Effects of DNA Superhelical Changes Induced by Ethidium Bromide on the DNA-degrading Activity of Two Antitumor Antibiotics, Bleomycin and Phleomycin

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ABSTRACT

The effects of changes in the conformational state of DNA on the single-strand and double-strand breakage activity of two antitumor antibiotics, bleomycin (BLM) A2 and phleomycin D1, have been studied by the gel electrophoretic analysis of the drug-degraded PM2 phage superhelical DNA pretreated with an intercalating agent, ethidium bromide (EB). Both the single-strand and double-strand breakage activities of BLM A2 increased as the negatively superhelical turns of native PM2 DNA were gradually removed by intercalation with increasing EB concentrations. The activities peaked when DNA was completely relaxed and gradually decreased as the higher concentrations of EB twisted DNA into the positively superhelical form. The decrease in breakage activity was not due to any inhibitory effect of EB at higher concentrations, since treatment of the relaxed Form I DNA with low EB concentrations also reduced the activity. In contrast to BLM A2, phleomycin D1 responded minimally to DNA conformational changes, which suggested further that the two drugs may react with DNA differently. The differential responses of BLM A2 activity towards different DNA conformational states may have biological implications, since DNA in cells may exist in different conformational states relating to various gene functions. The current study may serve as a model for studying combined effects of intercalative and nonintercalative antitumor antibiotics which are used frequently in combination treatments of cancer.

INTRODUCTION

BLMs are a family of glycopeptide antitumor antibiotics used in clinical treatment of certain human cancers (6). The antitumor activity of BLMs is thought to be related to their ability to induce SS and DS DNA breaks (15, 33, 48, 50). The NH2-terminal half-molecule of BLM may complex with Fe(II) and oxygen (8, 27, 46) and may produce free radicals which cause DNA strand breaks. The COOH-terminal half-molecule may interact with specific DNA sequences (9, 18, 23, 25, 26, 31, 49), in a specific manner (16, 18, 26), such that free radicals may cause either SS or DS (or both) DNA breaks.

We have reported previously the importance of the NH2-terminal BLM half-molecule portion, which presumably coordinates with Fe(II), in the BLM-DNA interaction and the degradation of DNA (17–19). Recently, a similar concept has also been proposed by Povirk et al. (42). Although the COOH-terminal BLM half-molecule portion has been suggested to interact with DNA, the DNA-degradative activity of several BLM analogues was not correlated with their interaction with DNA (18, 23) but was correlated with the Fe(II)-induced quenching effect on the BLM fluorescence (17). Structural modifications on the NH2-terminal BLM half-molecule portion affected not only the BLM interaction with DNA but also the extent of production of either SS or DS DNA breaks (19). These observations led us (17, 18) to suggest the importance of a proper conformation of the Fe(II)-BLM complex in the action of BLM on DNA. Similar emphasis has also been suggested by Sugiyama et al. (37) and Oppenheimer et al. (37). It is possible that the overall conformational state of the complex of BLM, Fe(II), and DNA is also important in the degradative activity.

The overall conformational arrangement of the drug/DNA/Fe(II) complex may also be affected by the conformational state of the DNA, i.e., at the level of either the superstructure (superhelix) or the double helix if a superhelical DNA is used. Thus, we have studied here the effects of the conformational changes of the superhelical PM2 DNA induced by a DNA intercalator, EB, on the DNA-degradative activity of BLM drugs such as BLM A2 and PLM D1. The observation that BLM A2 but not PLM D1 produced DS breaks, in conjunction with other observations, has led us to suggest that the 2 drugs may differ in the mode of interaction with DNA (19). The reduction of a double bond in the coplanar bithiazole moiety (for structure, see Ref. 17) renders the intercalation of PLM D1 with DNA more difficult. Thus, the EB-induced conformational changes in DNA can serve as a system for probing the differences between these 2 drugs more extensively.

Furthermore, we considered it important to study the effects of DNA conformation on the degradative activity of BLMs, since DNA in cells may exist in different conformational forms which are organized into various types of higher-ordered genomic structures such as nucleosomes and superhelical or solenoidal arrays of polynucleosomes (12, 16, 35, 44, 54) and nucleoids (5, 28). A portion of this study has been presented at a symposium (7).

