DNA Damage Induced by Bleomycin, Neocarzinostatin, and Melphalan in a Precisely Positioned Nucleosome

ASYMMETRY IN PROTECTION AT THE PERIPHERY OF NUCLEOSOME-BOUND DNA*

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The antitumor drugs bleomycin, neocarzinostatin, and melphalan all damage DNA by mechanisms which involve binding in the minor groove. In order to examine at high resolution the modulating effects of chromatin structure on the action of these drugs, an end-labeled DNA fragment from the *Xenopus laevis* 5 S rRNA gene was reconstituted with histone octamers to form a precisely positioned nucleosome. For each drug, DNA damage at specific sequence positions in the fragment was then compared for nucleosome-bound versus naked DNA. Reconstitution into nucleosomes resulted in a marked inhibition of the DNA cleavage induced by bleomycin (5-fold) and neocarzinostatin (2.4-fold) in the central region of nucleosomal DNA. However, at the periphery of nucleosome-bound DNA, a distinct asymmetry was apparent, with marked inhibition of cleavage toward the upstream side, but little if any inhibition toward the downstream side, which overlaps the binding site of the transcription factor TFIIIA. In the case of melphalan, alkylation at adenine N-3 was inhibited by nearly 2-fold throughout the nucleosome, whereas alkylation at guanine N-7 was either slightly inhibited or slightly enhanced, depending on sequence position. None of the drugs showed the 10-base pair periodicity characteristic of hydroxyl radical-induced cleavage of nucleosomal DNA. The results are consistent with a model in which minor groove sites in nucleosome-bound DNA remain relatively accessible to small molecules, even where the minor groove faces the histone core, and in which drug-induced DNA damage is inhibited by conformational constraints imposed on DNA by nucleosome structure. Furthermore, the degree of such constraints appears to be sequence-dependent, at least near the periphery of nucleosome-bound DNA.

Many chemotherapeutic drugs damage DNA; in fact, this damage is probably responsible for their therapeutic efficacy, as well as for adverse side effects such as myelosuppression and second malignancies in long term survivors of chemotherapy (Henne and Schmähl, 1985; Pratt and Ruddle, 1994). In order to clarify how chemical modification of DNA by chemotherapeutic agents affects DNA structure and function, and ultimately leads to both therapeutic and toxic effects, the interactions of these drugs with purified DNA have been studied extensively. However, in eukaryotic cells, chromatin structure imposes an additional level of specificity. Since this specificity can influence the degree to which damage is localized in essential DNA sequences or in sequences where damage is poorly repaired, it may be an important determinant of the biological effects of these drugs.

The basic subunit of chromatin is the nucleosomal core particle, which consists of approximately 146 bp of DNA coiled in a left-handed superhelix around an octamer of core histones. The structural organization of DNA into nucleosomes is considered an important factor in the regulation of gene expression, and nucleosomes have been shown to inhibit transcription *in vitro* (van Holde, 1989). Additionally, nucleosomes have been shown to precisely position around important regulatory regions, both *in vivo* and *in vitro* (Ramaswamy, 1986; Rhodes, 1986; Simpson and Stafford, 1983). For example, a nucleosome is positioned around the start site of the 5 S rRNA gene extending into the transcription factor binding site in a variety of species including *Lytechinus variegatus* (Moyer et al., 1989; Simpson and Stafford, 1983), *Xenopus borealis* (Hayes et al., 1990; Rhodes, 1986), and *Xenopus laevis* (Smith and MacLeod, 1993). In the present study, we have taken advantage of the precisely positioned nucleosome at the transcription start site of the *X. laevis* 5 S rRNA gene to determine, at single nucleotide resolution, the degree to which nucleosome structure modulates DNA damage by three antitumor drugs, bleomycin, neocarzinostatin, and melphalan.

Bleomycin and NCS are antibiotics which induce sequence-specific single and double strand breaks in DNA by free radical-based mechanisms (for a review, see Dedon and Goldberg (1992)). Bleomycin is a glycopeptide which forms a metal coordination complex with Fe**II**. The bleomycin:Fe**II** complex combines with oxygen to produce a highly reactive species which specifically abstracts hydrogen from the C-4' of the deoxyribose, ultimately leading to a strand break or abasic site. Early studies showed that bleomycin induces single strand breaks primarily at pyrimidines in G-C and G-T sequences. More recently, Steighnner and Povirk (Steighnner and Povirk, 1990) showed that bistranded cleavage resulted from a strand break at a primary site (which adhered to the expected G-C or G-T sequence specificity) followed by a secondary cleavage event on the opposite strand. Since the secondary cleavage sites, which occur either directly opposite the primary site or opposite the base immediately following it, are rarely G-C or G-T sequences, no true single strand cleavage occurs at most secondary sites, and thus cleavage at these sites can be taken as a measure of double strand cleavage.

