Effect of Nucleosome Structure on DNA Interstrand Cross-Linking Reactions †

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Diepoxybutane and Diepoxyoctane Interstrand Cross-Linking of the 5S DNA Nucleosomal Core Particle†

Julie T. Millard* and Erin E. Wilkes

Department of Chemistry, Colby College, Waterville Maine 04901

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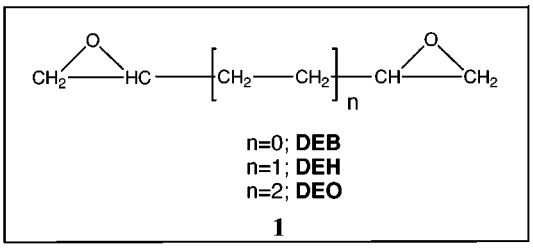
ABSTRACT: Diepoxyalkanes form interstrand cross-links in DNA oligomers preferentially at 5′-GNC sites. We have examined cross-linking by 1,2,3,4-diepoxybutane (DEB) and 1,2,7,8-diepoxyoctane (DEO) within a fragment of the 5S RNA gene of *Xenopus borealis* in both the free and nucleosomal states. Sites and efficiencies of interstrand cross-linking were probed through denaturing polyacrylamide gel electrophoresis and quantitative phosphorimagery. Both agents targeted 5′-GNC sites for cross-linking in the restriction fragment in its free state, and DEO also targeted 5′-GNNC sites. Monoalkylation occurred at all deoxyguanosines. The sites for both monoalkylation and interstrand cross-linking were similar in nucleosomal and free DNA, and cross-linked DNA was cleanly incorporated into the core particle structure. These findings suggest that the 5S core particle is able to tolerate any structural abnormalities induced by diepoxide cross-linking.

Mammalian detoxification systems convert hydrocarbons to water-soluble epoxide derivatives for excretion (*1*). For example, cytochrome P450 sequentially oxidizes butadiene to its mono- and diepoxides, 3,4-epoxy-1-butene and 1,2,3,4-diepoxybutane [**1**, DEB[1] (*2*, *3*)]. These oxidation events are



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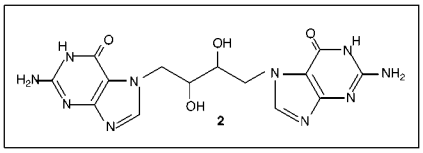
problematic for the organism because of the DNA alkylating capabilities of these two metabolites. Bifunctional alkylators such as DEB are frequently more carcinogenic than their monofunctional analogues, a fact that has been attributed to their formation of DNA interstrand cross-links (*4*, *5*). DEB in particular is suspected to be involved in the high incidence of lymphatic and hematopoietic cancers among those exposed to butadiene in industrial settings, where it is widely used (*6*−*8*). Indeed, interspecies differences in the carcinogenicity of butadiene have been attributed to metabolic differences in the formation of the diepoxide metabolite and its subsequent cross-linking activity (*9*, *10*). Differences also exist within the human population, with some individuals more susceptible to the formation of DEB-induced chromosomal deletions and exchanges, likely because of genetic differences in the enzymes responsible for metabolic activation of butadiene as well as for repair of the resulting DNA damage (*11*). Interestingly, DEB cross-linking can also be beneficial in some cases. For example, the antitumor prodrug treosulfan, used in the treatment of advanced ovarian cancer, is also converted *in vivo* to DEB, resulting in the interstrand cross-linking thought to be the biologically relevant event in its mode of action (*12*). The goal of this investigation is to further the understanding of the DNA damage induced by DEB and similar compounds and the role of this damage in carcinogenicity and antitumor potential.

DEB was demonstrated to act as a DNA cross-linking agent over forty years ago (*13*) forming linkages between deoxyguanosine residues at their N7 positions, leading to conjugate **2** (*14*). Bifunctional cross-linkers of five or fewer



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atoms were originally proposed to form interstrand linkages at 5′-GC sequences, which contain the minimal N7-to-N7 distance (*15*). However, more recent experimental evidence indicates that the five-atom nitrogen mustards in fact show a preference for cross-linking distal deoxyguanosines at 5′-GNC sequences (where N = A, G, C, or T) in short oligomers and longer restriction fragments (*16*−*19*). Furthermore, the four-atom DEB retains this sequence preference in short DNA oligomers (*20*), as do the longer-chained 1,2,5,6-diepoxyhexane (DEH; **1**) and 1,2,7,8-diepoxyoctane

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* To whom correspondence should be addressed at the Department of Chemistry, 5757 Mayflower Hill Dr., Colby College, Waterville, ME 04901. Phone (207) 872-3311; fax (207) 872-3804; e-mail jtmillar@colby.edu.

[1] Abbreviations: DEB, 1,2,3,4-diepoxybutane; DEH, 1,2,5,6-diepoxyhexane; DEO, 1,2,7,8-diepoxyoctane; bp, base pairs; HN2, mechlorethamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TE, 10 mM Tris buffer with 1 mM EDTA, pH 7.5; PMSF, phenylmethanesulfonyl fluoride; TBE, 0.045 M Tris−borate buffer with 0.001 M EDTA; dPAGE, denaturing polyacrylamide gel electrophoresis.

