Altered Gel Electrophoretic Mobility of Bleomycin-mediated Release of Nucleosomal DNA

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ABSTRACT

Nucleosomal DNA isolated from bleomycin-treated nuclei shows retarded electrophoretic mobility in neutral gels as compared with the sample isolated from micrococcal (or endogenous) nuclease-digested nuclei. The retardation in electrophoretic mobility is probably due to the presence of single-strand regions in the DNA duplex of bleomycin-treated samples as determined by single-strand-specific S1 nuclease digestion and buoyant density measurements. This observation argues against the possibility that the generation of nucleosomal DNA following drug treatment of nuclei is due to an activation of endogenous nuclease by bleomycin and strongly suggests that the drug has a unique feature of action on chromatin.

INTRODUCTION

The bleomycins, a group of glycopeptide antibiotics produced by Streptomyces verticillus, were isolated by Umezawa et al. (24). These antibiotics have been used in the treatment of human cancers (1, 3). The primary action of bleomycins that leads to cytotoxicity has been thought to be degradation of DNA. (For an extensive review of the action of bleomycin, see Ref. 7.) Bleomycins have been shown to cause both single- and double-stranded breaks (6, 14, 22). The breaks seem to occur preferentially at certain dinucleotide sequences depending upon the presence of ferrous ion (4, 23). Release of all 4 free bases from DNA and destruction of sugar moieties were also detected when DNA was incubated with high concentrations of bleomycin (8, 12). The action of bleomycin on DNA seems to involve physical binding (15).

We have shown previously that bleomycin can cause release of nucleosomes from isolated nuclei (10, 13). The amount of nucleosomal DNA produced is a function of the drug concentration as well as of time of incubation. These results, however, do not eliminate a possibility that production of nucleosomes in the drug-treated nuclei is due to an activation of endogenous nucleases. To rule out this possibility, further characterization of the bleomycin-released nucleosomal DNA must be undertaken. The present report shows that, using simple gel electrophoreses and buoyant density analyses, the bleomycin-generated nucleosomal DNA can be differentiated from the nuclease-released nucleosomal DNA.

MATERIALS AND METHODS

Cell Culture. Mouse L-cells and cactus mouse (Peromyscus eremicus) cells were grown in McCoy's Medium 5A supplemented with 20% fetal calf serum, harvested at late exponential phase of growth by rinsing the cultures with Hanks' solution without Mg2+ and Ca2+, and then trypsinized with the described Hanks' solution containing 0.01% of 3-times purified trypsin (Worthington Biochemical Corp., Freehold, N. J.). Cells were rinsed with the described Hanks' solution and immediately used for nuclei isolation.

Preparation of Nuclei, Conditions of Bleomycin Reaction, and Nuclease Digestion. Preparation of nuclei from the cultured cells was by the method described previously (10). Nuclei from laying hen erythrocytes and oviducts were isolated according to the procedures described by Wilhelm and Campagne (26) and Hewish and Burgoyne (9), respectively.

The bleomycin reaction was performed in a buffer containing 15 mM Tris-HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 0.34 M sucrose, 0.1 mM spermine, and 0.5 mM spermidine at 100 μg bleomycin per mg nuclei (DNA) per ml at 22° for 30 to 150 min. This reaction buffer was better than the system used previously (12) in terms of preventing nucleic acid lysis and inhibiting endogenous nuclease activity during incubation.

Digestion of isolated nuclei with micrococcal nuclease was also performed in the bleomycin reaction buffer containing 0.5 mM CaCl2 and using 100 units of enzyme per mg nuclei (DNA) per ml at 37° for different incubation times. Digestion of nuclei with endogenous nuclease was performed by incubating the nuclei in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl2, and 1 mM CaCl2 at 37° for 16 hr.

Isolation of DNA, Gel Electrophoresis, and Gel Elution of Nucleosomal DNA. The bleomycin reaction or nuclease digestion was terminated by addition of an equal volume of lysing solution containing 0.15 M NaCl, 15 mM EDTA, 50 mM Tris-HCl (pH 8.25), and 0.3% Sarkosyl NL 97. The DNA was extracted according to the procedure described previously (10). Neutral gel electrophoreses were performed in either 2% agarose or 2% polyacrylamide (acrylamide:bisacrylamide, 19:1, w/w) containing 0.5% agarose. Denaturing gels were prepared under alkaline conditions according to the method described by McDonell et al. (19). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed through a UV transilluminator. Elution of DNA from gel was after the procedure described by McDonell et al. (19).