* The abbreviations used are: bp, base pair(s); NCS, neocarzinostatin.
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NCS is one of a family of enediyne antibiotics, which contains a hydrophobic chromophore as the active component. Upon addition of thiol, the chromophore forms a highly reactive biradical species which can induce bistranded damage by simultaneously abstracting hydrogen from C-5' of deoxyribose in one DNA strand, and from C-1' or C-4' in the opposite strand. NCS thus induces frank double strand breaks at AGT-AC (5'→3',5'-→3') sequences, as well as abasic sites with closely opposed strand breaks at AGC-GCT sequences, which rapidly decompose in chromatin to yield additional double strand breaks (Bennett and Povirk, 1992; Dedon and Goldberg, 1990; Povirk et al., 1988). The drug also induces single strand breaks at nearly all T and at many A residues, and most of these breaks have 3'-phosphate and 5'-aldehyde termini (Kappen and Goldberg, 1983).

Adduct formation in nucleosomal DNA by the alkylating agent melphalan was also examined, primarily to determine whether adenine N-3 (Osborne and Lawley, 1989) and guanine N-7 alkylations are differentially inhibited in chromatin. Previous studies with other bulky adduct-forming agents have shown that core histones provide approximately equal protection of DNA from either benzo[b]furane diol epoxide, which binds in the minor groove at guanine N-2 (Smith and MacLeod, 1993) or aflatoxin B1 (Moyer et al., 1989), which binds in the major groove an guanine N-7 (Moyer et al., 1989); in each case there was a 2-3-fold decrease in covalent binding to the nucleosomal region compared with the linker region. However, since melphalan-induced alkylation at adenine N-3, a minor groove site, occurs mostly in A/T-rich tracts (Wang et al., 1991), and since A/T tracts tend to be oriented with the minor groove facing the nucleosome core, selective suppression of adenine alkylation might be expected a priori. Such selective suppression could explain the relatively low frequency of A-T to T-A transversions induced by this drug in an endogenous gene (Austin et al., 1991), despite the predominance of such mutations in a mammalian shuttle vector system (Wang et al., 1990).

In addition to determining how chromatin structure modulates the effects of specific DNA-damaging agents, examination of damage in chromatin may help to define the accessibility of both major and minor groove sites in nucleosomal DNA to interacting proteins, as well as the susceptibility of various domains of nucleosomal DNA to induced conformational distortion. Such information may provide insights into the interaction of larger molecules, such as DNA-binding proteins, with DNA in chromatin.

MATERIALS AND METHODS

Drugs—FeIII-bleomycin was prepared from clinical bleomaxine as described previously (Povirk and Houlgrave, 1988) and stored at -20 °C at a concentration of 6 mM. NCS chromophore was prepared by methanol extraction of clinical NCS (Povirk et al., 1981) and stored at -70 °C at a concentration of 220 μM. Prior to each experiment, NCS was diluted on ice in 20% methanol, 20 mM sodium citrate, pH 4, in the dark. Melphalan was dissolved at a concentration of 10 mM in 0.1 M HCl and stored at -20 °C.

Preparation of DNA Fragments—The plasmid pGTKX14 was a gift from M. C. MacLeod (Smith and MacLeod, 1993) and is numbered with respect to the natural transcription start point of the 5 S rRNA gene as indicated in Fig. 1. pGBKX14 was digested with AvaI and HindIII restriction endonucleases, and the resulting fragment was separated by agarose gel electrophoresis, electroeluted, and repurified by organic extractions and ethanol precipitation. DNA fragments were end-labeled by Klenow fill-in at the AvaI site with [α-32P]dATP.

Reconstitution—H1-stripped nucleosomal core particles were isolated from chicken erythrocyte nuclei as described previously (Laemmli, 1970; Smith and MacLeod, 1993). Core length DNA, which was used in control experiments, was purified from core particles by proteinase K digestion and organic extractions (Smith and MacLeod, 1993).