MATERIALS AND METHODS

Materials. BLM A2 and PLM D1 were obtained from Bristol Laboratories, Syracuse, N. Y. Covalently closed, circular, superhelical Form I PM2 phage DNA was isolated as described previously (19). The DNA preparations used contained at least 85% Form I superhelical DNA. The relaxed, circular, duplex Form I PM2 DNA was prepared by treatment of Form I DNA with calf thymus topoisomerase (32). EB, Tris, borate, disodium EDTA, and β-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Mo. Agarose-ME was purchased from Miles Laboratories, Elkhart, Ind.

Assays of DNA Breakage Activity of BLM and PLM by Gel Electro-
Details of the assay procedures have been described previously (19, 30). A reaction mixture (final volume, 0.15 to 0.20 ml) containing 50 mM borate buffer (pH 9.5), 66 mM NaCl, 110 to 140 μM PM2 Form I or Form II DNA preparations, and various amounts of EB, if added, was incubated for 5 min at room temperature. After incubation, 25 mM β-mercaptoethanol and a given amount of BLM A2 or PLM D3, from 17 to 30 nm, were added to start the DNA-degradative activity. After 30 min at room temperature, the degradative activity was terminated with the addition of an equal volume of a mixture containing 56% glycerol (v/v), 50 mM EDTA, and 0.5% bromophenol blue (w/v). Aliquots containing 0.8 to 1.5 μg DNA were layered onto a 0.9% agarose slab gel and were electrophoresed in a horizontal slab gel apparatus for 6 to 8 hr at room temperature with a 40 mM Tris-HCl buffer containing 5 mM sodium acetate and 1 mM EDTA, pH 7.8. After electrophoresis, gels were stained with EB (0.5 μg/ml) in the electrophoresis buffer for at least 2 hr. The stained gels were then excited with a transilluminator (Ultra-Violet Products, Inc.) and photographed with a Polaroid CU-5 Land Camera equipped with a No. 8 Kodak Wratten gelation filter (Eastman Kodak Co., Rochester, N. Y.) and Type 665 Land Films. The negative film of gel was used for determination of SS and DS DNA breakage activity by densitometry.

Quantitation of SS and DS DNA Breaks by Densitometric Scannings of Negative Films of Gels. The negative films of the EB-stained gel patterns of the drug-treated PM2 DNA were scanned with a recording Transdyne General Densitometer equipped with an automatic computing integrator. As illustrated in Chart 1, the production of the nicked, relaxed, duplex Form II DNA and the linear Form III DNA from the covalently closed, superhelical Form I was considered to be the result of SS and DS breaks, respectively (19, 25, 26, 30). The relative stainability of the Forms I, II, and III of DNA in the agarose gels was tested experimentally with known amounts of respective DNA preparations. In agreement with previous reports (25, 26), it was found that at the same amount of DNA, the EB fluorescence intensity of Form I DNA was only 70% of that of Form II or Form III DNA; thus, this factor was used to normalize all observations. In agreement with the report of Prunell et al. (43), at the EB-CsCl gradient to remove the small amount (<30%) of broken DNA (Forms II and III) resulting from the topoisomerase treatment. As performed routinely in the purification of Form I DNA from either PM2 phage (19) or pBR322 plasmid (30, 32), the EB in the EB-DNA complex from the EB-CsCl gradient was removed by extracting 5 times with NaCl-saturated isopropanol alcohol and 5 times with ether. Ether was then removed by bubbling air through solution. Form I DNA was then precipitated with 70% ethanol. The purified preparations contained 88 to 92% unbroken DNA as assayed from EB-agarose gel which separates all of the unbroken DNA molecules from Form II and Form III DNA. The DNA preparations were treated with BLM A2 (20 nm) in borate buffer (pH 9.5) at 37°C for 30 min and analyzed by agarose gels with or without EB.

