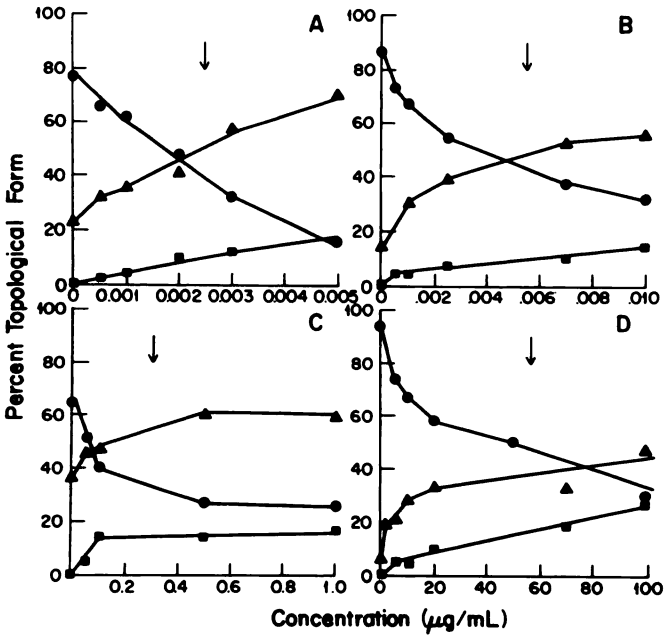

Fig. 1. BLM-induced cleavage of SV40 DNA and chromatin. Increasing concentrations of BLM were used to treat: A, naked SV40 DNA; B, isolated minichromosomes; C, isolated nuclei; and D, 35-mm dishes of SV40-infected BSC-1 cells (containing the equivalent of 6–10 μg of DNA). After treatment with BLM, supercoiled DNA (●), nicked circles (▲), and linear DNA (■) were separated on 1% agarose gels and analyzed as described in Materials and Methods. ↓, the drug concentration which caused a 50% reduction in supercoiled material. Endogenous endonuclease present in nuclei resulted in a lower percentage of supercoiled DNA from nuclear minichromosomes than in the other assays.

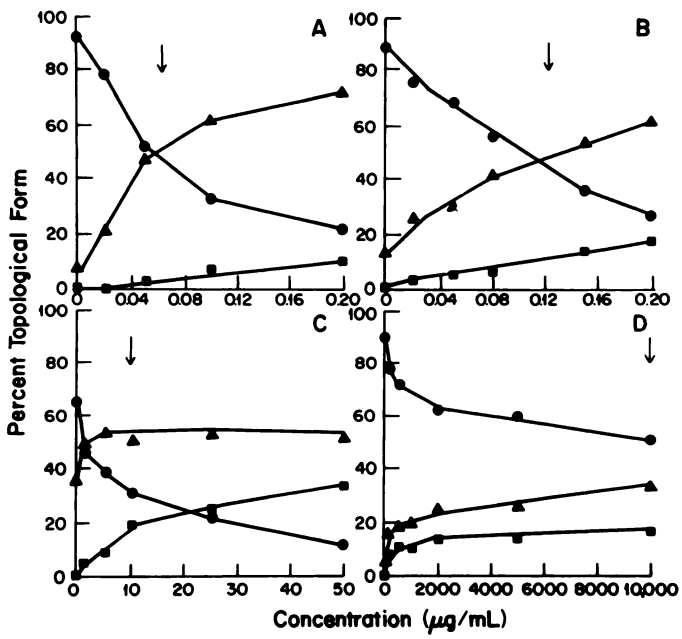

Fig. 2. NCS-induced cleavage of SV40 DNA and chromatin. Increasing concentrations of NCS were used to treat: A, naked SV40 DNA; B, isolated minichromosomes; C, isolated nuclei; and D, 35-mm dishes of SV40-infected BSC-1 cells (containing the equivalent of 6–10 μg of DNA). After treatment with NCS, supercoiled DNA (●), nicked circles (▲), and linear DNA (■) were separated and analyzed as described in Fig. 1.

isolated minichromosomes was compared, it was found that twice the concentration of BLM is required to cut isolated minichromosomes (Fig. 1B) as extensively as naked DNA (Fig. 1A) (0.0055 μg/ml compared with 0.0027 μg/ml, respectively).

When cell-free chromatin is cut with BLM, 100 times more DNA is treated in the assay using nuclei than in the assay using isolated minichromosomes (see Materials and Methods). Thus, it is probable that the 50-fold increase in BLM concentration needed to cut nuclear minichromosomes (Fig. 1C) to the same extent as isolated minichromosomes is due to the difference in the amounts of DNA in the systems.