Nucleosomes were reconstituted on the 32P-end-labeled 5 S DNA by the exchange of histones from chicken erythrocyte mononucleosomes (Moyer et al., 1989; Smith and MacLeod, 1993). Briefly, core particles and linear 32P-end-labeled 5 S DNA were combined at an approximate molar ratio of 100:1 in 670 μl of TE (10 mM Tris, pH 8, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). 5 μM NaCl was added by dropwise addition to a final concentration of 0.8 μM NaCl, and the samples were allowed to rest on ice for 20 min. The samples were placed in Spectrotrap microbore reversed phase tubing. The tubing was kept overnight at 4 °C. The samples were then dialyzed against TE containing 0.05 μM NaCl for 4 h at 4 °C. Control samples were prepared concurrently by performing a mock-reconstitution, substituting purified chicken core length DNA for the core particles. The efficiency of reconstitution was monitored by a gel mobility shift assay. Control and reconstituted samples were run on a 6% agarose gel at 100 V. The gel was stained by automated ethidium. Greater than 95% of the radiolabeled fragments became complexed with histones (Fig. 2).

Bleomycin Treatment of Reconstituted Fragments—Control or reconstituted 5 S DNA fragments (at a final DNA concentration of 50 μg/ml) were reacted with 0, 1, or 3 μM FeIII-bleomycin at 37 °C for 1 h in the presence of 25 mM 2-mercaptoethanol in a total volume of 150 μl. The reaction was terminated by addition of EDTA to 100 mM and the samples were cooled on ice. The salt concentration was adjusted to 0.1 M NaCl and the drug and histones were removed by organic extractions followed by ethanol precipitation of the DNA. The cleaved products were heat-denatured and analyzed on denaturing gels (8% polyacrylamide, 8 μv urea). Markers lanes were prepared according to the method of Maxam and Gilbert (Maxam and Gilbert, 1980).

NCS Treatment of Reconstituted Fragments—The nonprotein chromophore of NCS was added at final concentrations of 0, 3, or 6 μM to a solution containing the reconstituted chromatin and 1 mM glutathione to activate the drug, in a total volume of 150 μl. The reaction was incubated for 15 min at 22 °C in the dark. After the reaction was essentially complete within a few minutes, the histones were removed by organic extractions without prior inactivation of the drug followed by ethanol precipitation. The cleaved DNA products were analyzed on standard denaturing gels as described above.

Melphalan Treatment of Reconstituted Fragments—Melphalan was dissolved in TE and added to the reconstituted chromatin or DNA at final drug concentrations of 100 or 150 μM. Following incubation at 37 °C for 1 h, the NaCl concentration was adjusted to 0.8 M in order to stop the reaction and help dissociate DNA and histones. (In control experiments where melphalan was added just after addition of 0.8 M NaCl, no DNA damage was detected.) After removal of the histones by organic extractions, the DNA was ethanol-precipitated, dissolved in 0.1 ml of 50 mM Tris-HCl, 1 mM EDTA, heated at 70 °C for 1 h to deparafrene thermolabile adducts, again precipitated, and heated at 90 °C for 30 min in the presence of 10 μl 1 M piperidine to cleave the resulting shortening of DNA samples were then lyophilized, dissolved in formamide, and sminted to denaturing gel electrophoresis as above.

RESULTS

Chromatin Reconstitution—Salt exchange reconstitution of a 430-bp DNA fragment containing the X. laevis somatic-type 5 S rRNA gene has been shown to consistently form two precisely positioned nucleosomes, one extending from -95 to 50 and containing the start site of the gene (Fig. 1) and one extending from about position 160 to the end of the fragment (Smith and MacLeod, 1993). We have used the upstream (-95 to 50) in vitro positioned nucleosome to analyze the sequence-specific interaction of three chemotherapeutic drugs, bleomycin, neocarzinostatin, and melphalan, with intranucleosomal DNA.

