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BLEOMYCIN-INDUCED DNA LESIONS ARE DEPENDENT ON NUCLEOSOME REPEAT LENGTH

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Abstract—Treatment of cells or nuclei with bleomycin induces DNA lesions. We detect the presence of lesions as the release of fragments from bulk DNA when cells (or nuclei) are lysed in dilute alkali. To further characterize the lesions we have altered experimentally the average nucleosome repeat length and probed the lysate with nuclease S_1 in order to remove single-stranded DNA. In salt-incubated nuclei with short average nucleosome repeat length (140–145 base pairs) (and also with long nucleosome-free stretches of DNA) one can induced fewer DNA lesions in the nucleosome-containing DNA as compared to nuclei with 190–195 base pairs average nucleosome repeat length. Hence the ability of bleomycin to induce DNA lesions is dependent on nucleosome repeat length.

Bleomycin is one of the anti-neoplastic agents whose molecular pharmacology is well understood [1, 2]. When probing isolated DNA with bleomycin one can detect the release of DNA bases and the induction of single- and double-stranded DNA degradation. For example Fe(II) stimulates the reaction markedly whereas Cu(II) and Zn(II) have been shown to inhibit DNA degradation [2]. By the use of bithiazole derivatives it has been shown that bleomycin by intercalation promotes helix unwinding of closed circular DNA [3]. Furthermore treatment of isolated nuclei from Chinese hamster cells with bleomycin has been claimed to result in the release of nucleosomes by selectively degrading linker DNA [4].

To understand the process by which bleomycin induces cytotoxicity we need to increase our knowledge of how the drug interferes with chromatin. To examine chromatin we have developed an approach where cells (or nuclei) are lysed in dilute alkali in order to partly denature the DNA [5, 6]. During this process single-stranded DNA fragments are released into solution if the drug has induced DNA lesions. The fragments arise either due to the interaction between the drug and DNA or during the process of DNA repair. The single-stranded DNA fragments can then be separated from bulk DNA by agarose gel electrophoresis.

We report here data obtained from drug-treated cells or nuclei by the above described approach as well as a development of this approach where we probe the lysate with nuclease S_1 to digest single-stranded DNA. Furthermore we change the nucleosome repeat length by incubating nuclei in different salt-solutions. The data show that the ability of bleomycin to induce DNA lesions is dependent on nucleosome repeat length with fewer DNA lesions induced in chromatin with short repeat length [140–145 base pairs (bp)].

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MATERIALS AND METHODS

Cells, culture medium and labelling with [^{3}H]*thymidine*. A human colon adenocarcinoma cell line (WiDr), obtained from Flow Laboratories (Irvine, U.K), was grown as monolayers at 37° in 5% CO_2 in air. The culture medium was Eagle's MEM, containing 2 mM L-glutamine, 10% fetal calf serum and antibiotics. The culture medium was routinely changed twice weekly and the cells passaged every 4 to 6 days [5].

For experiments the cells were seeded in small culture dishes (35 × 10 mm) containing 3 ml medium 24 hr before the addition of 30 μCi [^{3}H]thymidine (20 Ci/mmol, Amersham, Bucks, U.K.) and the incubation performed for 24 hr. The cells were then washed free of thymidine and incubated in fresh medium for another 24 hr before analysis.

Bleomycin was obtained from a local pharmacy. W-7 was obtained from Boehringer-Mannheim (Hamburg, F.R.G.).

Cell lysis. The incubation medium was drained from the culture dish and the cells rinsed with cold phosphate-buffered saline. Cell lysis was performed in the dark at 0° by the addition of 2.25 ml of 0.03 M NaOH (pH 12.1). After 30 min the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH_2PO_4. The sample was then either digested with nuclease S_1 (see below) or immediately made 0.5% with regard to SDS [5, 7].

Lysis of nuclei was performed according to the same protocol.