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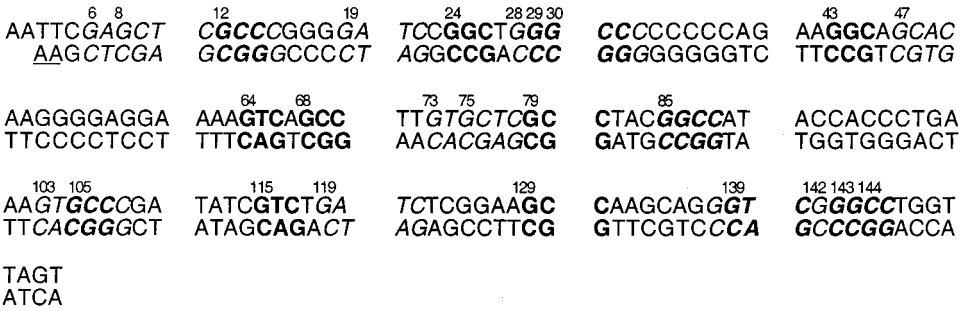

FIGURE 1: Sequence of the *Eco*RI−*Rsa*I restriction fragment of the *Xenopus borealis* 5S RNA gene and 5′-flanking region. Numbering is from 1 to 154 in the 5′−3′ direction of the top strand; this corresponds to positions −78 to +75 on the 5S RNA gene. Numbering of the bottom strand is in the same direction, but these residues are denoted with a prime (′). Position 16 in our sequence shows a C → G transversion from the wild-type sequence. Potential sites for cross-linking at 5′-GNC sequences are shown in boldface type; potential sites for cross-linking at 5′-GNNC sequences are shown in italic type. 3′-End radiolabeling is of the bottom strand and results in the incorporation of A3′ and A4′ (underlined) and the associated ^{32}P.

(DEO; **1**) (*21*). While elucidation of the preferred core target sequence in DNA oligomers is useful, it is unlikely to represent fully the situation *in vivo*. Other factors to consider may include efficiencies of reaction, flanking sequence effects, differential repair of lesions, and possible modulating effects of cellular proteins.

One class of cellular proteins that has been shown to impact DNA binding by a number of small molecules is the histones. These highly conserved proteins form an ionic complex with 165−245 bp of DNA, leading to nucleosomes, the repeating subunits of chromatin (for reviews, see refs *22*−*24*). Within the nucleosome are the "core particle" (146 bp of DNA wrapped one and three-quarters times around a histone octamer) and the "linker" region (20−70 bp of DNA bound to histone H1 and nonhistone proteins). Indeed, the bulk of eukaryotic nuclear DNA exists in a nucleosomal rather than in a free state. Differential reactivity within the nucleosome toward many external agents has been attributed to the drastic structural changes imposed on the DNA (for reviews, see refs *25* and *26*). Incorporation of DNA into a nucleosomal core particle results in a net curvature of about 45° per helical turn. Moreover, hydroxyl radical cleavage has demonstrated both a periodic narrowing and widening of the minor groove as well as underwinding at the central three helical turns of the core particle with a DNA helical repeat of 10.7 bp/turn and overwinding of the rest of the core particle with a helical repeat of 10.0 bp/turn (*27*, *28*). Singlet oxygen cleavage has also shown sharp bending at several sites (*29*).

An example of differential reactivity within the nucleosome is provided by DNA intercalators, which target the linker with about a 2-fold preference relative to the core DNA (*25*). For monoalkylating agents, the effect of nucleosome structure on DNA binding seems to relate to the size of the molecule. That is, relatively large molecules such as aflatoxin B1 and the diol epoxide metabolite of benzo[*a*]pyrene (BPDE) demonstrate a 2−3-fold reduction in binding to the nucleosome center (the dyad) with diminished protection at the nucleosome ends, while the relatively small dimethyl sulfate shows fairly uniform alkylation within the nucleosome (*30*−*33*). For bifunctional agents, the effect of nucleosome structure on DNA binding is less straightforward. Psoralen reacts exclusively with the linker and not with the core DNA of the nucleosome (*34*). At the other extreme, nitrogen mustards show only modest differences in the attack of defined sequence free versus nucleosomal DNA (*35*, *36*). Intermediate in their reactivities are mitomycin C and cisplatin, demonstrating some inhibition at the dyad relative to the ends of the nucleosome (*36*, *37*).

With the goal of further clarifying the factors responsible for the biological effects of the diepoxyalkane family, we have examined DNA interstrand cross-linking of defined sequence core particles by the four-carbon DEB and the eight-carbon DEO. It is likely that the different alkyl chain lengths of these compounds result in different degrees of DNA distortion upon cross-linking. A difference in the ability of the nucleosome to tolerate such structural changes could lead to different DNA sequences targeted in chromatin than in less highly constrained DNA oligomers, the only system for which the 5′-GNC target sequence has been reported for the diepoxyalkanes. Indeed, different adducts for the reaction of DEB with free and cellular DNA have been attributed to chromatin structure (*38*). Heterogeneity in mustard cross-link formation within different genes of the same cell line and within the same gene in different cell lines has likewise been attributed to chromatin structure (*39*), although the overall mustard alkylation patterns are remarkably similar in free and cellular DNA (*40*).

We present herein our findings for diepoxide reactivity within a 154 base pair fragment of the 5S RNA gene from *Xenopus borealis* (Figure 1) in both the free and nucleosomal state. The 5S DNA system has been widely used to study DNA−drug interactions because of its formation of a well-defined core particle (*27*, *31*, *36*, *37*, *41*, *42*). Previous work has shown that within the 5S core particle the nitrogen mustards mechlorethamine (HN2), chlorambucil, and melphalan retain the 5′-GNC sequence preference independent of variations in rotational (i.e., facing away from or toward the histone core) and translational (i.e., distance from the dyad) positions (*36*). We therefore likewise compared the interstrand cross-linking reactions of DEB and DEO within this 154 base pair fragment and found that not only is the 5′-GNC target sequence previously reported for synthetic DNA oligomers retained in the free restriction fragment but also that this target sequence is preserved within the core particle. Moreover, the secondary preference for 5′-GNNC demonstrated by DEO in short oligomers becomes significant in longer DNA, leading to a difference in the major cross-linked products for DEB and DEO.

MATERIALS AND METHODS

Purification of Core Particles. Core particles were isolated by a method similar to that of Libertini and Small (*43*) from chicken erythrocytes (Jason's Butcher Shop, Albion, ME) as previously described (*36*). Eighteen percent SDS−polyacrylamide gel electrophoresis (PAGE) on ∼30 μg of sample showed four protein bands of approximately equal intensities that comigrated with a commercially obtained sample of a histone mixture containing H3, H4, H2A, and H2B (Boehringer Mannheim).