The plasmid pGBKX14 was digested with AvaI and HindIII restriction endonucleases. The resulting 430-bp fragment was end-labeled at the AvaI site by Klenow fill-in and reconstituted under "Materials and Methods." As a control, DNA was purified from chicken erythrocyte mononucleosomes and substituted for core particles in the reconstitution protocol. As shown in Fig. 2, the control (C) and reconstituted (R) samples were analyzed by agarose gel electrophoresis under nondenaturing conditions. Under these conditions, DNA associated with the core histones exhibits a slower mobility than free DNA. Consistently, 95–100% of the end-labeled DNA in the reconstituted samples was shifted with respect to the control samples.
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DNA (lane C) agrees well with the pattern of DNA cleavage previously observed in other restriction fragments (Dedon and Goldberg, 1992). The pattern above nucleotide 11 in the lane corresponding to reconstituted chromatin (lane R) is quite similar to the pattern seen for uncomplexed DNA. However, below this region, bands corresponding to prominent bleomycin-induced cleavage sites in naked DNA were either absent entirely or much less intense in the reconstituted samples. This region is coincident with the placement of a positioned nucleosome over the start site of the gene as described by Smith and MacLeod (1993). This distinctive difference in the banding pattern between the control and reconstituted samples suggests that the nucleosomal DNA was protected to a large extent from attack by bleomycin. At the low bleomycin dose, there were some sites near positions 20–40 for which the bands were darker in the reconstituted sample lane; however, the autoradiogram showed that there was also substantially more full-length DNA in that lane. In order to evaluate cleavage quantitatively and correct for such differences in sample loading, the gel was analyzed with a Molecular Dynamics PhosphorImager. The integrated intensity of each observable band was calculated and divided by the total integrated intensity of the appropriate lane. This number represents the fraction of total molecules that were cut at that nucleotide. The cleavage frequency for each sequence position in the reconstituted sample was then normalized to the cleavage frequency at the same position in the unreconstituted sample (Drew and Calladine, 1987), and finally, this ratio was normalized to the average ratio in the linker region, based on the assumption that cleavage susceptibility in the linker would be unaffected by reconstitution. A plot of this normalized ratio (R/C) as a function of nucleotide position (Fig. 4A) reveals a dramatic suppression of cleavage within the central region of the nucleosome. Interestingly, a portion of the fragment approximately 40 bp from the end of the nucleosome (nucleotide positions 11–50) seemed to be largely unprotected from attack by bleomycin. Although the other end of the nucleosome (−95 to −56) contains a stretch of As and Ts which tend to isolate the predicted sites of cleavage, it is nevertheless clear that this region is more protected against cleavage than the region from 11 to 50, even though these two regions are approximately equidistant from the dyad axis of the nucleosome (see Fig. 5A). At several sites just inside and just outside the nucleosome boundary (positions 27–56), cleavage in the reconstituted sample actually appeared to be enhanced by 1.5–2-fold, although the unusually large experimental variations in R/C for this region cast some doubt on the significance of this effect.

In addition to single strand breaks, bleomycin is known to induce double strand breaks, as well as abasic sites with closely opposed breaks (Povirk and Houlgrave, 1988). As noted above (Introduction), single and double strand breakage by bleomycin can be determined separately by examining cleavage at primary and secondary sites, respectively, and these sites can be predicted on the basis of DNA sequence (Povirk et al., 1989). To determine the inhibition of breakage at primary and secondary cleavage sites in the nucleosome, we compared the integrated intensities of each for the region of maximum suppression of cleavage (positions −95 to 10), the end of the nucleosome (11 to 50), and the linker region (51 to 111). The degree of suppression of each primary and secondary site is shown in Fig. 4A, and the average suppression for each region is shown in Table I. There is approximately a 5-fold decrease in cutting of the central region of the nucleosome compared with the linker region or the end of the nucleosome (11 to 50). However, in these data, there appears to be no difference between the suppression of cutting at primary versus secondary sites, implying that single and

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**Fig. 1.** Partial sequence of linear 5 S DNA fragment. pGXls14 was digested with AarI and HindIII restriction endonucleases and end-labeled with \(^{32}P\) at the AarI site. A partial sequence of this fragment, including the positioned nucleosome used in this study is shown. The asterisk denotes the position of the label, ◊ represents positions of maximum hydroxy radical cleavage (Smith and MacLeod, 1993), and bold lettering represents the position of the nucleosome on the sequence. Numbering is based on the start site of transcription (+1) of the 5 S RNA gene. The nucleosome extends from 95 to 50 in the sequence.

**Fig. 2.** Electrophoretic mobility shift assay. The efficiency of reconstitution was monitored by electrophoresis under nondenaturing conditions. Complex formation resulted in a decreased electrophoretic mobility compared with the control fragment (free DNA). C = control sample subjected to mock reconstitution in the absence of histones; R = reconstituted sample. Greater than 95% of the end-labeled fragment becomes associated with core histones upon reconstitution.

**Bleomycin Cleavage of Intrunucleosomal DNA—In vitro reconstituted 5 S DNA was treated with 0, 1, or 3 μM bleomycin, and the cleavage products analyzed on standard denaturing polyacrylamide gels (Fig. 3A). There was very little cleavage in untreated DNA; the dark misshapen bands near positions 60–80 in the control lane apparently represent various forms of renatured or partially renatured full-length fragment, since they were much more prominent when the heat denaturation step was eliminated, and they were absent entirely in extensively cleaved DNA samples. The banding pattern resulting from sequence-specific bleomycin cleavage of uncomplexed...**

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**TABLE I**

<table>
<thead>
<tr>
<th>Region</th>
<th>Average Suppression</th>
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<tbody>
<tr>
<td>Control lane</td>
<td>1</td>
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<tr>
<td>Reconstituted</td>
<td>0.2</td>
</tr>
<tr>
<td>Linker region</td>
<td>0.5</td>
</tr>
<tr>
<td>End of nucleosome</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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**REFERENCES**


double strand cleavage were inhibited to equal extents within the protected regions.