Digestion with nuclease S_1. Immediately after neutralization of the dilute alkali used to lyse the cells (nuclei), 300 μl of 300 mM NaAc pH 4.6/0.5 mM ZnAc/750 mM NaCl was added. Two hundred IU/ml nuclease S_1 (Sigma Chemical Co., St Louis, MO) was then added and the mixture incubated for 30 min at 37°. The digestion was stopped by making the solution 1% SDS–0.02 M EDTA.

Nuclease S_1 is considered to be relatively insensitive to ionic strength [8–10]. Nevertheless the rate of enzyme digestion is reduced at increased salt

concentrations. At the highest salt concentration we use (0.6 M KCl) the rate of digestion is 40% of the rate at 0.1 M KCl. For this reason we chose a high concentration of enzyme (200 units/ml) to be used throughout the experiments. Furthermore we used two concentrations in the experiments with 0.6 M KCl (200 units/ml and 600 units/ml). The results were the same independent of the concentration of enzyme used (not shown).

Agarose gel electrophoresis. The labelled DNA was separated in 0.75% agarose gels using an LKB Multiphor electrophoretic system. The voltage gradient was 1 V/cm, using the following buffer system (0.04 M Tris–HCl pH 8.0, 0.04 M NaAc, 0.002 M Na_2EDTA, 0.2% SDS). The gels were cut into 1-mm thick slices and the radioactivity was measured in a toluene-based scintillation fluid containing 3% Soluene 100, using a Packard scintillation counter.

Isolation of nuclei in salt solutions. Isolated nuclei were suspended in buffer (0.25 M sucrose, 50 mM Tris–HCl pH 7.5, 25 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). KCl was then added to a final concentration of 0.3 or 0.6 M KCl. The nuclei were then incubated at 37° in order to reduce the nucleosome repeat length according to Ref. 11. The average nucleosome repeat length of nuclei suspended in buffer is 190–195 bp. In nuclei incubated in buffer supplemented with 0.3 M KCl the average repeat length is 160–170 bp and in nuclei incubated in 0.6 M KCl it is 140–145 bp. We have repeated these experiments using micrococcal digestions and obtained the same results (not shown). In experiments with histone H1-depleted nuclei the sample was first incubated at pH 3.0 (citric acid–sodium phosphate buffer) at 0° to remove histone H1 [12] and then resuspended in buffer and salt as described above.

RESULTS

We use a procedure of alkaline cell lysis to partly denature the DNA [5]. During the cell lysis the double helix of DNA starts to uncoil in the alkaline milieu. The uncoiling, which is initiated at single-stranded gaps (and/or alkali-labile regions) results in denaturation of 20 kb regions of the DNA [13]. For the large DNA molecules of mammalian cells the time needed to complete the uncoiling can be measured. When the solution is then neutralized after 30 min the DNA strands larger than 20 kb renature and form double-stranded DNA whereas DNA smaller than 20 kb remain in solution as single-stranded DNA.

Treatment of cells with drugs often results in the presence of alkali-labile regions in the DNA. The labile regions arise either by direct interaction between the drugs and DNA or during the process of DNA repair. Therefore during the uncoiling of the DNA in the alkaline medium DNA fragments are released. The fragments can be separated from the high molecular weight DNA by agarose gel electrophoresis. We have earlier described the appearance of DNA fragments in cells treated with methotrexate or 5-fluoropyrimidines [6] and in cells incubated with drugs which induce DNA cross-links [14].

Treatment with bleomycin

Cells with pre-labeled DNA are treated with bleomycin (10 μg/ml) for 30 min and then either immediately lysed, or lysed after incubation in fresh medium for 30 min, 60 min or 24 hr (Fig. 1A and B). The gel electrophoretic separation shows initially mainly high molecular weight DNA (DNA at the same location in the gel as in control cells) but also a small amount of DNA fragments (slices 24–34).

The longer the duration of the post-incubation period in fresh medium the higher levels of DNA fragments (slices 24–34). In parallel the high molecular weight DNA is reduced. In cells not treated with bleomycin one does not detect the DNA fragments at slices 24–34. When the dose of bleomycin is increased one can detect higher levels of DNA fragments (Fig. 1C).