Isolation of 5S DNA Fragment. The plasmid pXP-11 (*42*), containing a portion of the 5S RNA gene of *Xenopus borealis*, was kindly supplied by the Tullius laboratory (Boston University). We used pXP-11 to transform *Escherichia coli* and performed plasmid purifications with Qiagen p-2500 purification columns. *Eco*RI−*Rsa*I double digestion was followed by 3′-end radiolabeling of the *Eco*RI site under standard conditions (*44*). Radiolabeled 154-mer was purified through 6% native PAGE (37.5:1 acrylamide:bisacrylamide) followed by the crush-and-soak procedure (*44*).

Reconstitution. Lyophilized, radiolabeled DNA fragment was reconstituted into chicken core particles by one of two methods. For the "dilution" method (*45*), lyophilized, radiolabeled 154-mer (∼2 μg) in a siliconized microcentrifuge tube was incubated with variable amounts of core particles in 1 M NaCl, TE [10 mM Tris buffer (pH 7.5) and 1 mM EDTA], phenylmethanesulfonyl fluoride (PMSF; 0.50 mM from a 50 mM stock in 2-propanol) for 1 h on ice (20 μL total volume). The salt concentration was then gradually decreased by adding aliquots of TE/PMSF (+ 5 μL, 1 h incubation; + 5 μL, 1 h incubation; + 170 μL, 15 min incubation; +200 μL, minimum of 15 min incubation or storage at 4 °C). This method resulted in a mixture of free and nucleosomal DNA (∼20 μg of core particles produced approximately an equal amount of each). For the "dialysis" method (*46*), lyophilized, radiolabeled 154-mer (∼0.07 μg) was mixed with 20 μg of core particles in TE/PMSF/1 M NaCl (final volume of 100 μL), incubated on ice for 45 min, and then transferred to a Slide-a-Lyzer Mini (Pierce). The salt concentration was then gradually decreased by dialyzing the mixture at 4 °C for 1 h periods against 300 mL portions of TE/PMSF successively containing 0.8 M NaCl, 0.6 M NaCl, and 0.05 M NaCl. This method resulted in ca. 90% nucleosomal DNA. Formation of nucleosomal DNA was monitored through 6% native PAGE (37.5:1 acrylamide: bisacrylamide) containing 5% glycerol run in 0.5× TBE at 4 °C.

Cross-Linking Reactions. Radiolabeled DNA reconstituted to yield a ca. 1:1 ratio of free to nucleosomal product was incubated with 250 mM DEB or 250 mM DEO (final volume of 100 μL) at 37 °C for 15 min (DEB) or 1 h (DEO). Native loading dye [6× (0.05% xylene cyanol in 50% glycerol)] was added to these samples, which were then subjected to 6% native PAGE as described above. Nucleosomal and free DNA were purified by the crush-and-soak procedure (*44*). Alternatively, free radiolabeled DNA in TE buffer was incubated under similar reaction conditions and then ethanol-precipitated. HN2 (50 μM) reactions were in 40 mM sodium cacodylate (pH 8) at 37 °C for 1.5 h as previously described (*36*).

Separation of Cross-Link. Cross-linked samples were resuspended in 10 μL of 5 M aqueous urea/0.1% xylene cyanol and loaded onto a 6% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide, 42% urea, 30% formamide, 0.35 mm thick, 41 × 37 cm), run on a Hoefer thermojacketed Poker Face gel stand at ca. 60 W and ambient temperature to minimize loss of the heat-labile adducts. Autoradiography/phosphorimagery (Bio-Rad GS-505 imaging system with Molecular Analyst version 2.1.2) revealed a family of bands of reduced mobility relative to single strands, corresponding to interstrand cross-linked isomers (*47*).

Piperidine Cleavage of Diepoxide-Alkylated DNA. Diepoxide cross-linked and monoalkylated DNA samples were recovered from denaturing polyacrylamide gels by the crush-and-soak procedure (*44*). These samples were cleaved at sites of guanine N7 alkylation by heating at 90 °C in 10% aqueous piperidine for 15 min (*48*). Control (not alkylated) samples showed virtually no degradation under these conditions.

Sequencing Gel Analysis. Samples were dissolved in 10 μL of 5 M urea/0.1% xylene cyanol and loaded onto an 8% denaturing gel (19:1 acrylamide:bisacrylamide, 50% urea) run at ca. 65 W and 55 °C. After drying and autoradiography, bands were assigned with reference Maxam−Gilbert G-lanes (*48*).

RESULTS

To determine whether the 5′-GNC cross-linking consensus sequence previously found in short synthetic oligomers is retained in longer DNA, a 154-base pair radiolabeled restriction fragment was incubated independently with 250 mM DEB or DEO. Analysis of these reactions by 6% denaturing PAGE revealed primarily single-stranded DNA with a family of bands of reduced mobility corresponding to interstrand cross-linked isomers (Figure 2). This pattern was noticeably different for the two diepoxides, suggesting some differences in the sites and/or efficiencies of cross-linking for the two agents.

The low-mobility bands were purified from wet denaturing gels in order to locate the sites of cross-linking within the 154 bp fragment. Additionally, the single-stranded regions of the gel, containing both unreacted DNA and monoadducts, were also purified. Both cross-links and monoadducts were treated with 10% aqueous piperidine at 90 °C to cleave at sites of N7 alkylation. The resulting fragments were run on 8% dPAGE and the cleavage bands were assigned via reference Maxam−Gilbert G reactions (Figure 3). Monoalkylation occurred at every deoxyguanosine site with some variations in intensity between sites. Virtually no cross-linked DNA remained intact, supporting N7 of deoxyguanosine as the major site of alkylation, with major cleavage occurring at every 5′-GNC site for both DEB and DEO. Additionally, significant cross-linking also occurred at every 5′-GNNC site for DEO. For example, cleavage also occurred at G50′, G76′, G78′, and G106′ in the DEO samples, suggesting cross-linking at 5′-GCAC, 5′-GTGC, 5′-GCTC, and 5′-GTGC, respectively. Furthermore, purification and piperidine cleavage of individual cross-linked bands verified that the least mobile band corresponded to central cross-links. That is, the band indicated by the arrow in Figure 2 comprised DNA cross-linked at G64−G66′, G68−G70′, G79−GG81′, G85−G87′, and G86−G88′ (with the first residue on the top strand

