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

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ABSTRACT: Antitumor agents of the nitrogen mustard family and mitomycin C form interstrand cross-links in duplex DNA. To provide information about the cellular mechanism by which these compounds exert their cytotoxic effects, we examined cross-linking of a nucleosomal core particle formed on a fragment of the 5S RNA gene of *Xenopus borealis*. For the mustards mechlorethamine, chlorambucil, and melphalan, both sites of monoalkylation and interstrand cross-linking were similar in nucleosomal and free DNA. Some small (two- to three- fold) differences in intensity of cross-linking at some sites were apparent. However, these differences did not appear to correlate with rotational or translational positioning. For mitomycin C, cross-linking was inhibited five- to ten-fold at the nucleosomal dyad and showed attenuation of inhibition toward the ends. Furthermore, rotational positioning also appeared to be a factor, with sites facing inward in the nucleosome less accessible for mitomycin cross-linking. None of these agents demonstrated the 10-base pair periodicity exhibited by hydroxyl radical cleavage of nucleosomal DNA.

DNA interstrand cross-linking agents bind covalently to both strands of the double helix, inhibiting both replication and gene expression. Such bifunctional alkylating agents have been recognized for many years to be among the most powerful antitumor tools. However, although the nitrogen mustards, cisplatin, and mitomycin C (MC)[1] are indeed widely used human chemotherapeutics (*1*), numerous livestock deaths in the Northwest result from ingestion of plant material containing deadly pyrrolizidine alkaloids (*2*). Furthermore, diepoxides, which are widespread in nature, produce chromosomal aberrations and the induction of cancer (*3*). The mechanisms by which different cross-linkers elicit profoundly different biological responses remain unclear.

Goldacre et al. (*4*) first suggested that the cytotoxicity of the nitrogen mustards, the first clinically useful anticancer drugs, might be directly related to cellular cross-linking reactions. Subsequent in vitro and in vivo studies demonstrated conclusively the formation of DNA interstrand cross-links for these and other highly toxic bifunctional electrophiles (*5*−*8*). Moreover, interstrand cross-linking has been found to correlate with mustard cytotoxicity (*9*) and may be critical in the action of other antitumor drugs.

The in vitro cross-linking of free DNA by many agents has been described extensively in the literature. However, in eukaryotic cells, the majority of nuclear DNA does not exist in a free state but is complexed with histones. The result is chromatin, a polymer composed of repeating "nucleosome" subunits (for reviews, see refs *10*−*13*). The nucleosome can be further subdivided into a "core particle" and a "linker" region: the "bead" and the "string," respectively, of the "beads-on-a-string" model. The core particle contains 146 bp of DNA wrapped around a histone octamer [one $(H3, H4)_2$ tetramer plus two (H2A, H2B) dimers], and the linker contains DNA of variable length (20−70 bp), histone H1 (and H5 in avian erythrocytes), and various nonhistone proteins. The resulting structure not only facilitates DNA packaging in the nucleus but has also been implicated in regulatory events. Variations in histone sequence (*14*−*16*) and chemical modification patterns (*17, 18*) also contribute to regulation of gene expression.

Incorporation of DNA into the nucleosome results in substantial structural alterations relative to free DNA. Nucleosomal DNA undergoes a rather dramatic curvature of ∼45° per helical turn as it wraps around the histone octamer. Core particles are symmetric about a dyad axis, but the DNA does not follow a regular path. Instead, it sharply bends at several sites and demonstrates periodic narrowing and widening of the minor groove (*19*−*23*). Furthermore, the three helical turns of DNA at the center are underwound relative to B-DNA, with a repeat of 10.7 bp/turn, whereas the rest of the DNA within the core particle is overwound, with a repeat of 10.0 bp/turn (*24*). These structural deviations result in differential reactivity within the nucleosome toward singlet oxygen (*25*), hydroxyl radical (*24; 26*), and a variety of other external agents (*27*).

The effect of nucleosomal structure on DNA cross-linking reactions is not well understood. Whereas psoralen photoreacts exclusively with linker regions, attributed to restricted intercalation into nucleosomal core DNA (*28*−*30*), the preferred site of binding of cisplatin within the nucleosome depends on the level of binding. At low binding ratios ($r <$ 0.05, where $r =$ moles of drug/mole of nucleotide base pairs),

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[1] Abbrevations: mitomycin C, MC; mechlorethamine, HN2; chlorambucil, CBC; melphalan, L-PAM; phenylmethylsulfonyl fluoride, PMSF; polyacrylamide gel electrophoresis, PAGE; sodium dithionite, SDT.