In contrast, treatments of nuclear minichromosomes and intracellular SV40 DNA utilize similar quantities of chromatin. However, even without large differences in the amount or structure of the drug target, a 200-fold increase in BLM concentration is needed to cut 50% of the supercoiled material in intracellular DNA (Fig. 1D) as extensively as nuclear chromosomes.

Degradation of supercoiled SV40 DNA by NCS and AUR. Naked SV40 DNA, isolated minichromosomes, nuclear minichromosomes, and intracellular SV40 DNA were also treated with NCS and AUR. The results are shown in Fig. 2 (NCS) and Fig. 3 (AUR). Comparison of the NCS-induced cutting of naked DNA (Fig. 2A), isolated minichromosomes (Fig. 2B), and nuclear minichromosomes (Fig. 2C) reveals that the fraction of supercoiled molecules decreases with increasing drug concentration in all three of these cutting assays. In contrast, most of the breaks made by NCS on intracellular SV40 DNA (Fig. 2D) occur at the lower drug concentrations used in this assay, and little additional cutting is seen as the

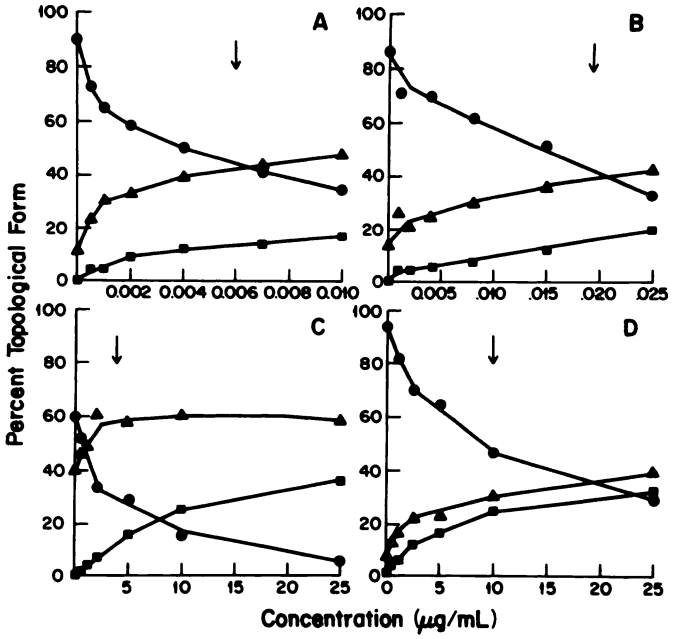

Fig. 3. AUR-induced cleavage of SV40 DNA and chromatin. Increasing concentrations of AUR were used to treat: A, naked SV40 DNA; B, isolated minichromosomes; C, isolated nuclei; and D, 35-mm dishes of SV40-infected BSC-1 cells (containing the equivalent of 6–10 μg of DNA). After treatment, supercoiled DNA (●), nicked circles (▲), and linear DNA (■) were separated and analyzed as described in Fig. 1.

NCS concentration is increased. Incubating the cells with drug for a longer period of time or an additional drug dose after 30 min of incubation does not significanlty increase the cutting of supercoiled material.[1] This pattern of cutting suggests that

[1] Unpublished observation.

binding sites necessary for NCS activity are being saturated by this drug. The nature of these putative sites is not yet resolved, but they could be involved in entry of the drug into the cell or in drug activation. AUR treatment resulted in dose-dependent decreases in the amount of supercoiled SV40 DNA in all of the four cutting assays.

The amount of NCS and AUR required to cut 50% of the supercoiled DNA in each of these four systems was evaluated (the *arrows* in Fig. 2, A–D, and Fig. 3, A–D designate these concentrations). As was seen with BLM, the NCS concentration required to cut naked supercoiled DNA must be doubled to cut isolated minichromosomes to the same extent. The 100-fold increase in NCS concentration that is needed to achieve equivalent cutting of nuclear and isolated minichromosomes is also similar to the results seen with BLM. Surprisingly, the cleavage of intracellular SV40 DNA by NCS requires more than 1000 times the drug concentration needed to cut nuclear minichromosomes. This is an even greater difference than was observed when BLM was used in these two cutting assays.

Like BLM and NCS, AUR also cuts naked DNA more extensively than isolated minichromosomes, although the AUR concentrations used in these two systems differ by 3-fold, rather than 2-fold as was seen with NCS and BLM. In addition, the increase in the amount of drug target also results in a greater AUR concentration being required to cut nuclear minichromosomes as extensively as isolated minichromosomes. In sharp contrast to the results obtained using BLM and CNS, AUR cuts intracellular SV40 DNA at drug concentrations only 3 times higher than the concentration used to cut nuclear minichromosomes.