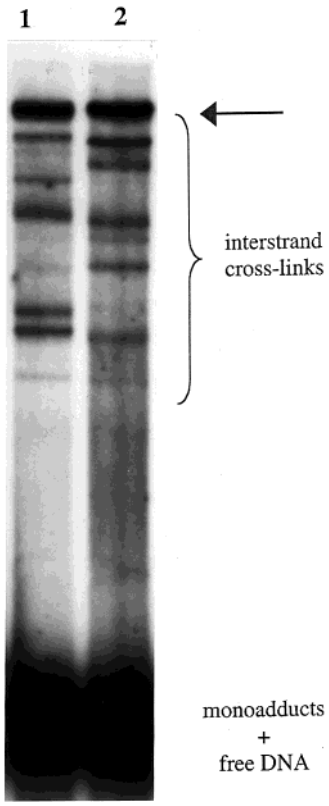

FIGURE 2: Denaturing PAGE analysis of diepoxide-cross-linked, radiolabeled DNA. Bands of lower mobility than single strands correspond to interstrand cross-links. Lane 1, DEO-treated (250 mM) free DNA; lane 2, DEB-treated (250 mM) free DNA. Arrow denotes lowest mobility cross-linking band subsequently determined as corresponding to central cross-links.

linked to the second residue on the bottom strand as depicted in Figure 1).

We next wanted to test whether the cross-linking sites would be preserved if the 154-mer were in the form of a nucleosomal core particle rather than as free DNA. We first performed a series of reconstitution reactions under different conditions to generate nucleosomal DNA. 3′-Radiolabeled 154-mer was incubated with variable amounts of purified chicken erythrocyte core particles under conditions of high salt (1 M NaCl) in order to disassemble the chicken core particles. The salt concentration was then successively lowered through dilution, allowing re-formation of the histone octamer onto the desired 154-mer. Incorporation of the free DNA into the nucleosome was monitored via 6% native PAGE, which yielded two major bands upon autoradiography: a higher mobility band corresponding to free DNA and a lower mobility band corresponding to nucleosomal DNA (*42*, *45*, *36*, *37*). The amount of nucleosomal product was a function of the core particle-to-DNA ratio, with higher ratios leading to the formation of more radiolabeled nucleosomes (Figure 4). We found that incubation of ~40 μg of chicken core particles with ~0.07 μg of radiolabeled 154-mer yielded the maximum yield of radiolabeled nucleosomal DNA (~94%), whereas 1.25 μg of core particles with ~0.07 μg of radiolabeled 154-mer yielded a mixture of approximately equal amounts of free and nucleosomal DNA. We and others have previously demonstrated that the nucleosomal DNA in this system is well-defined positionally via hydroxyl radical cleavage analysis (*27*, *42*, *36*, *37*).

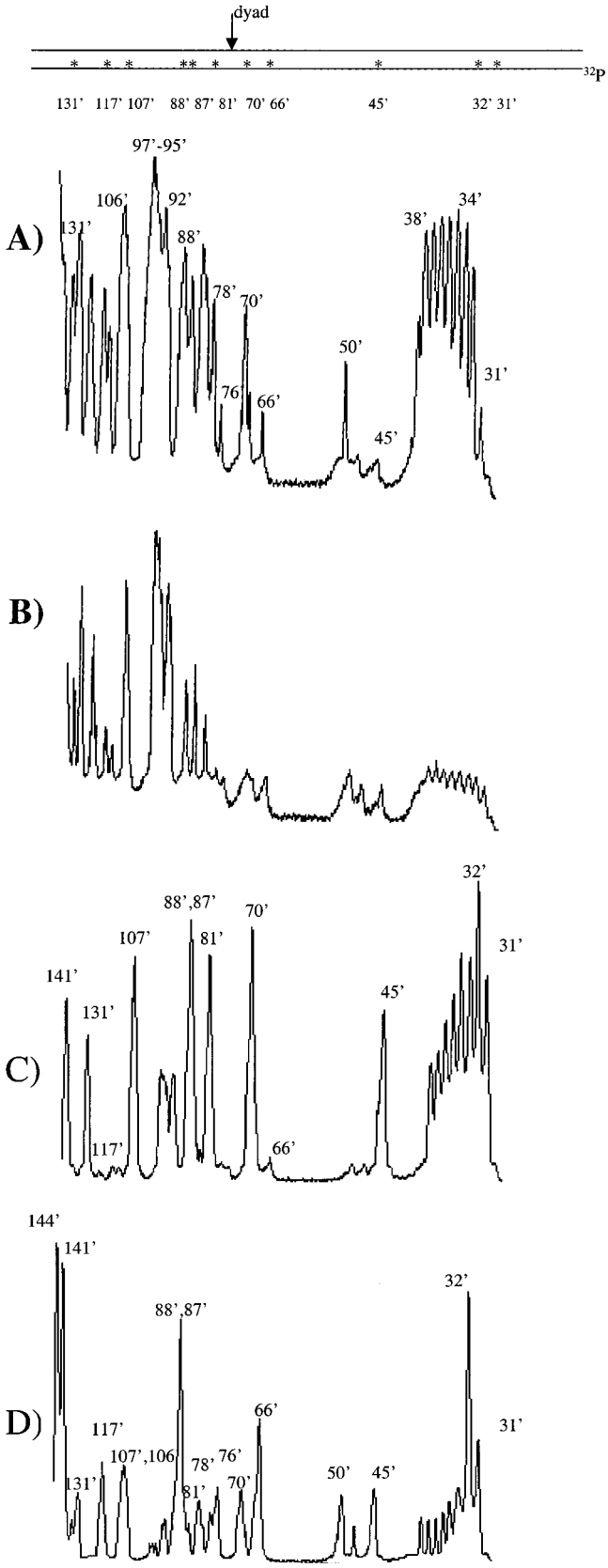

FIGURE 3: Two-dimensional scans of the phosphorimage of the denaturing PAGE analysis of piperidine-cleaved monoalkylated and cross-linked free 5S DNA. Potential sites of 5′-GNC cross-linking are shown as asterisks on the cartoon map of the 154 bp fragment, which is reversed from the depiction in Figure 1. (A) DEB-monoalkylated DNA; (B) DEO-monoalkylated DNA; (C) DEB-cross-linked DNA; (D) DEO-cross-linked DNA.