S1 Nuclease Digestion of Nucleosomal DNA. To nucleosomal DNA that was dissolved in a buffer containing 0.2 mM NaCl, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.8), and 2 mM EDTA were added an equal volume of solution containing 0.8 M NaCl, 0.4 M sodium acetate (pH 4.5), and 0.5 mM ZnCl2. S1 nuclease (Miles Laboratories, Inc., Elkhart, Ind.) was then added, and the reaction mixture was incubated at 37° for 1 hr. The amount of S1 nuclease added was sufficient to digest only single-stranded DNA but not the duplex DNA. This has been checked by using restricted fragments from Hind III digests of SV40 DNA which contain single-stranded termini as substrate.
Analysis of Buoyant Density of DNA in CsCl. Buoyant density analyses were performed using a Beckman Model E analytical ultracentrifuge equipped with a high-intensity light source, scanning system, and multiplexer. The gradients were scanned after a 72-hr run at 25°. The buoyant density was calculated as described by Mandel et al. (17).

RESULTS AND DISCUSSION

Retardation of Electrophoretic Mobility of Nucleosomal DNA from Bleomycin-digested Nuclei in Neutral Gel. It was shown previously from this laboratory that bleomycin can cause release of nucleosomes by introducing double-stranded breaks in the linker regions (10, 13). In this study, further characterizations of the nucleosomal DNA released by bleomycin were performed. A direct comparison of gel electrophoretic mobilities in nucleosomal DNA’s isolated from bleomycin-treated nuclei and that isolated from either micrococcal nuclease- or endogenous nuclease-digested nuclei (not shown) revealed drastic differences. Fig. 1, A and B, shows that the electrophoretic mobility of nucleosomal DNA isolated from bleomycin-treated chicken erythrocyte or oviduct nuclei in neutral gel is slower than the corresponding samples from the micrococcal nuclease-digested nuclei.

The difference in mobilities in the nucleosomal DNA between the bleomycin- and micrococcal nuclease-digested samples can be seen at least up to nucleosomal octomers. This suggests that the differences in gel electrophoretic mobilities of nucleosomal DNA’s are not due to different extents of digestion. It has been found that the nucleosomal DNA released by bleomycin is always retarded in neutral gel electrophoreses when the comparison is made with the nucleosomal DNA samples isolated from a wide range of concentrations of micrococcal nuclease digestion (not shown). Gel electrophoresis of bleomycin-released nucleosomal DNA mixed with micrococcal nucleosomal DNA revealed a combined pattern of the 2 different DNA samples (Fig. 1C). These results suggest that the shift in electrophoretic mobility in the nucleosomal DNA of bleomycin-treated samples cannot be attributed to simple electrophoretic artifacts. Moreover, the DNA isolated from the nuclei which were treated with bleomycin under the condition described previously (12) also showed different electrophoretic mobility in neutral gel as compared with that isolated from the micrococcal nuclease-treated nuclei. This result rules out the possibility that the difference in electrophoretic mobility of bleomycin and micrococcal nuclease digestion products merely reflects extent of digestion in an isolated experimental condition.

The drastic difference in electrophoretic mobilities in neutral gel between bleomycin- and micrococcal nuclease-released nucleosomal DNA, as shown in Fig. 1, however, cannot be detected when the same samples are analyzed in denaturing gels. Fig. 2A shows that the electrophoretic mobility of bleomycin-released nucleosomal DNA from chicken erythrocyte nuclei is similar to that of the micrococcal nuclease-generated nucleosomal DNA in an alkaline gel, with an exception of mononucleosomal DNA, which migrates somewhat more slowly. Similar results can be observed when hen oviduct nuclei are used (Fig. 2B). Another denaturing gel system, i.e., 5% polyacrylamide in 7 M urea, also was used to confirm this finding (not shown).