To further analyze the pattern of cleavage within the nucleosomal region, the phosphorimaging intensity of the control and reconstituted samples of the entire lane was plotted versus the relative position in the gel (Fig. 5). This intensity is proportional to the level of radioactivity in a particular band and corresponds to the frequency of cutting at that site. Since bleomycin is thought to bind in the minor groove, a 10-bp periodicity in the cleavage frequencies (similar to that seen with hydroxyl radical) might have been expected in the reconstituted samples, with cleavage being preferentially inhibited at sequence positions where the minor groove faces the nucleosome core. However, even in the central region, the pattern of residual cleavage in the reconstituted sample (dotted line) mimics that of the control lane (heavy line), suggesting that the reduction in cleavage seen in the reconstituted samples is not due to mere accessibility of the minor groove of DNA in the nucleosome. Thus, it appears that the sequence specificity of bleomycin cleavage supersedes minor groove accessibility and that, remarkably, cleavage sites are suppressed to approximately equal extents whether the minor groove is facing outward or inward with respect to the nucleosome at any particular site.
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**Fig. 4. Quantitative comparison of drug-induced cleavage.** The integrated phosphorimage intensity of each observable band was divided by the total intensity in the appropriate lane to give the fraction of the total represented by each band (the fractional intensity). At each position, the ratio of the fractional intensity for the reconstituted sample (R) to that for the control sample (C) is plotted versus nucleotide position. A, bleomycin-treated nucleosomes. The open bars represent secondary cleavage sites, and the solid bars represent primary cleavage sites. B, NCS-treated nucleosomes. C, melphalan-treated nucleosomes. Open and solid bars represent adenine and guanine residues, respectively. Each data point represents the average R/C ratio from four experiments, normalized to the average R/C for the linker region. The bar above each graph represents the nucleosome position, and the ◦ represents positions of maximum hydroxyl radical cleavage.

**NCS Cleavage of Intr nucleosomal DNA**—In vitro reconstituted nucleosomal core particles or control samples were treated with 0, 3, or 6 μM NCS in the presence of glutathione and analyzed on a standard denaturing gel as described above. Suppression of cleavage again was apparent in the region coincident within the central region of the nucleosome (positions -96 to 10, data not shown).

To analyze this phenomenon quantitatively, the gels were scanned as described above, using the PhosphorImager. The pattern of NCS-induced cleavage is complicated by that fact that the nucleoside 5'-aldehyde moieties present at the termini of most but not all NCS-induced breaks retard the mobility of the resulting fragments by the equivalent of two nucleotide positions with respect to the 5'-phosphate-terminated markers (Kappen and Goldberg, 1983). However, since it is not possible to predict with certainty which bands correspond to 5'-phosphate and which to 5'-aldehyde termini, the data are presented according to apparent band position, without correction for the aldehyde termini (Fig. 4B). Despite this complication, it is apparent that cleavage was suppressed in the central region (55 to 10) and in the upstream periphery (-95 to -56) of the nucleosome. As with bleomycin, the region of the nucleosome from 11 to 50 exhibited no protection from cleavage by NCS. The calculated average R/C values were 0.429 ± 0.23 for the region of greatest suppression of cleavage (-51 to 10), and 1.140 ± 0.14 for the region of no suppression of cleavage (11 to 50). Thus, there was approximately a 2.4-fold decrease in the frequency of cleavage in the central region and the upstream periphery of the nucleosome, as compared with the linker.

To examine the intranucleosomal region of DNA, the entire lane was scanned and the relative intensity plotted as a function of position in the gel as described in Fig. 5. Within each of the regions described above, the pattern of residual NCS-induced cleavage in the reconstituted samples paralleled that of the control samples (Fig. 5B). Consistent with the results obtained with bleomycin, and in contrast to those obtained with hydroxyl radicals (Smith and MacLeod, 1993), there was no indication of a 10-bp periodicity in the protection of nucleosomal DNA from NCS. It is possible that a weak periodicity might have been obscured by the presence of both 5'-phosphate and 5'-aldehyde-terminated fragments and by the fact that NCS-induced breaks tend to be clustered in the relatively A/T-rich tracts where the minor groove faces the nucleosome. It is nevertheless apparent that near the center of the nucleosome cleavage is inhibited even at sites where the minor groove faces outward and that in all regions cleavage continues to be dominated by the inherent sequence specificity of the drug rather than by the rotational positioning of the minor groove on the nucleosome.