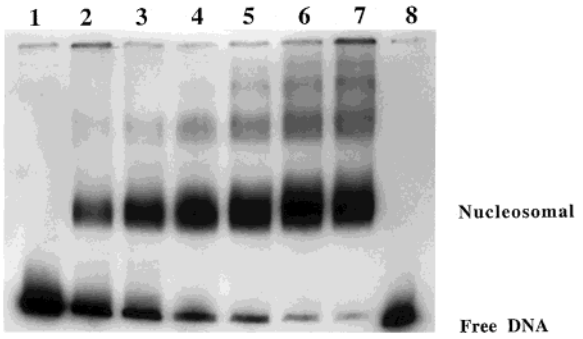

FIGURE 4: Native gel separation of free (lower band) and nucleosomal (upper band) 5S DNA. Radiolabeled 5S DNA (~0.07 μg) was reacted with variable amounts of histones. Lane 1, no histones; lane 2, 1.25 μg of histones; lane 3, 2.5 μg of histones; lane 4, 5 μg of histones; lane 5, 10 μg of histones; lane 6, 20 μg of histones; lane 7, 40 μg of histones; lane 8, control free DNA.

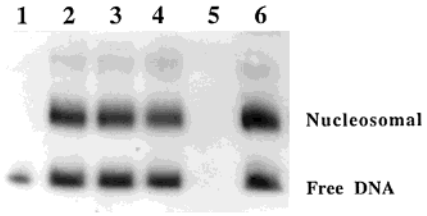

FIGURE 5: Native gel separation of free (lower band) and nucleosomal (upper band) 5S DNA fragment reconstituted under conditions designed to produce approximately equal amounts of free and nucleosomal DNA. Lane 1, control free DNA; lane 2, reconstituted DNA incubated with 250 mM DEB; lane 3, reconstituted DNA incubated with 250 mM DEO; lane 4, reconstituted DNA incubated with 50 μM mechlorethamine; lane 5, blank; lane 6, control reconstituted DNA.

We then proceeded with cross-linking studies of nucleosomal DNA, performing reconstitution under conditions that produced a mixture of approximately equal amounts of free and nucleosomal DNA in order to ensure identical cross-linking reaction conditions for the two. Aliquots of this reconstitution reaction were then incubated with either DEB, DEO, or HN2 prior to the separation of free and nucleosomal DNA through 6% native PAGE. Two major bands were again visible upon autoradiography: a high-mobility band corresponding to free DNA and a lower mobility band corresponding to nucleosomal DNA (Figure 5). Control reconstitution aliquots were indistinguishable from DEB-, DEO-, or HN2-cross-linked reconstitution aliquots on these gels, indicating that the integrity of the core particle was not disrupted upon cross-linking and electrophoresis. The bands corresponding to free and nucleosomal DNA were independently isolated from these gels for subsequent analysis of cross-linked product through 6% dPAGE. Again, autoradiography revealed primarily single strands and a family of low-mobility bands (Figure 6) that appeared similar for the free and nucleosomal samples of each pair. However, the pattern for each agent was slightly different, again suggesting subtle differences in the cross-linking of this DNA fragment by DEB, DEO, and now HN2. We performed quantitative phosphorimagery and found that all three agents produced similar cross-linking efficiencies for free and nucleosomal DNA (~17% for the DEB samples, ~10% for the DEO samples, and ~17% for the HN2 samples). Two-dimensional profile scanning of each gel lane was also performed for a quantitative comparison of the relative

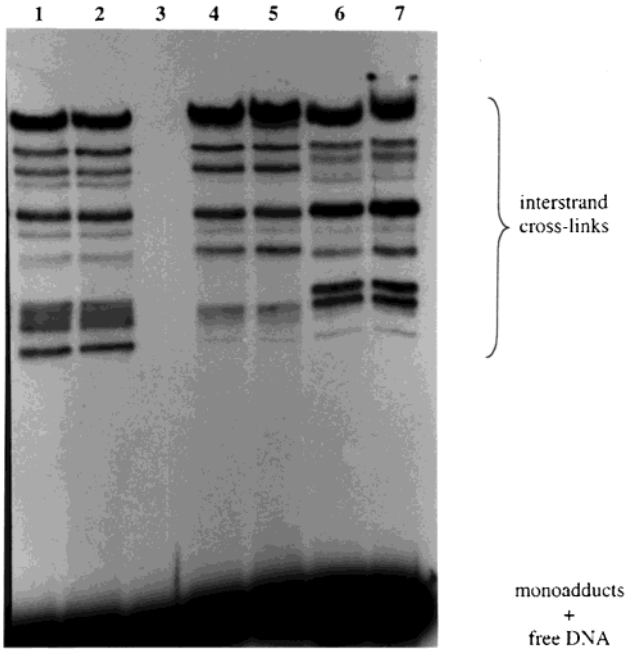

FIGURE 6: Denaturing PAGE analysis of cross-linked free and nucleosomal DNA. Bands of lower mobility than single strands correspond to interstrand cross-links. Lane 1, HN2-treated (50 μM) free DNA; lane 2, HN2-treated (50 μM) nucleosomal DNA; lane 3, control (not cross-linked) DNA; lane 4, DEB-treated (250 mM) free DNA; lane 5, DEB-treated (250 mM) nucleosomal DNA; lane 6, DEO-treated (250 mM) free DNA; lane 7, DEO-treated (250 mM) nucleosomal DNA.

intensities of the cross-linking patterns for the free and nucleosomal DNA (data not shown). This analysis verified that there were no significant differences between the free and nucleosomal patterns of cross-linked bands for DEB, DEO, or HN2.