Absence of Protein-DNA Cross-Linking in Bleomycin-released Nucleosomal DNA. What is the mechanism that caused electrophoretic retardation of nucleosomal DNA isolated from bleomycin-treated nuclei? Two possibilities have been investigated. The first one could be the protein association in the nucleosomal DNA, while the second one could be the presence of single-stranded regions in the nucleosomal DNA. It has been reported that the nucleosomal DNA isolated from formaldehyde-“fixed” SV40 minichromosomes migrates more slowly than does normal nucleosomal DNA in a neutral gel, presumably due to cross-linking of protein to DNA during fixation. The cross-linked proteins are resistant to sodium dodecyl sulfate, Pronase, and chloroform treatments. The DNA isolated from the fixed minichromosomes showed a decrease in buoyant density in neutral CsCl density gradient (25). We and others (12, 23) have reported previously that, in the bleomycin-treated purified DNA, a malonic aldehyde-like compound is produced. The production of this compound is presumably due to the damage of sugar moiety in the DNA. It is quite possible that the aldehyde may also be produced in our bleomycin-treated nuclei and participates in cross-linking of proteins in the nucleosomal DNA.

To investigate this possibility, isolated mouse L-cell nuclei were digested with bleomycin. The DNA was extracted and electrophoretically fractionated into monomer, dimer, . . . and high-molecular-weight DNA as shown in Chart 1. The DNA with different repeated units was eluted from the gels, and the buoyant densities (ρ) of different DNA fractions were determined in a neutral CsCl gradient using bacteriophage 2C as

![Chart 1](chart1.png)

Chart 1. Neutral CsCl density gradient analysis of different fragment sizes of nucleosomal DNA isolated from bleomycin-treated mouse L-cell nuclei. The control sample was the profile of DNA obtained from the gel of the nuclei treated with no bleomycin. Center, a densitometer tracing profile of the gel of DNA isolated from the bleomycin-treated nuclei. The gel segments corresponding to monomer (1), dimer (2) . . . were cut, and the DNA was eluted and analyzed in the neutral CsCl gradient. The buoyant densities of bacteriophage 2C (ρ = 1.742 g/ml) and satellit DNA (ρ = 1.699 g/ml) and the satellite DNA (ρ = 1.690 g/ml) in the control sample are indicated. Bars, corresponding to (from the top) 8548, 4200, 2115, 1750, 924, 371, and 272 base pairs, indicate the position of Haelll-digested PM2 DNA fragments run in the parallel gel. Note that the satellite DNA fraction in the nucleosomal DNA cannot be clearly discerned. Also, the buoyant densities of nucleosomal DNA are shifted toward the position of bacteriophage 2C DNA.
reference \( (p = 1.742 \text{ g/ml}) \). The genomic DNA isolated from undigested mouse L-cell nuclei was used as a comparison. The mouse genomic DNA comprises a main-band DNA with \( p = 1.699 \text{ g/ml} \) and a fraction (approximately 10%) of satellite DNA with \( p = 1.690 \text{ g/ml} \).

In comparison with that in the control sample, the buoyant density profiles for the bleomycin-released nucleosomal DNA's are generally broader (Chart 1). This is because the sizes of the nucleosomal DNA's are smaller. Furthermore, the satellite DNA peaks in the nucleosomal DNA samples cannot be clearly discerned. In addition, the \( p \) values for the "main-band DNA" in the fractionated bleomycin-generated nucleosomal DNA are higher than 1.699 g/ml.

Similar results were obtained when the nuclei isolated from cactus mouse were used. The genomic DNA of cactus mouse contains \( (G + C) \)-rich satellite DNA \( (p = 1.705 \text{ g/ml}) \) in addition to the main-band DNA \( (p = 1.700 \text{ g/ml}) \). The buoyant density profiles for the bleomycin-generated nucleosomal DNA with different repeated units were found to shift toward the bottom of the gradients (Chart 2).

Taken together, these results argue against the involvement of protein cross-linking in the bleomycin-generated nucleosomal DNA. The protein-DNA complex would band at a position where the buoyant density is lower than that for the purified DNA.