The data are compatible with earlier published data showing the appearance of single- and double-stranded DNA breaks in bleomycin-treated cells [1, 15, 16].

Nuclease S_1 treatment of nuclei with altered nucleosome repeat length

The pH of the alkaline solution used to lyse cells is 12.1. It is known that at this pH histone H1 is removed from the nucleosome complex whereas histones H2–4 remain intact [17]. Histone H1 is located in the internucleosomal region and its removal facilitates the denaturation of the internucleosomal DNA, resulting in a mixture of double-stranded nucleosomal DNA and single-stranded inter-nucleosomal DNA.

Nuclease S_1 is an enzyme known to digest single-stranded but not double-stranded DNA. When control cells with pre-labelled DNA are lysed in dilute alkali and the DNA then digested with nuclease S_1 we find that the pre-labelled DNA appears as small fragments of double-stranded DNA [5]. This double-stranded DNA shows up in a 0.75% agarose gel as a population of molecules migrating not from from the front. The molecular size of this DNA (70–200 bp single-stranded DNA after denaturation in formamide) is not changed by a subsequent digestion with micrococcal nuclease, as expected for nucleosomal-sized DNA [5]. Hence generation of double-stranded nucleosomal-sized DNA fragments occurs in mature chromatin not affected by drugs.

The nucleosome repeat length can be altered experimentally by incubating intact nuclei or H1-depleted nuclei in salt solutions. The incubation conditions are patterned after Ref. 11. By incubating the nuclei in KCl one can reduce the average repeat length from 190–195 bp to 165–170 bp and finally 140–145 bp. We have repeated the quoted work and found the same nucleosomal repeat lengths (not shown). In parallel one also creates longer nucleosome-free stretches of double-stranded DNA.

The lysis of the nuclei is performed by adding NaOH to the KCl solution. It has been established earlier that the presence of salt in the alkaline solution increases the rate of uncoiling of the DNA when cells are lysed [13]. The presence of KCl does not increase the number of gaps in the DNA. However, the length of single-stranded DNA formed from each