Sites of monoalkylation and cross-linking for DEB and DEO in both the free and nucleosomal samples were verified via piperidine cleavage and 8% dPAGE following purification from wet denaturing gels. Again, monoalkylation occurred at every deoxyguanosine residue and cross-linking occurred at 5′-GNC sites for DEB and at both 5′-GNC and 5′-GNNC sites for DEO (Figure 7). There were some small differences in the intensities of cleavage between the free DNA shown in Figure 3 and these nucleosomal DNA samples. Subtle differences in reaction conditions between these samples and/or incomplete recovery during gel purification could have contributed to these differences.

To investigate whether DNA that had previously been treated with diepoxide could be incorporated into the structure of the nucleosome, radiolabeled 154-mer was mixed with core particles as for a reconstitution to yield ~90% nucleosomal DNA except for the absence of NaCl to disassemble the chicken core particles. DEB or DEO was then added to the sample and, following the appropriate reaction time at 37 °C, NaCl was added to a final concentration of 1 M. Samples were incubated at 4 °C to disassemble the core particles and then dialyzed against TE/PMSF buffer containing successively less NaCl. We simultaneously reconstituted samples that had been treated with DEB or DEO and then ethanol-precipitated, as well as a control sample that was immediately treated with DEB or DEO after reconstitution. Native 6% PAGE demonstrated that similar levels of reconstitution occurred in all of these samples

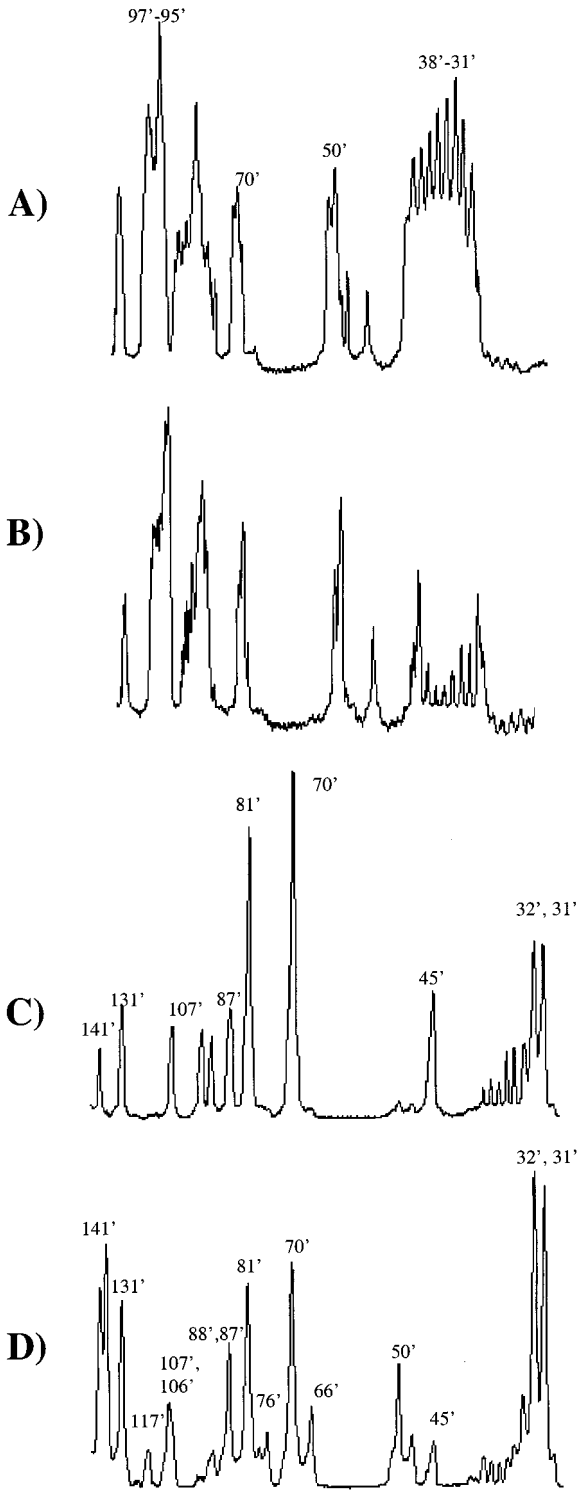

FIGURE 7: Two-dimensional scans of the denaturing gel analysis of piperidine-cleaved monoalkylated and cross-linked nucleosomal DNA. (A) DEB-monoalkylated nucleosomal DNA; (B) DEO-monoalkylated nucleosomal DNA; (C) DEB-cross-linked nucleosomal DNA; (D) DEO-cross-linked nucleosomal DNA.

(Figure 8), suggesting both that histones are not adversely affected by diepoxide treatment and that diepoxide-cross-linked DNA can be incorporated into the core particle structure.

DISCUSSION

Early reports suggested that bifunctional alkylators must span at least 8 Å to link deoxyguanosines at the sequence

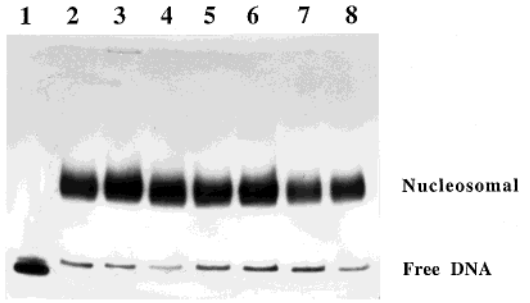

FIGURE 8: Native gel analysis of nucleosomal and free diepoxide-treated 5S DNA. Lanes 2 and 3 contain samples that were first reconstituted with histones and then reacted with diepoxide. Lanes 4 and 5 contain samples that were first reacted with diepoxide and then ethanol-precipitated prior to reconstitution with histones. Lanes 7 and 8 contain samples that were reacted with diepoxide in the presence of histones prior to reconstitution. Lane 1, free DNA; lane 2, 250 mM DEB-reconstituted first; lane 3, 250 mM DEO-reconstituted first; lane 4, 250 mM DEB-reacted, ethanol-precipitated, and then reconstituted; lane 5, 250 mM DEO-reacted, ethanol-precipitated, and then reconstituted; lane 6, control reconstitution; lane 7, 250 mM DEB-reacted in the presence of histones and then reconstituted; lane 8, 250 mM DEO-reacted in the presence of histones and then reconstituted.