RESULTS

DNA Breakage Activity of BLM A2 with EB-treated PM2 DNA. Chart 1 illustrates the EB/PM2 DNA system used for studying the effects of DNA conformational changes on the DNA-degradative activity of BLM A2. The addition of low concentrations of EB causes the unwinding of the double-helix structures and the reduction of the number of the negatively supercoiled turns of the native Form I (or I) PM2 DNA. Addition of more EB eventually removes all the negatively supercoiled turns of DNA to form the relaxed, double-stranded, circular Form II DNA. Upon further addition of EB, the Form II DNA supercoils in the opposite direction to become positively supercoiled Form I+ DNA. This type of superhelical change is a relatively specific effect of intercalative agents on covalently closed, circular DNA (2).

The system used for studying the SS and DS breakage activities of BLM A2 and PLM D3 has been described previously (19, 25, 26, 30). The introduction of a SS break (nick) converts Form I, II, or III DNA to the nicked, relaxed, circular Form II DNA. The introduction of a DS break converts Form I+, II+, or III DNA to a linear Form III DNA. These forms of PM2 DNA differ in mobility in agarose gels. In a 0.9% gel, Forms II+ and III have the fastest mobility, followed by Form III, then by Form II, and then by Form II DNA which comigrates with Form II+. Thus, the production of Form III DNA results primarily from the DS breaks. Fig. 1 shows the agarose gel electrophoretic pattern of the PM2 DNA products after DNA was pretreated with increasing concentrations of EB and then degraded with a fixed concentration of BLM A2 (20.7 nm). Lane a shows the untreated, superhelical Form I DNA preparation which primarily contained Form I DNA and a trace amount of Form II DNA. When treated with BLM A2 in the absence of EB (Lane b), both the nicked Form II (upper band) and the linear Form III (middle band) were produced at the expense of Form I DNA (lower band). Lanes c to m show the BLM A2-degraded DNA products when DNA was pretreated with increasing concentrations of EB. These results show that, as the EB concentration was increased, the production of both Form II and Form III DNA increased and then decreased after reaching a maximum at an EB/DNA ratio of between 0.10 (Lane f) and 0.11 (Lane g).

The gel pattern of PM2 form I DNA after treatment with EB at EB/DNA equal to ratios used in Fig. 1, but without addition of BLM A2, was also obtained (not shown). No production of either Form II or Form III DNA from Form I DNA was detected. With the increasing concentration of EB, the mobility of the Form I DNA band was slightly reduced and was then restored as more EB was added, in a manner similar to that shown by the residual Form I DNA band after treatment with EB and BLM A2 (Fig. 1).
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Fig. 1. Agarose gel electrophoretic pattern of DNA products after treatment of PM2 Form I DNA (11.5 µg) with a fixed amount of BLM A2 (20.7 µM). PM2 Form I DNA was pretreated with increasing concentrations of EB. Gel mobility, top to bottom: fast-moving band, Form I (or 1) DNA; slow-moving band, Form II DNA; intermediate band, Form III DNA. Lane a, untreated Form I DNA; Lanes b to m, BLM A2-degraded DNA which was pretreated with EB at EB/DNA concentration ratios of 0 (Lane b), 0.02 (Lane c), 0.04 (Lane d), 0.07 (Lane e), 0.10 (Lane f), 0.11 (Lane g), 0.13 (Lane h), 0.15 (Lane i), 0.17 (Lane j), 0.19 (Lane k), 0.22 (Lane l), and 0.26 (Lane m).

Chart 2. Percentage distribution of DNA conformational forms after treatment of Form I DNA (115.8 µg) with 20.7 µM BLM A2. DNA was pretreated with increasing concentrations of EB. Percentage distribution was measured from the gel electrophoretic pattern shown in Fig. 1. □, Form I DNA; △, Form II DNA; ○, Form III DNA.

Intercalation of EB induces superhelical relaxation and thus should change the gel electrophoretic mobility of superhelical DNA. However, the mobility changes are usually not obtained in slab gels because of the dissociation of bound EB from DNA during electrophoresis. To observe these changes, disc gels have to be used, and each of the gels has to contain equivalent amounts of EB throughout the electrophoresis to retain the bound EB (11).