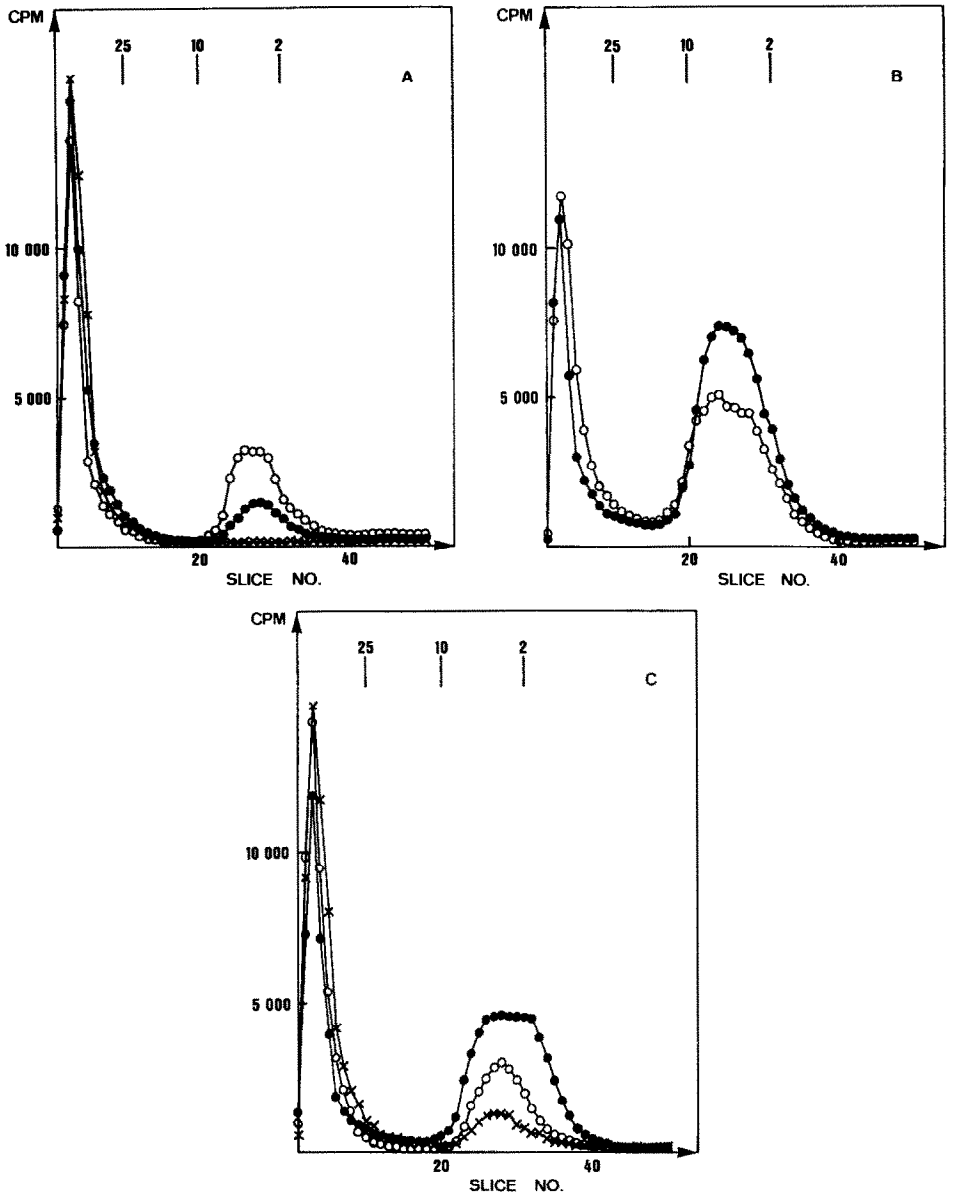

Fig. 1. Fragmentation of DNA by bleomycin. (A) Human colon adenocarcinoma cells with pre-labelled DNA were treated with bleomycin (10 μg/ml) for 30 min. The cells were either immediately lysed (-●-) or lysed after incubation in fresh medium for 30 min (-○-). Cells not drug-treated (-x-). Cell lysis was performed in dilute alkali and the DNA then separated in 0.75% agarose gels. The numerals across the top (25, 10 and 2) denote the size (in kb) and location of single-stranded DNA markers. (B) Cells treated with bleomycin (10 μg/ml) for 30 min and then incubated in fresh medium for 60 min (-○-) or 24 hr (-●-). (C) Cells treated with bleomycin (10 μg/ml -x-; 20 μg/ml -○-; 50 μg/ml -●-) for 30 min and then immediately lysed.

initiation point is increased. Remaining in the solution is double-stranded DNA with altered nucleosome repeat length.

Figure 2 show experiments on nuclei with steady-state labelled DNA which are treated with various concentrations of KCl, lysed in dilute alkali and the DNA then treated with nuclease S_1. The double-stranded DNA is completely fragmented when the average repeat length is 190–195 bp (Fig. 2A). In the gel the labelled DNA fragments show a broad distribution located between slices 25–35. There is no high molecular weight DNA. The results are essentially the same as those seen in lysed cells [5]. In a parallel lane of the same gel we have also separated DNA treated in the same manner but not digested with nuclease S_1. Here one can detect only high molecular weight DNA located at slices 3–6.

When the average repeat length is 165–170 bp the fragmentation of double-stranded DNA by nuclease S_1 is reduced. The gel separation now shows some