with the minimal N7-to-N7 distance in B-DNA, 5′-GC (*15*, *49*). Paradoxically, such a requirement would preclude reactivity for agents of less than seven carbon atoms in length despite experimental evidence that such cross-linking occurs (*50*). In fact, the five-atom nitrogen mustards and four-atom DEB form interstrand cross-links at 5′-GNC sequences, suggesting that considerable DNA distortion must accompany an N7-to-N7 cross-link between deoxyguanosines on opposite strands (*17*, *18*, *20*, *51*). Indeed, DNA bending upon mustard interstrand cross-linking has been experimentally verified (*52*). The ability of the relatively short mustard and DEB tethers to cross-link a distance of about 9 Å provides evidence for considerable conformational flexibility of DNA during these reactions. Hopkins et al. (*51*) have suggested that DNA propeller twisting and kinking may combine to narrow the major groove, thereby driving the relevant N7 lone electron pairs together to facilitate mustard cross-linking. This hypothesis is supported by the finding of a major-groove-compressing bend in the crystal structure of an oligomer containing the mustard/diepoxide recognition sequence, 5′-GGCC (*53*). It is reasonable to speculate that the constraint imparted by histones in nucleosomal DNA could preclude the necessary conformational flexibility required for cross-linking the canonical 5′-GNC sequence, thereby altering the diepoxide mode of attack from that demonstrated with free DNA. Indeed, different adducts for the reaction of DEB with free and cellular DNA have been reported and attributed to chromatin structure (*38*).

Our previous studies of interstrand cross-linking by the diepoxides DEB and DEO were limited to reporting sequence preferences for interstrand cross-linking in short DNA oligomers (*20*, *21*). 5′-GNC is the principal target for both agents, but 5′-GNNC is a secondary target, reacting at ~50% of the frequency of 5′-GNC for DEB and ~77% of the frequency of 5′-GNC for DEO when both sequences are presented in a single DNA duplex. In an attempt to understand further the biological outcomes of diepoxides, we have extended these diepoxide studies to a longer DNA: a 154 base pair restriction fragment from the 5S DNA of *X.*

borealis that has the added benefit of forming well-positioned nucleosomal core particles.

We used 6% denaturing polyacrylamide gel electrophoresis to monitor cross-linking. Interstrand cross-linked isomers are separated on denaturing gels as distinct bands with central cross-links having the lowest mobility (*47*). When free radiolabeled 154-mer was incubated with either DEB or DEO, a family of low-mobility bands was visible after electrophoresis and autoradiography. The patterns of interstrand cross-links were slightly different for the two diepoxides, suggesting differences in the sites and/or efficiencies for cross-linking within this restriction fragment. Sites of alkylation were pinpointed through piperidine cleavage of gel-purified monoadducts and cross-links followed by further dPAGE. Fairly uniform monoalkylation occurred with one exception being the G-rich tract from G31′ to G38′, which showed significant enhancement in the DEB samples. Many agents that alkylate the N7 position of guanine, including nitrogen mustards, exhibit such preferential alkylation at runs of contiguous guanines (*54*, *40*). Moreover, the sequence preference for cross-linking differed for the two agents: DEB showed significant cross-linking only at 5′-GNC sites, while DEO showed significant cross-linking at both 5′-GNC and 5′-GNNC sites. Thus, despite showing quite similar reactivity within short DNA oligomers, when a multitude of sites are presented within a restriction fragment of DNA and reaction products are examined under conditions of single hit (an average of less than one alkylation event per molecule), the cross-linking preferences of these two agents differ. Furthermore, the longer alkyl chain length of DEO appears to reduce the absolute cross-linking specificity, as demonstrated by the two sequences preferentially cross-linked. Alley et al. (*55*) have demonstrated this effect for 2,5-bis(1-aziridinyl)-1,4-benzoquinone relative to mechlorethamine, with the flexibility of its 10-atom chain cited as the cause of the observed 5′-GN$_n$C specificity, where n= 0, 1, or 2. The extended chain length of DEO is approximately 9 Å long (*50*) as compared to the approximately 9 Å 5′-GNC interatomic distance and the 11 Å 5′-GNNC interatomic distance to link the N7 positions of deoxyguanosines on opposite strands of B DNA (*56*), further suggesting that the degree of distortion required to accommodate this tether may be reduced relative to that for DEB.

We then compared cross-linking of both free and nucleosomal DNA for DEB, DEO, and the nitrogen mustard mechlorethamine. The 154 base pair fragment of the 5S DNA was presented as a mixture containing approximately equal amounts of the free and nucleosomal forms in order to maintain identical reaction conditions within the two samples for better comparison of the products. This fragment forms a uniquely positioned core particle with 16 5′-GNC sites and 16 5′GNNC sites distributed along the DNA (Figure 1). These sites are presented in a variety of rotational settings, from those that face the octamer and contain a compressed minor groove to those that face away from the histone core and contain a compressed major groove (*27*). These sites also vary in the identity of the central N(s) and the flanking sequences, which have been shown to modulate the overall cross-linking efficiency of such agents as mitomycin C (*57*, *58*).