Formation of double-stranded breaks by BLM, NCS, and AUR. The degradation of supercoiled material described above reflects both single- and double-stranded break formation by BLM, NCS, and AUR. It was also of interest to evaluate how chromatin structure, drug metabolism, and other cytoplasmic factors could influence the rate of formation of double-stranded breaks. The ability of these drugs to make this type of lesion was quantitated by measuring the fraction of linearized molecules made when 50% of the supercoiled molecules had been cut. These data are shown in Table 1. When BLM was used as the cutting agent, no significant difference in the amount of linear SV40 DNA made at 50% cutting of supercoiled material was seen in the four systems. In contrast, treatment of isolated minichromosomes with NCS resulted in 3 times more linearized molecules being made than were formed by NCS cleavage of naked DNA. Cutting of nuclear and intracellular viral chromatin also resulted in 1.5–2 times more SV40 DNA being linearized than was observed after NCS treatment of isolated minichromosomes. There is little difference in the number of linear molecules formed by AUR treatment of naked SV40 DNA, isolated minichromosomes, or nuclear minichromosomes. However, an approximately 2-fold increase in linear molecules is seen when intracellular SV40 DNA is treated with AUR compared with AUR-induced linearization in the other three cutting assays.

TABLE 1

Percentage of total SV40 DNA in the form of linear molecules after BLM, NCS, or AUR treatment of naked SV40 DNA, isolated minichromosomes, nuclear minichromosomes, and intracellular SV40 DNA

The experimental conditions and calculation of the percentage of linear DNA were as described in Materials and Methods. The fraction of linear DNA was determined at drug concentrations which caused 50% reduction in supercoiled DNA. Experiments were run a minimum of three times, and standard deviations are shown in parentheses.

Drug	Naked SV40 DNA	Isolated minichromosomes	Nuclear minichromosomes	Intracellular SV40 DNA
BLM	10 (±4)	9 (±4)	15 (±3)	16 (±4)
NCS	3 (±2)	10 (±2)	17 (±3)	20 (±3)[a]
AUR	13 (±3)	16 (±3)	11 (±2)	23 (±2)

[a] Fifty per cent cleavage of supercoiled DNA was not achieved when intracellular SV40 DNA was treated with NCS. This value was taken when 44% of the supercoiled DNA had been cut.

Repair of BLM-, NCS-, and AUR-induced damage to intracellular SV40 DNA. NCS, BLM, and AUR have been shown to stimulate unscheduled DNA synthesis in eukaryotic cells (31–33), and repair of double- and single-stranded cleavage of plasmid DNA has been described following BLM treatment of *Escherichia coli* transformed with the plasmid pBR322 (21). Thus, the amount of damage detected in the intracellular SV40 DNA cutting assay may be altered by the cellular processes which effect DNA repair. To assess the ability of the eukaryotic BSC-1 cell line to repair drug-induced damage to SV40 DNA, virus-infected cells were treated with BLM, NCS, or AUR for 30 min, washed free of drug, and then incubated in drug-free media for 0 min, 15 min, 30 min or 1 hr. After incubation, cells were lysed and the fraction of supercoiled, nicked circular, and linear SV40 molecules was determined. The results of this assay are shown in Fig. 4. No change in the amount of DNA in each topological form is detected up to 1 hr after removal of BLM (Fig. 4A), NCS (Fig. 4B), or AUR (Fig. 4C).

Discussion

In the studies presented in this paper, the DNA tumor virus SV40, in the form of naked DNA, isolated minichromosomes,