**Melphalan Adduction in Intr nucleosomal DNA**—The same end-labeled reconstituted and unreconstituted DNA fragments were treated with the alkylating agent melphalan. Following heat depurination of thermolabile adducts and cleavage of the resulting abasic sites in alkali, strand breaks were analyzed as above. Like most other damaging agents, melphalan produced less damage in the region of DNA bound to the core particle than in the linker region. As shown in Fig. 4C and Table II, adenine alkylation (presumably at N-3, a minor groove site (Osborne and Lawley, 1993)) was suppressed by about 40%, with relatively little variation between individual adenines in the sequence. There was less suppression of guanine N-7 alkylation, with an average decrease of about 16% and more variation between individual nucleotide positions; in fact, certain guanine residues were more susceptible to cleavage in nucleosomal than in naked DNA. In contrast to results obtained with bleomycin and NCS, there was little difference between the
A. **Nucleosome Damage by Bleomycin, Neocarzinostatin, and Melphalan**

**FIG. 5. Cleavage pattern in free versus nucleosomal regions of DNA.** Following electrophoresis on a sequencing gel, the radioactivity profiles for control (solid line) and reconstituted (dotted line) samples from one experiment were quantitated using software supplied by Molecular Dynamics (ImageQuant), which constructs line graphs equivalent to densitometer tracings. A, bleomycin-treated samples (3 μM). B, NCS-treated samples (6 μM). Electrophoresis is from left to right (as denoted by the arrow), and positions of selected bands are noted.

**TABLE I**

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Primary sites</th>
<th>Secondary sites</th>
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<tbody>
<tr>
<td>-95 to 10</td>
<td>0.235 ± 0.15</td>
<td>0.187 ± 0.11</td>
</tr>
<tr>
<td>11 to 50</td>
<td>1.360 ± 0.28</td>
<td>1.531 ± 0.67</td>
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<tr>
<td>51 to 111</td>
<td>0.985 ± 0.52</td>
<td>0.897 ± 0.15</td>
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**TABLE II**

<table>
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<tr>
<th>Nucleotide position</th>
<th>As</th>
<th>Gs</th>
</tr>
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<tbody>
<tr>
<td>-95 to 10</td>
<td>0.602 ± 0.028</td>
<td>0.887 ± 0.060</td>
</tr>
<tr>
<td>11 to 50</td>
<td>0.809 ± 0.044</td>
<td>0.758 ± 0.059</td>
</tr>
<tr>
<td>51 to 81</td>
<td>0.879 ± 0.075</td>
<td>1.160 ± 0.115</td>
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**DISCUSSION**

The cytotoxicity of chemotherapeutic drugs is thought to be a consequence of DNA damage, either covalent DNA modification or direct strand breaks. Although the interactions of these drugs with isolated DNA have been studied extensively, less is known of the influence of chromatin structure on the nature, extent, and genomic localization of drug-induced damage. However, the finding that active genes are more vulnerable to bleomycin-induced damage than nonexercised DNA (Kuo, 1981) suggests that chromatin structure affords at least some protection from DNA damage by this drug. In addition, numerous lines of evidence, including the cleavage of chromatin into oligonucleosome size fragments by both bleomycin and NCS (Bennett *et al.*, 1993; Kuo and Hsu, 1978), and the decreased susceptibility of DNA in cells where the nucleosomal repeat length has been reduced (Lönn *et al.*, 1989), indicate that these drugs preferentially target internucleosomal linker DNA, implying that DNA within the nucleosome core is relatively protected.

Nucleosome structure has been studied extensively and has been shown to play an important role in gene regulation, transcription, and DNA repair (Hayes and Wolffe, 1992a). In many cases, the rotational and translational positioning of nucleosomes on DNA is sequence-dependent. In general, rotational positioning is determined by preferential placement of short runs of (A, T) with the minor groove facing toward the octamer and short runs of (G, C) with the minor groove facing away from the octamer (Drew and Travers, 1985). Translational positioning depends at least in some cases on specific sequences around the dyad (Ramsay, 1986). The 5S rRNA gene from a variety of
species has been shown to precisely position a nucleosome around the start site of the gene both in vivo and in vitro (Drew and Calladine, 1987; Rhodes, 1986; Simpson and Stafford, 1983; Smith and MacLeod, 1993). We have used a fragment containing this gene in which the rotational and translational position of DNA on a nucleosome has been determined previously (Smith and MacLeod, 1993) to examine the binding and cleavage patterns of bleomycin, neocarzinostatin, and melphalan, at single nucleotide resolution.