Following cross-linking, we independently isolated free and nucleosomal DNA from native gels and analyzed the reaction products via dPAGE. The patterns of DEB and HN2 cross-links were similar with some subtle differences in the relative intensities of some bands, but that of DEO was distinct. Moreover, the patterns for the free and nucleosomal sample were identical for each agent within the pair. Piperidine cleavage verified fairly uniform monoalkylation at all deoxyguanosines and cross-linking at 5′-GNC sites for DEB and DEO and cross-linking at 5′-GNNC sites for DEO in the nucleosomal samples, consistent with the pattern of the free 154-mer. Some small differences in the cleavage intensities for specific sites of cross-linking were observed for the free and nucleosomal samples shown in Figures 3 and 7, for example, the decreases in cross-linking at G87′ and G66′ and the increase at G31′ in the nucleosomal samples. The former two sites undergo maximal cleavage by hydroxyl radical, indicating that they face away from the histone core and contain a compressed major groove (*27*). Furthermore, these sites are each approximately one helical turn from the nucleosomal dyad. Interestingly, inhibition of cross-linking at both G87′ and G66′ by the nitrogen mustard chlorambucil has also been observed for nucleosomal DNA, as has enhancement at G31′ (*36*). However, these differences are modest, as verified by the identical patterns of cross-linked bands seen in Figure 6 for free and nucleosomal samples that experienced identical reaction conditions.

The conservation of reactivity within the 5S nucleosome relative to free DNA has been previously reported for HN2 (*36*), but we wished to compare its cross-linking pattern to the diepoxides directly within the longer DNA. While much higher concentrations of diepoxide were necessary for reaction (250 mM diepoxide versus 50 μM for HN2), reaction times to achieve no more than a single average alkylation per molecule were shorter for the diepoxides (15 min for DEB, 1 h for DEO, and 1.5 h for HN2). Longer reaction times for the diepoxides led to a blurring of the cross-linking pattern (data not shown), and reduced concentrations led to recovery of insignificant amounts of cross-linked material (data not shown). Reduced DEB cross-linking efficiency relative to mustards has been documented in DNA oligomers (*20*), in purified T7 DNA (*59*), and in herring sperm chromatin (*13*). This inefficiency has been attributed to the short alkyl chain length of DEB (*59*), yet the longer-chained DEO is clearly less efficient still, suggesting that other factors are responsible. One relevant factor is likely the distinct mechanisms of epoxide and mustard reactions, which are S_N1 and S_N2, respectively (*60*−*62*). The rate-determining step in mustard cross-linking corresponds to formation of the aziridinium active intermediate (*63*). The S_N2 mechanism of diepoxide cross-linking, on the other hand, is first-order in both DNA and diepoxide, suggesting a large concentration effect. Under our reaction conditions, both DEB and HN2 produced comparable levels of cross-linking that were the same for free and nucleosomal DNA (∼17%), but DEO produced only about 60% as much cross-linking, even with substantially longer reaction times than DEB. The relative reactivity of diepoxyalkanes decreases with increasing chain length (*60*), which is likely to account for the reduced efficiency of DEO relative to DEB at similar concentrations even at longer reaction times.

Despite the fact that DEB interstrand cross-linking is likely to induce considerable structural changes in DNA, our findings suggest that there are no significant differences in

the attack of nucleosomal and free DNA. It appears that access to the major groove is not greatly impaired by the histone core and that core particle DNA tolerates the conformational changes induced by DEB and DEO cross-linking. To verify that the latter is true, we also performed reconstitution experiments on already cross-linked DNA. We found that labeled 154-mer was equally well incorporated into the core particle structure after treatment with diepoxide in both the presence and the absence of the histones. These reconstitutions looked identical to a control reconstitution and also to a control reconstitution that was treated with DEB/DEO following its reconstitution. Previously, we demonstrated that both cisplatin and its *trans* isomer react with histones to negatively impact nucleosomal core particle formation. Although epoxides alkylate proteins (*64*) and produce DNA−protein cross-links (*65*), under our conditions either the histones do not react, or their reaction does not impede subsequent dismantling and re-formation of the core particle structure.

We considered the possibility that monoadducts could convert to cross-links when histones are removed during the purification of nucleosomal DNA. However, because there is no obvious loss of monoalkylated products at cross-linked sites when comparing nucleosomal to free DNA samples, we consider that it is unlikely that conversion of monoadducts to cross-links occurs during electrophoresis and purification in the nucleosomal samples. We are confident, therefore, that our findings for the conservation of cross-linking sites are not merely artifacts of our procedures. The nucleosome must be able to tolerate the structural distortions required for diepoxide cross-linking, consistent with a flexible model of chromatin that "breathes", allowing exposure of sites that would otherwise be blocked by the histone core.

In general, attack of nucleosomal DNA appears to be affected by the size and kinetics of the reacting agent. Relatively small compounds such as dimethyl sulfate react similarly with nucleosomal and free DNA, whereas relatively large molecules such as aflatoxin demonstrate a 2−3-fold reduction in binding to the center of the nucleosome relative to free DNA, with protection diminishing at the ends of the nucleosomes (*30*, *31*). Monoalkylators do not demonstrate the 10−11 bp periodicity demonstrated by agents that may react quickly on the time scale of core particle breathing, such as hydroxyl radical (*27*, *28*), some enediynes (*42*, *66*), and UV light (*67*, *68*). That is, rotational positioning does not appear to impact alkylation even for bulky compounds such as BPDE, with sites facing both toward and away from the histone octamer targeted (*32*, *33*). This also seems to be true for many of the interstrand cross-linking agents examined to date; for example, the mustards, cisplatin and its trans isomer, and now DEB and DEO (*36*, *37*). Mitomycin C seems to be an exception, with both rotational and translational position impacting its cross-linking efficiency at its preferred 5′-CG site, a fact that has been attributed to its rapid reaction rate relative to the time scale of nucleosome breathing (*36*).

In conclusion, we have found that the 5′-GNC consensus sequence for DEB and DEO interstrand cross-linking previously found in short DNA oligomers is conserved in a fragment of the 5S DNA in both the free and nucleosomal state. The secondary 5′-GNNC site for DEO also becomes significant in this longer DNA, suggesting that while short DNA oligomers are good preliminary model systems, they may not be sufficient to describe completely the sites targeted in the genome. Our results are further support for the accessibility of DNA within the 5S nucleosome, which appears to be a relatively minor inhibitor of DNA interstrand cross-linking reactions. However, we have not yet accounted for products other than interstrand cross-links, different DNA sequences in the core particle, or chemical modification of histones, nor have we included linker DNA/histones in our system, all of which may provide a system more representative of the higher level chromatin structure found in vivo.

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