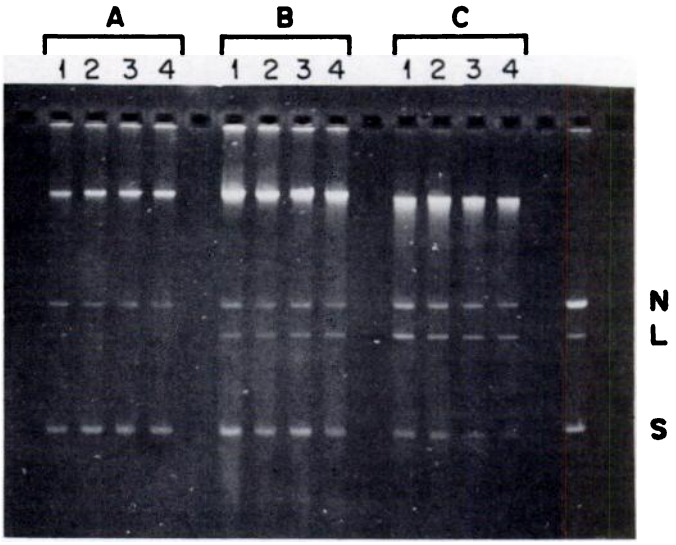

Fig. 4. Agarose gel analysis of repair of drug-treated SV40 DNA in BSC-1 cells. SV40-infected cells were treated for 30 min with: A, 20 μg/ml of BLM; B, 100 μg/ml of NCS; or C, 10 μg/ml of AUR. After treatment, cells were incubated for various periods of time in drug-free media and lysed as described in Materials and Methods. *Lanes: 1,* 0 min after removal of drug; *2,* 15 min of repair; *3,* 30 min of repair; *4,* 1 hr of repair. Markers for supercoiled (*S*), nicked circular (*N*), and linear SV40 DNA (*L*) are in the last lane. Genomic BSC-1 cell DNA appears as the band above the nicked circular SV40 DNA.

nuclear minichromosomes, and intracellular DNA was used as the target of BLM, NCS, or AUR. Comparison of drug action in these four systems was done to help differentiate influences of chromatin structure from the effects of other factors associated with eukaryotic cells such as cellular permeability, drug metabolism, and repair of drug-induced damage on DNA cleavage.

The concentration of drug required to reduce the amount of DNA in the supercoiled fraction by 50% was used to evaluate the total cleavage of SV40 DNA in the four cutting assays. The cutting of purified SV40 DNA and isolated minichromosomes was compared to examine the effect of nucleoproteins on drug activity in cell-free systems. The increased concentrations of BLM, NCS, and AUR needed to cut isolated minichromosomes as extensively as naked SV40 DNA, as well as the strong preference for the linker region betwen nucleosomes demonstrated by these drugs (11–13), strongly suggest that nucleosomal proteins are protecting the DNA from drug attack.

In addition to the presence of nucleoproteins, the DNA in minichromosomes differs from purified SV40 DNA in having a much lower degree of torsional stress (34), and it is possible that this may also affect drug activity. However, for BLM, at least, the increase in drug concentration needed to cut minichromosomes is probably not related to superhelical density because supercoiled pBR322 DNA is reported to be less sensitive to BLM-induced cutting than relaxed DNA (35). Differing responses to torsional stress, as well as varying abilities of these drugs to cut DNA adjacent to the nucleosome core, could explain why the increases in drug concentration needed to cut SV40 minichromosomes are similar for the three drugs, but not identical.

Although environmental and structural differences between the SV40 molecules in isolated and nuclear minichromosomes could contribute to the observed extent of DNA damage, it is not possible to make rigorous comparisons between drug action in these two systems since the latter contains nearly 100 times more DNA. In the nuclear system, the 50–200-fold increase in the dosage of all three drugs required to induce scission levels comparable to isolated minichromosomes does, however, suggest a nearly linear relationship between DNA damage and content.

To examine differences in drug activity in cell-free and intracellular systems, the cutting of nuclear minichromosomes and intracellular SV40 DNA was compared. In spite of similarities in total DNA content and SV40 chromatin structure, striking differences in cleavage were seen when BLM or NCS was used as the cutting agent (200- and 1000-fold, respectively). Since no repair of the damage to intracellular SV40 DNA caused by any of these drugs was detected, repair processes do not appear to affect the measurement of intracellular SV40 DNA cleavage. Cytoplasmic activation of BLM and NCS may be mechanistically different from the exogenous activation used when treating isolated nuclei with these two drugs, and it is possible that less drug is being activated inside cells than in the nuclear minichromosome cutting assay. However, it appears that decreased activation can only partially account for the 1000-fold difference in the cutting of nuclear minichromosomes and intracellular SV40 DNA by NCS, because when nuclei isolated from infected cells are treated with NCS using no added activators, the concentration of drug needed to cut 50% of nuclear minichromosomes increased by only a factor of 10.[2] The most probable explanations for the remaining 100-fold difference in the cutting of intracellular SV40 DNA and nuclear minichromosomes by NCS include decreased drug entry into the cell and/or cellular drug inactivation. The relative influences of cell permeability and drug metabolism on the cutting of intracellular DNA by BLM are also not clearly defined.