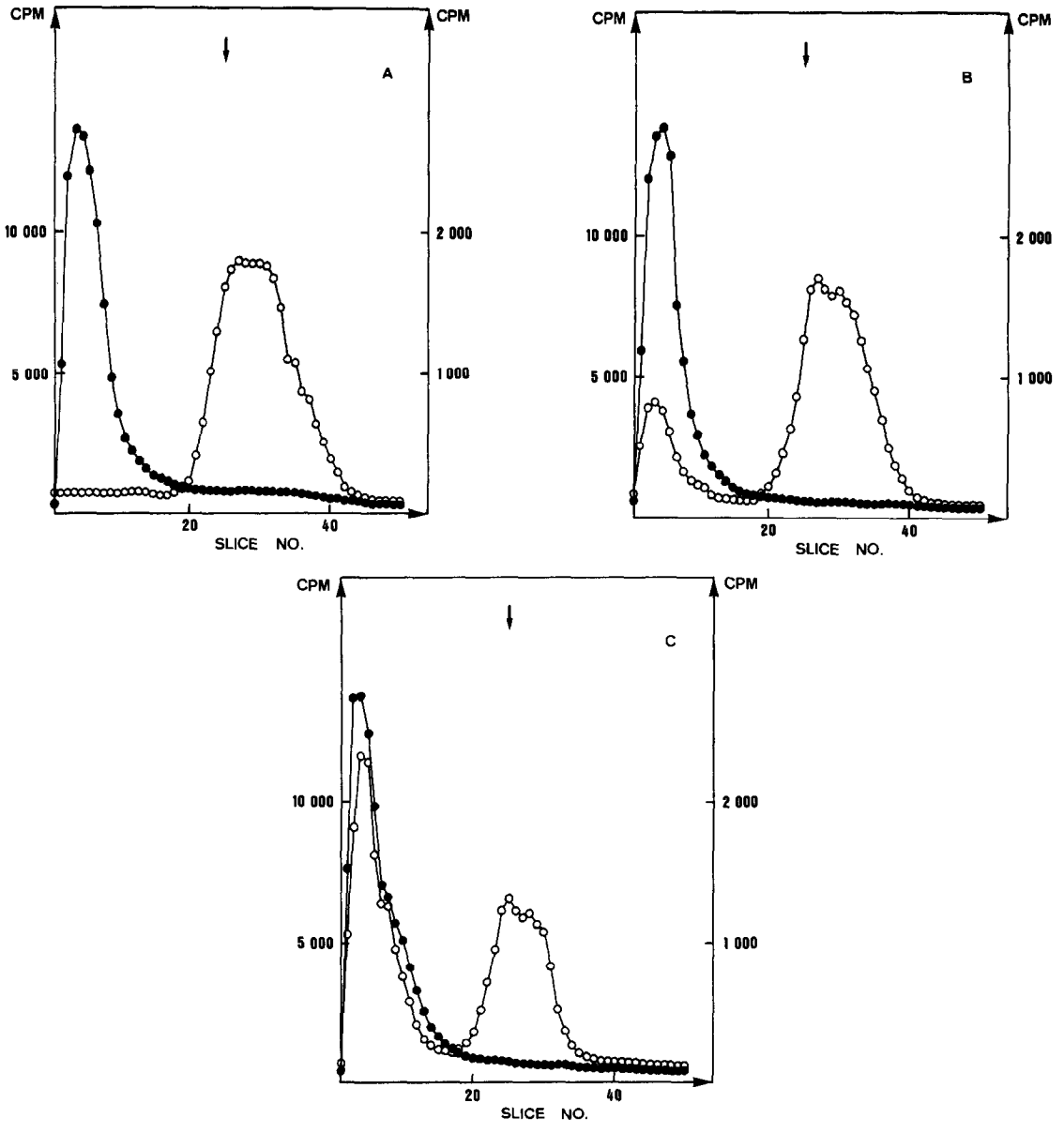

Fig. 2. Effect of salt-induced changes in nucleosome length on nuclease S_1-digestion. Nuclei, isolated from cells with pre-labelled DNA, were either (A) lysed without prior salt incubation (average nucleosome repeat length 190–195 bp); (B) lysed after prior incubation in 0.3 M KCl (average nucleosome repeat length 160–165 bp) or (C) lysed after prior incubation in 0.6 M KCl (average nucleosome repeat length 140–145 bp). After lysis the DNA was digested with nuclease S_1 (–○–). The DNA of another sample incubated in parallel was not digested with nuclease S_1 (–●–). Samples with the same average nucleosome repeat length were then separated in the same 0.75% agarose gel. The arrow indicates the location of a single-stranded DNA marker (2 kb) used to standardize the separation. The scale to the left refers to (–●–) and the scale to the right to (–○–).

high molecular weight DNA (slices 3–6) as well as DNA at slices 25–35 (Fig. 2B). When the repeat length is 140–145 bp the level of fragmented double-stranded DNA is reduced still further. Now roughly half of the label appears as DNA fragments and half as high molecular weight DNA (Fig. 2C).