The gel pattern shown in Fig. 1 was analyzed quantitatively, and the results were shown in Chart 2, in which the percentage distribution of each DNA conformational form was plotted against EB/DNA ratios. The untreated DNA preparation contained 90% Form I DNA and 10% Form II DNA. Degradation of DNA with BLM A2 in the absence of EB pretreatment resulted in 61% Form I, 33% Form II, and 6% Form III DNA. The maximal production of Form II DNA (54%) occurred at an EB/DNA ratio of between 0.10 and 0.11, whereas that of Form III (15%) occurred at a ratio of 0.10. These results show an approximately 2-fold increase in the production of both Form II and Form III DNA as compared to that in the absence of EB treatment, and the SS/DS DNA break ratio did not change significantly. At higher ratios, the production of Forms II and III decreased gradually. At a ratio of 0.26, there were 39% Form II DNA and 7% Form III DNA.

Degradation of Form I DNA with BLM A2. To exclude the possibility that EB at concentrations higher than a ratio of 0.10 inhibited the DNA-degradative activity of BLMs and thus contributed to the decline in the activity of BLM A2 shown in Chart 2, we have studied (Fig. 2) the degradation by BLM A2 of the EB-pretreated PM2 Form I DNA which was produced by treatment of PM2 Form I DNA with calf thymus topoisomerase (32). Since Form I DNA produced by topoisomerase is virtually totally relaxed, the addition of a small amount of EB (EB/DNA < 0.1) turns Form I to 1' DNA. Lane a shows that untreated Form I DNA which, due to the presence of a high concentration of EB (0.5 µg/ml) in the gel electrophoretic system, has become positively superhelical and thus migrated to a position equivalent to that of a native superhelical DNA such as Form I DNA. In a gel without EB, Form I DNA would comigrate with Form II DNA. In Lane b, the Form I DNA was degraded with BLM A2 in the absence of EB pretreatment. The Form I DNA was degraded almost completely and produced Form II (slow-moving band) and Form III DNA. Lanes c to j show the pattern of degraded DNA products from BLM A2 treatment of Form I DNA which has been pretreated with increasing amounts of EB (from an EB/DNA ratio of 0.022 to 0.20). It is clear that when PM2 Form I DNA was pretreated with increasing concentrations of EB, the production of both Form II and Form III DNA decreased, and the retention of the undegraded Form I increased.

The quantitative relationship of the changes among 3 DNA forms described in Fig. 2 is shown in Chart 3. The untreated PM2 DNA preparation shown in Fig. 2, Lane a, contains 85% Form I DNA, 15% Form II DNA, and virtually no Form III DNA. The degradation by BLM A2 of PM2 Form I DNA which was not pretreated with EB resulted in 7% DNA in Form I, 59% DNA in
Form II, and 34% DNA in Form III species. Treatment of DNA with a low concentration of EB, e.g., an EB/DNA ratio of 0.022, caused a significant inhibition of DNA-degradative activity of BLM A<sub>2</sub> and resulted in 28% DNA in Form I<sup>0</sup>, 52% in Form II, and 20% in Form III species. Pretreatment of Form I<sup>0</sup> DNA with increasing concentrations of EB increased the extent of inhibition on the degradative activity of BLM A<sub>2</sub>. As a result, the percentage of DNA remaining in the undegraded Form I<sup>0</sup> increased, whereas that of Forms II and III decreased significantly. Thus, the inhibition by low concentrations of EB on the BLM A<sub>2</sub>-induced degradation of Form I<sup>0</sup> DNA contrasts with the stimulatory effect of low EB concentrations (EB/DNA < 0.1) on the degradation of the negatively superhelical Form I DNA (Chart 2). The observation is consistent with the interpretation of results shown in Fig. 1 in which the increase in positive superhelical turns of DNA reduced the degradative activity of BLM A<sub>2</sub>. Effects of Topoisomerase-induced Superhelical Relaxation on Degradative Activity of BLM A<sub>2</sub>. To substantiate our conclusion that decreasing negative superhelical turns increases the degradative activity of BLM A<sub>2</sub>, we have prepared circular pBR322 DNA preparations which were relaxed to different extents by the treatments of increasing amounts of calf thymus topoisomerase. All preparations were repurified through EB-CsCl gradient to remove the small amount (<30%) of Forms II and III produced as a result of topoisomerase treatments. These preparations are shown in Fig. 3. Each of the topoisomerase-treated preparations shows a series of DNA bands differing by one superhelical turn. Treatment with more topoisomerase resulted in more reduction of the gel mobility of DNA due to more superhelical relaxation. These preparations were then treated with the same amount of BLM A<sub>2</sub> and analyzed with an EB gel (not shown), which converted all of the unbroken DNA molecules into the positively superhelical form and thus separated these from Form II and III DNA molecules produced as a result of BLM A<sub>2</sub> treatment. The results are shown in Table 1, which shows an increase in the production of Form II DNA with treatments of increasing amounts of topoisomerase. These results are consistent with those obtained from the EB-treated DNA preparations.