When control and reconstituted samples were treated with equal concentrations of bleomycin, a 5-fold suppression of cleavage was observed in the central region of the nucleosome, which extends from -56 to +10, according to the translational and rotational positioning determined by Smith and MacLeod (1993). Interestingly, one end of the nucleosome exhibited little or no suppression of cleavage (11 to 50), whereas the opposite end (-95 to -56) showed suppression comparable with the central region. Similarly, NCS-induced cleavage showed suppression within the central region of the nucleosome (approximately 2.4-fold compared with the linker region) and the same asymmetric pattern of cleavage on the ends. For the central region, the degree of suppression of NCS-induced cleavage is similar to the suppression observed previously for adduct formation by aflatoxin B1 (Moyer et al., 1989) and benzo[a]pyrene diol epoxide (Smith and MacLeod, 1993), whereas the suppression of bleomycin-induced cleavage is substantially greater. However, even within the central region, the residual cleavage of nucleosomal DNA by both bleomycin and NCS preserved the sequence specificity seen in naked DNA; a similar result was obtained with benzo[a]pyrene diol epoxide (Smith and MacLeod, 1993).

The lack of protection against bleomycin- and NCS-induced cleavage seen at one end of the nucleosomal DNA is consistent with the results of Bennett et al. (1992) who found that although treatment of reconstituted chromatin with either of these drugs produced a typical nucleosomal ladder, in each case the bands were less well defined than those generated by micrococcal nuclease digestion. Drug-induced cleavage just inside the boundaries of nucleosome-bound DNA (e.g. positions 11 to 50 in the 5S sequence) could account for the smearing of these bands and could also explain the NCS-induced cleavage of chromatin into fragments of ~125 bp, slightly shorter than the length of DNA bound by the nucleosome core (McHugh et al., 1982; van Holde, 1989). This region of unprotected DNA within the nucleosome is also roughly coincident with a transitional region described by Smith and MacLeod (1993) (positions 28–44), in which benzo[a]pyrene diol epoxide binding was less suppressed than in the central region of the nucleosome. A similar transitional region was reported for aflatoxin B1 binding (Moyer et al., 1989), but in neither case was there such a distinct asymmetry in protection as that seen with bleomycin and NCS.

The apparent susceptibility of the unprotected region (11 to 50) to damage can be correlated to the weaker interactions of DNA with the H2A-H2B dimers at the ends of the nucleosomes.

The intact nucleosome is a tripartite structure in which the periphery of nucleosomal DNA (approximately 20–25 bp on each end) is bound to the H2A-H2B dimers, whereas the central 100 bp is bound, much more tightly, to a central (H5-H4) tetramer (Thoma, 1992). Transcription in the presence of bound tetramers is inhibited by addition of dimers, and it is believed that the dimers are displaced during transcription in vitro (Hayes and Wolfe, 1992a). The loosely bound dimers can be preferentially released from core particles by treatment with dimethylmaleic anhydride or high concentrations of ethidium bromide, and in each case the first dimer is released more readily than the second (McMurray and van Holde, 1991; McMurray et al., 1991; Nieto and Palacian, 1988). It may be significant that the region which exhibits the lack of protection from bleomycin- and NCS-induced cleavage within the nucleosome is the end closest to the TFIIB binding site (positions 45–91), the internal control region of the gene. Previous studies have shown that binding of the octamer over this region inhibits transcription in vitro, but that when a tetramer is bound (without dimers), transcription proceeds (Hayes and Wolfe, 1992b). Furthermore, transcription also proceeds when TFIIB is added prior to nucleosome formation, suggesting that the presence of the dimer impedes binding of the transcription factor. Thus, it is possible that the asymmetry in cleavage at the ends of the nucleosome is due to a weaker binding of DNA to the H2A/H2B dimer in this region (11 to 50), compared with its binding to the other dimer on the opposite end of the nucleosome (-95 to -56). This weaker binding may be necessary to prevent displacement of TFIIB by the nucleosome.