Next we examined nuclei devoid of histone H1 by incubation of the nuclei at pH 3 [12]. This treatment induces a minor reduction in the level of enzyme-induced DNA fragmentation when the nucleosome repeat length is 190–195 bp. Furthermore when the H1-depleted nuclei are then incubated with KCl one can detect a higher level of high molecular weight DNA both at the 165–170 bp and the 140–145 bp repeat length than in nuclei with histone H1 (Fig. 3).

Hence the data show that by reducing the average nucleosome repeat length and in parallel creating nucleosome-free stretches of DNA the ability of nuclease S_1 to fragment DNA is reduced. Furthermore we have shown in an earlier publication

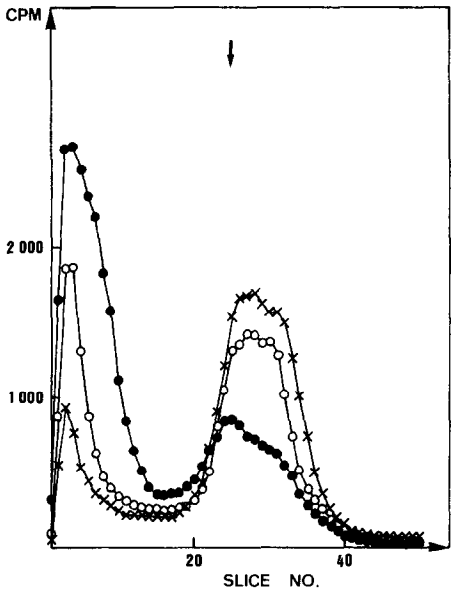

Fig. 3. Histone H1-depleted nuclei. Nuclei, from cells with pre-labelled DNA, were depleted of histone H1 by incubation at pH 3. Thereafter the nuclei were either not treated with salt (average nucleosome repeat length 190–195 bp) (–x–), treated with 0.3 M KCl (average nucleosome repeat length 160–165 bp) (–○–), or treated with 0.6 M KCl (average nucleosome repeat length 140–145 bp) (–●–). After lysis the DNA was digested with nuclease S_1 and then separated in 0.7% agarose gels. The arrow indicates a single-stranded DNA marker (2 kb) used to standardize the gel.

that when cross-links are induced in DNA during treatment of cells, the ability of nuclease S_1 to fragment DNA is reduced due to reduced denaturation of the DNA during the alkaline treatment [6].

Importance of nucleosome repeat length in nuclei treated with bleomycin

Nuclei with pre-labelled DNA are isolated and incubated with salt-solutions to vary the average nucleosome repeat length, according to the protocol of Fig. 2. The nuclei are then incubated with bleomycin (10 μg/ml) for 30 min, lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. The gel separation shows high molecular weight DNA and DNA fragments (slices 25–35).

The DNA fragments do not exist in nuclei not treated with bleomycin. Furthermore they appear in bleomycin-treated nuclei at the same location in the agarose gel as in experiments involving lysis of cells. The data show that the shorter the average nucleosome repeat length (and in parallel the presence of increasing amounts of nucleosome-free DNA) the less drug-induced DNA fragmentation is detected (Fig. 4a). Significantly fewer DNA fragments are formed in nuclei with nucleosome repeat length of 140–145 bp, as compared to the 190–195 bp nucleosome repeat length.

The salt-induced sliding of the histones create long nucleosome-free stretches of DNA. These regions should be hyper-reactive to bleomycin. Therefore we tested the amount of cold TCA-precipitable radioactivity in treated and control nuclei. In bleomycin-treated nuclei the amount of precipitable radioactivity is on the average 87% and 78% of that in the control nuclei when the average repeat length is 165–170 bp and 140–145 bp, respectively.