A direct measurement of protein content in the purified nucleosomal DNA from bleomycin-treated nuclei was performed using \(^{[3H]}\text{thymidine and [35S]methionine doubly labeled mouse L-cells. The results show that protein is not detectable in the nucleosomal DNA (not shown).}

It is difficult to determine the differential effects of the satellite DNA and the main-band DNA to the action of bleomycin from the results as shown in Charts 1 and 2. The main reason for this is because, in the bleomycin-released nucleosomal DNA, the satellite and main-band DNA fractions cannot be clearly separated. However, we have demonstrated that bleomycin can preferentially destroy active chromatin (11). Satellite DNA sequences are generally considered to be genetically inactive and to support a very low level of transcription activity (2); therefore, they would be more resistant to the action of bleomycin.

**Single-stranded Regions in the Nucleosomal DNA from Bleomycin-treated Samples as a Probable Cause of the Electrophoretic Retardation.** We next investigated whether the single-stranded regions in the nucleosomal DNA are responsible for electrophoretic retardation as shown in Fig. 1. In fact, the aforementioned increase of the buoyant density in the bleomycin-released nucleosomal DNA in neutral CsCl gradient is consistent with an explanation that the nucleosomal DNA contains single-stranded regions. Single-stranded DNA usually bands at a position below the double-stranded DNA in a neutral CsCl gradient.

It was shown previously that the high-molecular-weight nucleosomal DNA isolated from the bleomycin-treated Chinese hamster nuclei contains single-stranded regions (10). This was demonstrated by a comparison of the same DNA sample electrophoresed under nondenaturing and denaturing conditions. The nicks are spaced regularly with a multiplicity of the nucleosomal repeat unit, so that the high-molecular-weight nucleosomal DNA converts into low-molecular-weight nucleosomal DNA when the sample is analyzed under denaturing conditions (10).

In this report, we confirm and expand this finding by using single-strand-specific nuclease digestion. The nucleosomal DNA isolated from the bleomycin-treated chicken erythrocyte nuclei was digested with Si nuclease under the condition that only the single-stranded regions are removed. The digested DNA was then analyzed by nondenaturing gel electrophoresis. As shown in Fig. 3, after Si nuclease digestion, the electrophoretic mobilities of the bleomycin-released nucleosomal DNA's become increased. The mobility differences between the Si-treated (Lane 2) and untreated (Lane 1) samples can be seen at least up to several multiplicity units. This result suggests that the bleomycin-released nucleosomal DNA contains single-stranded termini.

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**Control experiments** were performed using nucleosomal DNA isolated from micrococcal nuclease-digested chicken erythrocyte nuclei. As expected, Si nuclease digestion of micrococcal nucleosomal DNA creates a slight change of electrophoretic mobilities (Fig. 4) because the nucleosomal DNA contains single-stranded staggerers (5). Furthermore, no obvious conversion of high-molecular-weight nucleosomal DNA into low-molecular-weight DNA was found (Fig. 5). This is consistent with the notion that the cuts introduced by micrococcal nuclease into the chromatin are across the double-stranded DNA (5).
Taken together, these results indicate that bleomycin can introduce both single- and double-stranded breaks into nuclear DNA \textit{in situ}. It remains to be investigated whether the double-stranded breaks are an accumulation of extensive single-stranded breaks, although it has been shown that both types of breaks can be produced simultaneously in the reaction of bleomycin with purified DNA (14). It also remains to be investigated, in addition to stranded breakages, whether or not release of free bases (formation of apurinic and apyrimidinic sites) and production of malonic aldehyde-like compounds also take place in the bleomycin-digested nuclei.

The presence of single-stranded regions in the bleomycin-released nucleosomal DNA is most likely to be responsible for causing electrophoretic retardation in nondenaturing gel. This interpretation is based on the observation that single-stranded nucleosomal DNA migrates more slowly than the double-stranded nucleosomal DNA in neutral gel. As shown in Fig. 5, the electrophoretic mobility of heat-denatured nucleosomal DNA isolated from micrococcal nuclease-digested nuclei is slower than that of the native nucleosomal DNA.