Degradation of EB-treated Form I DNA with PLM D<sub>1</sub>. Fig. 4 shows the gel pattern, and Chart 4 shows the quantitative analysis of the DNA products after the EB-treated Form I DNA was degraded with a fixed amount of PLM D<sub>1</sub>. The results confirmed our previous observation (19) that, under the conditions used, PLM D<sub>1</sub> caused primarily SS DNA breaks, resulting in the production of Form II DNA. In contrast with the observations for BLM A<sub>2</sub> activity (Chart 2), the production of Form II DNA by PLM D<sub>1</sub> was not affected significantly by the pretreatment of DNA with increasing amounts of EB.

Degradation of EB-treated Form I<sup>0</sup> DNA with PLM D<sub>1</sub>. Chart 5 shows the quantitative analysis of the production of Forms II and III DNA from EB-pretreated Form I<sup>0</sup> DNA which was produced by topoisomerase. The data were obtained from the scans of EB-containing gel patterns of the PLM D<sub>1</sub>-degraded DNA products (not shown). The results show clearly that pretreatment with EB had little effect on the extent of Form I<sup>0</sup> DNA degradation by PLM D<sub>1</sub>.

**DISCUSSION**

Results in this study show that both the SS and the DS breakage activities of the BLM A<sub>2</sub> responded to the alterations in the superhelicity of PM2 DNA. Relaxed DNA form was significantly more sensitive to degradation by BLM A<sub>2</sub> than was either the negative or the positive superhelical DNA. When Form I DNA was treated with EB, the sensitivity to degradation by BLM A<sub>2</sub> increased until an EB/DNA ratio of 0.10 to 0.11 was used, at
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which point Form I DNA was maximally relaxed (i.e., Form I DNA). That the decrease in sensitivity to degradation by BLM A₂ observed at EB/DNA ratios > 0.11 was due to the introduction of the positive superhelicity in the DNA rather than to the inhibitory effect of higher concentrations of EB on the degradative activity is demonstrated by the following observations: (a) The EB/DNA ratio of 0.10 (Chart 2) required to induce the maximal degradative activity of BLM A₂ approximates the ratio of 0.091 we have reported previously (20) for EB to completely remove the superhelical turns of PM2 DNA; and (b) the induction of the relaxed Form I DNA by EB at EB/DNA ratios > 0.10 into positive superhelical form resulted in a reduction of the degradative activity of BLM A₂. In agreement with these interpretations, we have also observed that BLM A₂ was more active towards the more relaxed pBR322 DNA prepared by topoisomerase treatment.

The reason for the increase in DNA-degradative activity of BLM towards the superhelically relaxed DNA is unclear to us at present. The relaxation in the DNA tertiary structure may have facilitated the interaction of BLM A₂ with specific sites of DNA molecules, thus increasing the rate of degradation. It is also possible that the relaxation of the DNA superhelical structure may open new sites in DNA molecules for degradation by BLM A₂. Whether the site-sequence specificities of the BLM A₂-induced DNA breaks are altered by the changes in DNA superhelicity is currently under study.