We also examined lysates where the DNA was digested with nuclease S_1 before gel electrophoresis. The results show that with the shortest nucleosome repeat length (140–145 bp) the same level of DNA fragmentation (slices 24–34) is present after the nuclease S_1-treatment as in nuclei not treated with bleomycin (Fig. 4b). The total amount of TCA-precipitable labelled material shows a similar difference between treated and control nuclei as detected above.

Control experiments: post-incubation with the calmodulin-inhibitor W-7

The repair of bleomycin-induced DNA lesions is reduced when calmodulin is inhibited with W-7 [18]. Also the repair of the DNA lesions we detect here is reduced when the cells are post-incubated with W-7. Figure 5 shows the release of DNA fragments from cells incubated in the presence or absence of W-7 for 24 hr after the bleomycin-treatment. The data show, as expected, a higher level of DNA fragmentation after incubation with W-7. The higher level of DNA fragmentation is due to reduced DNA repair [18–20].

DISCUSSION

Bleomycin induces DNA lesions which are visualized in our assay as the release of DNA fragments from bulk DNA. Control experiments show that the level of DNA fragmentation is increased when the repair of the lesions is reduced when the cells are post-incubated in the presence of the calmodulin inhibitor W-7.

We carried out experiments with salt-incubated nuclei to examine the importance of the nucleosome repeat length for the ability of bleomycin to induce DNA lesions. By altering the salt concentration one can alter the average nucleosome repeat length and in parallel create longer nucleosome-free stretches of DNA [11]. We find that the shorter the average nucleosome repeat length the fewer bleomycin-induced DNA lesions are present in the DNA containing nucleosomes. The data are furthermore supported by a new experimental approach involving the digestion of the DNA with nuclease S_1.

Nuclease S_1 digests single-stranded but not double-stranded DNA. The rate limiting step in the digestion is the introduction of the first nick at the site of locally denatured DNA. When the molecule contains a single-stranded incision it is quite efficiently cleaved by the nuclease [8–10]. The enzyme needs at least three unpaired bases to recognize an incision site [21].

In cells not treated with drugs we have found that nuclease S_1 removes all single-stranded DNA replication intermediates as well as short single-stranded stretches of DNA present in the internucleosomal DNA regions [5]. The pre-labelled