Several unpublished observations suggest that the damages introduced by bleomycin are present in the linker regions rather than in the nucleosomal cores. (a) When the nucleosomal particles isolated from the bleomycin-treated nuclei were digested with DNase I and the extracted DNA was analyzed in 12% acrylamide:7 M urea gel system (21), a typical 10.4-nucleotide repeat can be detected (16). If bleomycin destroys DNA in the nucleosomal core, one would not expect to see such typical nucleotide repeats. (b) Mononucleosomal DNA isolated from the bleomycin-treated nuclei contains double-stranded regions longer than 140 base pairs as analyzed by S1 nuclease digestion. (c) Mononucleosomal DNA isolated from the bleomycin-treated nuclei was resistant to the treatment of 1 M piperidine (90°; 15 min) alone or 1 M piperidine plus 0.1 n NaOH (90°; 15 min). Hot piperidine or alkaline treatment would fragment DNA which contains modified bases and apurinic or apyrimidinic sites (18).

The result of different electrophoretic mobilities between bleomycin-released nucleosomal DNA and nucleosome-generated nucleosomal DNA reported here strongly rules out the possibility that the nucleosomal DNA produced in the bleomycin-treated nuclei is due to an activation of the cellular nucleases by the drug. Controversial results have been reported concerning the effects of bleomycin on the action of nucleases. Yamaki et al. (27) first demonstrated that bleomycin stimulated DNase action in the isolated system, while Müller et al. (20) reported that the drug inhibited DNase I action. Müller et al. (20) also reported that bleomycin has minimal effects on DNase II activity. The present results seem to argue against the former report, although there is not sufficient evidence to show that the bleomycin inhibits nuclease activity.

The unique features of drug action on chromatin structure described in this report are probably related to the unique mode of action of bleomycin on purified DNA (7) and also to the characteristic cytological response of mitotic chromosomes to bleomycin treatment (10). It was reported that, when mitotic cells were held at metaphase for a prolonged period, bleomycin caused a unique type of chromosomal damage which we termed chromosomal disintegration. To our knowledge, no other agents including DNases can reproduce such a damage (10).

In conclusion, this report has described a unique feature of the action of bleomycin on chromatin. The results presented provide significant evidence that the action of this antibiotic on chromatin is different from those of the known nucleases (including endogenous nuclease). This may render the importance of using this antibiotic as a probe to study chromatin structures in eukaryotes.

REFERENCES

Bleomycin-mediated Release of Nucleosomes

Fig. 1. Neutral gel electrophoresis of nucleosomal DNA from bleomycin- and micrococcal nuclease-digested chicken erythrocyte (A) and oviduct nuclei (B). In each case, the bleomycin-digested sample is flanked by 2 micrococcal nuclease-digested samples. Bleomycin digestion was under the conditions described in "Materials and Methods" for 1 hr at 22°C. C, mixing experiment of nucleosomal DNA from bleomycin- and micrococcal nuclease-treated chicken erythrocyte nuclei. Lane 1, DNA from micrococcal nuclease-digested nuclei; Lane 2, mixed DNA of Lanes 1 and 3; Lane 3, DNA from bleomycin-digested nuclei. A and B were analyzed in 2% acrylamide plus 0.5% agarose gel; C was run in 2% agarose gel.

Fig. 2. Alkaline gel electrophoresis of nucleosomal DNA from bleomycin- and micrococcal nuclease-digested chicken erythrocyte (A) and oviduct (B) nuclei. The samples in this figure correspond to those in Fig. 1.
Fig. 3. Nondenaturing agarose gel electrophoresis of S1 nuclease-digested nucleosomal DNA isolated from the bleomycin-treated chicken erythrocyte nuclei. Lane 1, nucleosomal DNA from bleomycin-digested nuclei; Lane 2, S1 nuclease-treated DNA as shown in Lane 1; Lane 3, nucleosomal DNA from micrococcal nuclease-digested nuclei.

Fig. 4. Nondenaturing agarose gel electrophoresis of S1 nuclease-treated nucleosomal DNA from micrococcal nuclease-digested chicken erythrocyte nuclei (A) and oviduct nuclei (B). In each case, the S1 nuclease-treated sample is flanked by untreated sample.

Fig. 5. Nondenaturing agarose gel electrophoresis of heated nucleosomal DNA from micrococcal nuclease-digested chicken erythrocyte nuclei. Lane 1, native nucleosomal DNA (5 μg); Lanes 2 and 3, heated (100°C; 5 min) nucleosomal DNA (5 and 10 μg, respectively).
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