The phenomenon is not unique to EB, since we have observed a similar effect induced by a bifunctional intercalative agent, BBM-928A (20, 21). The maximal increase in the BLM A₂ degradative activity was observed at a concentration of BBM-928A which induced a maximal superhelical relaxation. Thus, the conformation of the DNA clearly affected the activity of BLM A₂.

In genetic structures of eukaryotic systems, DNA may assume specific conformational states such as superhelical or solenoidal forms found in higher-ordered structures such as nucleosomes and their polynucleosomal arrays (12, 14, 16, 35, 38, 44, 54) and nucleoids (26, 28). Nucleoids were suggested to contain superhelical DNA with topological constraint similar to that of circular DNA. Unfolding or relaxation of nucleosomes by EB has been well studied (39, 55). These superstructures can provide a way of controlling gene activities such as transportation, repair, and replication (1). Changes of these structures have been proposed to be associated with certain gene functions and regulations. For example, transcriptionally active genomic structures may be partially unfolded or may be different from transcriptionally inactive structures (13, 34, 40, 53). A decrease in number of topological turns was associated with differentiation in nucleoids of Friend erythroleukemia cells (28). Relaxation of supercoiling was related to initiation and elongation of DNA synthesis (29). Superhelix-altering enzymes such as topoisomerases (including gyrase) have been suggested to play an important role in gene function (4, 22, 24, 36, 51). Thus, the differences in the degradative activity of BLM A₂, depending on the conformational states of genomic DNA, may have different biological consequences.

In addition, the combined effects of an intercalating agent, EB, and a DNA-degrading agent, BLM A₂, may be of interest when considering the use of combination chemotherapy involving BLM and DNA-intercalative antitumor antibiotics. Several groups of intercalative antibiotics or synthetic compounds with antitumor activities have been reported, and some of them have been used in clinical treatment of certain cancers. These include the monofunctional agents such as anthracyclines (10) and the bifunctional agents such as the diacridines (3), pyridocarcabzone dimer (45), echinomycin (52), and BBM-928A (20, 21).

In contrast with BLM A₂, the activity of PLM D₁ was not affected significantly by the EB-induced DNA superhelical conformational changes. The reason for this difference is unclear. PLM D₁ is actually 7,8-dihydrobleomycin B₂, and one of the double bonds in one of the 2 coplanar thiazole rings is reduced by hydrogenation which renders the intercalation with DNA difficult. The different responses between BLM A₂ and PLM D₁ may have resulted from the thiazole ring modification, since BLM B₂ behaved in a manner similar to that of BLM A₂ (data not shown). Several differences between BLM and PLM in the interaction with DNA have been summarized (19). For example, both BLM A₂ and PLM B₂ produced SS and DS breaks in a similar manner, whereas PLM D₁ produced essentially only SS breaks (19).

Recent studies of Povirk et al. (42) also suggested differences between BLM and PLM in their interaction with DNA. The destruction of the coplanar bithiazole rings could affect the spatial arrangement of the 2 rings and of the COOH-terminal amine relative to the rest of the drug molecule. Consequently, the intercalation of bithiazole with DNA was prevented, and the interaction of COOH-terminal amine with DNA was altered. Thus, it is possible that the overall conformational arrangement of DNA-drug complex is different between BLM A₂ and PLM D₁, and as a result these 2 drugs responded differently to changes in DNA superhelicity.

Another possible difference in the spatial arrangement of the DNA-drug complex between the 2 drugs could be that, while interacting with DNA, BLM A₂ may form dimers through stacking of the coplanar bithiazole moieties, whereas PLM D₁ may fail to do so. The activity of the dimeric BLM A₂ molecules, the existence of which has been suggested by Lloyd et al. (26) to account for the production of SS as well as DS breaks, may be more sensitive to the superhelical changes, whereas the activity of the monomeric PLM D₁, which produced mainly SS breaks (19), may be less sensitive.