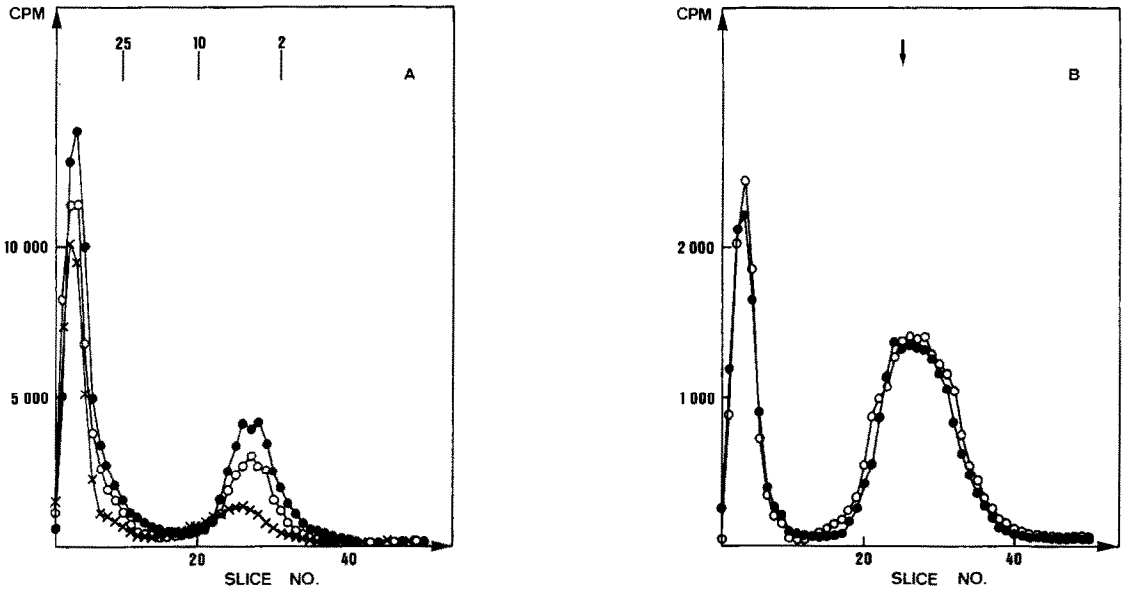

Fig. 4. Treatment of nuclei with bleomycin. Nuclei were isolated from cells with pre-labelled DNA. The nuclei were incubated in solutions favouring the average nucleosome repeat length of 190–195 bp (–●–), 160–165 bp (–○–) or 140–145 bp (–x–). The nuclei were treated with bleomycin (10 μg/ml) for 30 min, lysed in dilute alkali and the DNA separated in 0.75% agarose gels either immediately (A) or after digestion with nuclease S_1 (B). (B) shows only nuclei at 140–145 bp average nucleosome repeat length. (–●–) represents bleomycin-treated nuclei, (–○–) untreated nuclei. In (A) 25, 10 and 2 denote the size (in kb) and location of single-stranded DNA markers. In (B) the arrow denotes the single-stranded DNA marker (2 kb) used to standardize the separation.

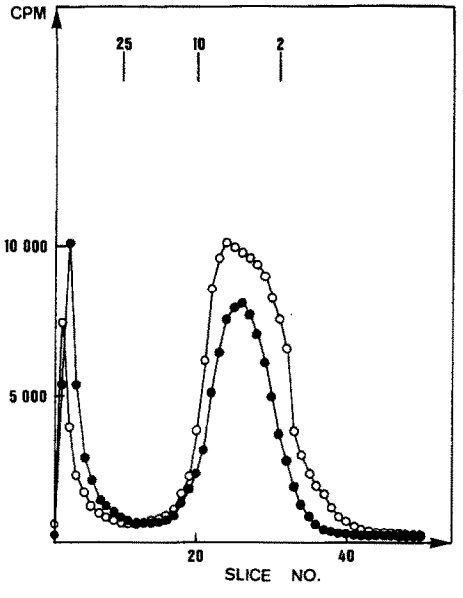

Fig. 5. Treatment with W-7. Cells with pre-labelled DNA were treated with bleomycin (10 μg/ml) for 30 min and then incubated for 24 hr in medium containing W-7 (10 μM) (–○–) or in fresh medium (–●–). The cells were lysed and the DNA then separated in 0.75% agarose gels. The 25, 10 and 2 denote the size (in kb) and location of single-stranded DNA markers.

DNA therefore appears as small fragments of double-stranded DNA of nucleosomal size. The reason for this is that the treatment with alkali at pH 12.1 preferentially removes histone H1 [17]. As expected, when the DNA is cross-linked to prevent denaturation in dilute alkali, the ability of nuclease S_1 to fragment the DNA is reduced [14].

Bleomycin interacts less strongly with the short nucleosomal repeat length DNA whereas the nucleosome-free regions of the DNA are degraded to small oligonucleotides. This supports the view that the cytotoxicity of the drug is dependent on chromatin topology and that chromosomes are cleaved into nucleosomes [22]. In contrast it has been shown that bleomycin-induced DNA fragmentation of plasmid-DNA (which lacks protein) is almost randomly dispersed [23].

Chromatin topology varies considerably in different stages of the cell cycle. During DNA synthesis one can detect alterations in the nucleosome repeat length with initially a shorter repeat length than in mature chromatin [24]. When examining the repair of e.g. UV-irradiated chromatin, extensive alterations in nucleosome configurations have been found. Immediately after repair synthesis the DNA is in a non-nucleosomal state. The DNA later presumably folds into nucleosomal structures [25].

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