In sharp contrast to the large difference seen in the cutting of nuclear and intracellular DNA by BLM and NCS, the AUR concentration used to cut nuclear minichromosomes and intracellular SV40 DNA differs by only 3-fold. AUR has a lower requirement for exogenous activator than BLM or NCS, and AUR activation inside cells may not be quantitatively different from cell-free drug activation. Membrane permeability to AUR or a small amount of drug inactivation could explain the comparatively slight difference in cutting.

Single-stranded breaks are the predominant type of initial lesion made by BLM, NCS, and AUR, whereas double-stranded breaks occur at somewhat higher drug dosages (2, 8, 10). Increased formation of double-stranded breaks on chromatin relative to purified DNA may result from the focusing of single-stranded breaks of nucleoproteins. In addition, it is possible that the sequence preference or distribution of DNA breaks caused by drugs activated in a cell-free system is different from the cutting by drugs activated intracellularly. To evaluate the effect of environment on drug-induced double-stranded breaks, the percentage of total SV40 DNA in the form of linear molecules was measured when 50% of supercoiled material had been cut.

In these assays, comparison of the linearization of purified DNA and isolated minichromosomes reveals that only NCS-induced double-stranded break formation is increased by the presence of nucleosomal proteins. These results are not entirely consistent with previous reports which demonstrated that nucleosomes influence drug action by focusing the breaks made by these three drugs in the linker regions of chromatin (11–13). In these reports, the degradation of bulk chromatin by BLM, NCS, or AUR results in formation of mononucleosomes, which requires that one double-stranded break be made at intervals of 170–180 base pairs. However, in the SV40 systems, the DNA molecules were linearized with only one double-stranded break occurring in the 5243-base pair SV40 genome. Thus, the extent of drug cleavage of SV40 DNA in these assays is lower than the cleavage levels in the studies on bulk chromatin and may not be extensive enough to detect an influence due to the presence of nucleoproteins on BLM- or AUR-induced formation of double-stranded breaks. Possibly, the effect of nucleosomal proteins on NCS-induced cleavage is more pronounced and, thus, detectable by our assays. In addition, the slight increase in linearization seen when comparing NCS-induced linearization of nuclear and isolated minichromosomes may reflect this drug's ability to recognize more complex structural differences in chromatin.

Cytoplasmic metabolism appears to increase linearization of SV40 DNA by AUR but has little effect on double-strand breakage by BLM and NCS. AUR also differs from NCS and BLM in its decreased requirement for exogenous activation and in the increase in drug concentration required to cut intracellular SV40 DNA as extensively as nuclear minichro-

[2] Unpublished data.

mosomes. The reasons why this drug, which is structurally more similar to NCS than is BLM, should differ from NCS and BLM in these ways are not yet clear. NCS and AUR are more similar to each other than to BLM in having higher double-stranded cleavage activities (due to nucleoproteins or cytoplasmic metabolism) on intracellular SV40 DNA than on purified SV40 DNA.

No repair of single- and double-stranded breaks made by BLM, NCS, and AUR in intracellular SV40 DNA was detected up to 1 hr after removal of drug from the cells, although treatment of eukaryotic cells with BLM (31), NCS (32), and AUR (33) has been reported to stimulate unscheduled DNA synthesis, and repair of BLM-induced damage to pBR322 in *E. coli* has also been reported (21). The absence of detectable repair on intracellular SV40 DNA reflects the inability of the eukaryotic BSC-1 cells to carry out one or more of the processes (which include excision, gap filling, ligation, and nucleosome assembly) needed to regenerate intact SV40 chromosomes.

This paper describes the cleavage activity of BLM, NCS, and AUR on SV40 DNA in different environments. In addition, examining double-stranded break formation on purified DNA, cell-free chromatin, and intracellular chromatin can provide information on the distribution of single-stranded breaks induced by these drugs in the SV40 genome. Previous reports have suggested that the site of drug-induced breaks may be affected by localized changes in the structure of chromatin, such as those associated with actively transcribing genes. In addition, the choice of activator (sulfhydryl or ferrous) used to stimulate BLM cleavage *in vitro* can affect the sequence preference of this drug (16). Currently, the locations of preferred breaks made by these three drugs on naked SV40 DNA, nuclear minichromosomes, and intracellular SV40 DNA are being mapped. The cutting sites of these drugs on the SV40 genome in these different environments are being compared to determine the effect of chromatin structure and drug metabolism on the sites of drug cleavage.

Acknowledgments

The authors would like to thank Frank Rauscher III for generously supplying purified auromomycin, Sheila Flanagan for assistance with the nuclei assays, and Mary McHugh, Richard Beckmann, and Nina Ruth Wright for aid in editing the manuscript.

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