Our observation that the relaxed PM2 DNA was more sensitive to the BLM A₂ degradative activity differs from those reported...
by Lloyd et al. (26) and Povirk et al. (41). Using a gel electrophoretic system, Lloyd et al. (26) found that Form I PM2 DNA was slightly more sensitive than Form I DNA to the clinical preparation of bleomycins. By studying the sedimentational properties, Povirk et al. (41) reported the same preference to argue in favor of the presence of the intercalation of the bithiazole moiety with DNA. Currently, we have no clear explanation for this discrepancy. However, we want to emphasize that our conclusions were derived from studies of the effects on the BLM A2 activity with systematic changes in superhelicity of the same DNA preparation. In our hands, we observed some variations in the DNA-degradative activity of BLM A2 depending upon the Form I DNA preparations. We have observed that the concentration of BLM A2 to cause breakage of 50% of DNA molecules could vary from 15 to 32 μM in different Form I DNA preparations (19). We also observed variations in the degradative activity of BLM A2 on different preparations of purified Form I DNA, depending very much on the amounts of the accompanying Form II DNA in the Form I preparations. This type of variation may complicate any direct comparison between Form I and Form I DNA preparations. Nevertheless, we have approached this problem by using preparations of pBR322 DNA treated with varying amounts of topoisomerase and purified by EB/CsCl gradient centrifugation. These studies confirm the observations made using EB treatment.

Furthermore, the present system using EB to induce superhelical changes encompasses the changes in negative as well as positive superhelicities of DNA. EB-free circular DNA preparations with different degrees of negative superhelicity can be prepared by the treatment of topoisomerase in a manner similar to that for our Form I DNA, but in the presence of varying amounts of EB, which is removed subsequently. However, such a procedure cannot produce positively superhelical DNA forms. In our study, the presence of EB during the digestion of DNA by BLM may lead to the possibility that EB at low concentrations may directly stimulate and, at high concentrations, inhibit the BLM A2 activity dependent on the decrease or increase in superhelicity of DNA rather than on the changes of the EB concentration. The senseness of superhelicity seemed to be irrelevant. Thus, we think that the present system provides a simple system for studies of the effects of DNA superhelical changes and is confirmed by the studies using topoisomerase.

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Fig. 2. Gel electrophoretic pattern of DNA products after treatment of PM2 Form I DNA (113.6 µm) with a fixed amount of BLM A2 (30.7 µm). Isolated Form I DNA was pretreated with increasing concentrations of EB. Gel mobility, top to bottom. Both gel and electrophoretic buffers contain EB (0.5 µg/ml) to convert the undegraded Form II DNA into positively supercoiled form which has a higher mobility than that of Form II or Form III DNA. Slow-moving band, Form II DNA; intermediate band, Form III DNA. Lane a, control; EB/DNA concentration ratios: 0 (Lane b), 0.022 (Lane c), 0.038 (Lane d), 0.050 (Lane e), 0.066 (Lane f), 0.080 (Lane g), 0.124 (Lane h), 0.164 (Lane i), 0.201 (Lane j).

Fig. 3. Gel electrophoretic pattern of DNA products of pBR322 plasmid Form I DNA preparations (25 µg) relaxed partially by treatments with increasing amounts of calf thymus topoisomerase I (1 hr at 37°). Before being applied to gels, all DNA preparations were treated with EB/CsCl gradients to remove Forms II and III DNA. Slow-moving band, contains Form II DNA; intermediate band, Form III DNA. Lane a, control; EB/DNA concentration ratios: 0 (Lane b), 0.022 (Lane c), 0.038 (Lane d), 0.050 (Lane e), 0.066 (Lane f), 0.080 (Lane g), 0.124 (Lane h), 0.164 (Lane i), 0.201 (Lane j).

Fig. 4. Gel electrophoretic pattern of DNA products of treatment of PM2 Form II DNA with a fixed amount of PLM D (20.7 µm) in the presence of increasing EB concentrations. Gel mobility, top to bottom. Lane A, untreated Form I DNA; Lanes B to M, EB/DNA ratios of 0 (Lane B), 0.020 (Lane C), 0.045 (Lane D), 0.070 (Lane E), 0.10 (Lane F), 0.12 (Lane G), 0.14 (Lane H), 0.16 (Lane I), 0.17 (Lane J), 0.18 (Lane K), 0.22 (Lane L), and 0.26 (Lane M).
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