It is remarkable that of the several agents for which damage to nucleosomal DNA has been examined (aflatoxin B1, dimethyl sulfate, benzo[a]pyrene diol epoxide, bleomycin, melphalan, and NCS), only one, hydroxyl free radical, induces damage with a 10-bp periodicity corresponding to the rotational positioning of DNA on the nucleosome. Although damage by NCS, bleomycin, and benzo[a]pyrene diol epoxide is suppressed within the central region of nucleosomal DNA, the sequence specificity of the residual damage closely mimics that seen with naked DNA. Since all three of these agents are relatively small (much smaller than a helical turn of DNA) and all attack DNA from the minor groove, it is quite surprising that damage is not preferentially suppressed at sites where the minor groove faces the histone core and equally surprising that damage is suppressed at all sites where the minor groove faces outward.

To explain the finding that the extent of damage is not dictated simply by the accessibility of the minor groove, several mechanisms may be considered. First, structural distortions induced by binding DNA around nucleosomes may disrupt the normal interactions of these agents with the minor groove. However, if this were the case, substantial alterations in the sequence specificity of the residual cleavage would be expected, and this is clearly not observed. Second, interaction of these agents with DNA may involve an initial nonspecific binding in the minor groove, followed by sliding along the groove until a high-affinity binding site is found. Since most of the agents are positively charged, the partial neutralization of DNA phosphate charge by histone amines could reduce the initial nonspecific binding step without affecting the "sliding" step, which determines sequence specificity. However, this proposal would not explain the similar results obtained with benzo[a]pyrene diol epoxide, which is uncharged. A third possibility is that binding of the drugs to DNA prior to cleavage may involve induced local changes in DNA conformation and that these constraints imposed by nucleosome structure may prevent these conformational changes. In the case of NCS, the critical conformational change could be intercalation of the naphthoate moiety of the chromophore, which appears to be involved in the binding of this agent to DNA (Galat and Goldberg, 1990; Povirk and Goldberg, 1981). By analogy to the ethidium bromide studies mentioned above, it would be expected that any reaction involving intercalative binding would be severely suppressed in nucleosomal DNA and that any such binding would be largely restricted to regions near the ends of nucleosomal DNA. The apparently weaker binding of DNA to histones at one end the nucleosome (positions 11–50), as discussed above, may make this region more susceptible to reactions involving intercal-
Nucleosome Damage by Bleomycin, Neocarzinostatin, and Melphalan

tion or other conformational changes.

For bleomycin, no such simple model can be proposed, since its mode of binding to DNA is much less certain. Although early evidence suggested that intercalation of the bithiazole rings was involved in binding (Povirk et al., 1979), more recent models have favored binding of the bithiazole in the minor groove, following the curvature of the groove in a manner similar to distamycin. Modeling studies indicated that this mode of binding could be accommodated with very little perturbation of DNA helical structure (Kuwahara and Sugiiura, 1988). However, it is difficult to reconcile this proposal with the severe suppression of bleomycin-induced DNA damage which is seen at every potential cleavage site in the central region of the nucleosome, especially since this suppression is greater than that reported for any other low molecular weight DNA-damaging agent. These results suggest that bleomycin-induced DNA damage may in fact require intercalation or some type of induced conformational change in DNA prior to hydrogen abstraction. The finding that bleomycin binding renders certain adjacent DNA bases more susceptible to modification by diethylypyrocarbonate (Fox and Grigg, 1988) also supports this proposal. The finding that double and single strand cleavage are inhibited to equal extents (Table I) confirms results obtained by directly quantitating single and double strand breaks in supercoiled plasmids (Bennett et al., 1993) and is consistent with the view that the two breaks involved in double strand cleavage occur during a single bleomycin binding event (Povirk et al., 1989; Steighner and Povirk, 1990).

In the case of melphalan, major induced conformational changes in DNA seem unlikely, but nucleosome structure may also reduce random thermal motions of the helix which may be required to accommodate melphalan. Modeling studies of Bauer et al. (1993) suggest that melphalan binding to adenine N-3 requires a slight widening of the minor groove. In the case of guanine N-7 alkylation, the degree of protection afforded by nucleosome structure appears to reflect the bulkiness of the alkylator ( aflatoxin B1 > melphalan > dimethyl sulfate). Although adenine alkylation by melphalan is suppressed more than guanine alkylation, this difference alone is not nearly sufficient to explain the difference between the endogenous aprt gene and the pZ189 shuttle vector in the proportion of melphalan-induced mutations which occur at A-T base pairs; thus, other factors such as differential DNA repair must be involved.

In conclusion, the view of nucleosome structure which is emerging from studies of DNA-damaging agents is one in which both the major and minor grooves are remarkably accessible to small molecules. Resistance of nucleosomal DNA to conformational change seems to be a more important constraint on interactions with other molecules than does accessibility. Moreover, the degree of conformational constraints appears to be sequence-dependent, at least at the periphery of nucleosome-bound DNA.

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