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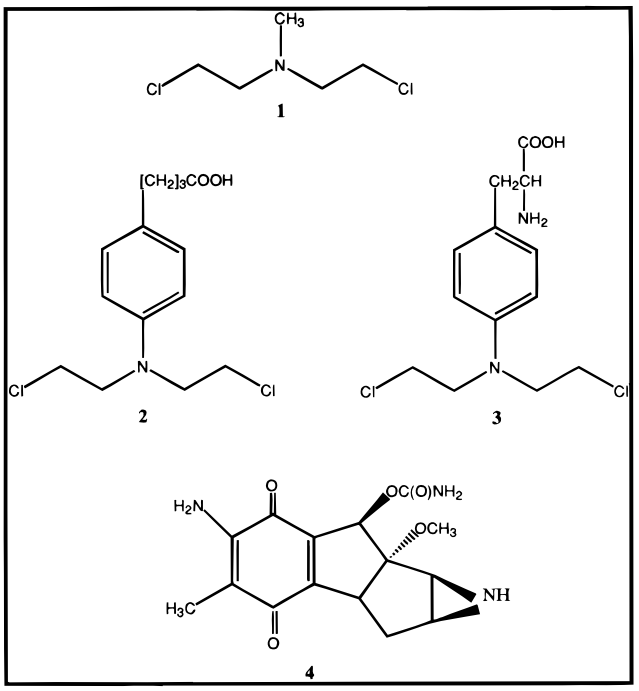
FIGURE 1: Sequence of the *Eco*RI-*Rsa*I restriction fragment of the *Xenopus borealis* 5S RNA gene and 5′-flanking region. Numbering is from 1 to 154 in the 5′-3′ direction of the top strand; this corresponds to positions −78 to +75 on the 5S RNA gene. Numbering of the bottom strand is in the same direction, but these residues are denoted with a prime (′) in the text. Position 16 in our sequence shows a C→G transversion from the wild-type sequence. Putative mustard cross-linking sites at GNC sequences are shown in bold; putative mitomycin C sites at CG are underlined. The 5′-end labeling was of the top strand; whereas the 3′-end labeling was of the bottom strand.

cisplatin also shows preferential binding to linker DNA (*31*, *32*). This preference has been attributed to histone-induced constraint on the core DNA, which could block the necessary distortion for cisplatin binding. At higher levels of drug ($r < 0.1-0.2$), linker DNA does not react preferentially (*33*). *N*-Methylmitomycin A demonstrates ~20-fold less cross-linking for defined sequence core particles relative to free DNA (*34*), again indicating a preference for the linker region. However, in all of these studies, interstrand cross-links were not analyzed independently from all other adducts.

On the other hand, the alkylation patterns of nitrogen mustard, uracil mustard, and quinacrine mustard are remarkably similar in free DNA and cellular DNA (*35*), suggesting little effect of nucleosomal structure on mustard binding. Furthermore, in defined sequence nucleosomes, the mustard melphalan shows only slightly less reactivity toward core DNA than toward the linker, with little overall alteration in nucleotide sequence (*36*). However, once again, only total alkylation was examined in these studies. The distribution of mustard interstrand cross-links exclusively within the nucleosome has not been reported. Because mustard cross-linking reactions are less favorable than monoalkylation and produce only a small subset of the total products (*37*), it is possible that a difference in cross-linked adducts will be masked by similar monoalkylation patterns in free and nucleosomal DNA when total products are studied.

We report here the interstrand cross-linking of defined sequence core particles by mustards and MC. These core particles were produced by replacing genomic chicken DNA in isolated core particles with a fragment of the 5S RNA gene from *Xenopus borealis*. The 5S nucleosome has been widely used to study DNA−drug interactions because the 5S DNA complexes to the histone octamer in a unique setting with the start site of the gene close to the dyad (*24*, *38*). Such a well-defined system allows an easy comparison between the sites targeted in free versus nucleosomal DNA. We examined interstrand cross-linking by the nitrogen mustards mechlorethamine (HN2; **1**), chlorambucil (CBC; **2**), and melphalan (L-PAM; **3**) as well as MC (**4**). (See structures **1**−**4**). Mustards preferentially alkylate the N7 position of deoxyguanosine residues on opposite strands in the duplex sequence 5′-GNC (where N is any base) (*39*−



41), whereas MC targets the N2 position of deoxyguanosine residues on opposite strands in the duplex sequence 5′-CG (*42*−*44*). Considerable global DNA distortion has been postulated to accompany mustard cross-linking (*40; 45; 46*), whereas MC interstrand cross-links induce only slight local conformational changes (*47; 48*). Both the putative mustard 5′-GNC and mitomycin 5′-CG cross-linking sites are present in the 5S DNA of *Xenopus borealis* in a variety of rotational (i.e., facing away from or toward the histone core) and translational (i.e., distance from the dyad) settings (Figure 1). The nucleosomal dyad is at about residue C76 of this sequence (*38*). A comparison of the interstrand cross-linking reactions of these drugs with nucleosomal versus free DNA may help to define further not only the relative accessibilities of the major and minor grooves but also the susceptibility to conformational alterations in chromatin. In addition, these studies may provide information about DNA cross-linking by antitumor agents in vivo.

MATERIALS AND METHODS

Purification of Core Particles. Core particles were isolated by a method similar to Libertini and Small (*49*) from chicken erythrocytes (Acme Poultry; Seattle, WA). Frozen cells were lysed through thawing and addition of 0.01 M Tris (pH 7.2), 0.15 M NaCl, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The broken cells were then centrifuged at 2000 x *g* for 10 min to pack the nuclei. The supernatant was quickly removed by vacuum aspiration. The nuclei were then washed 4−5 times with the same buffer containing 0.2−0.05% Igepal (Sigma) and with decreasing centrifuge speeds to avoid irreversible nuclei packing. Nuclei lysis buffer [0.01 M Tris (pH 7.8), 0.2 mM EDTA, and 0.5 mM PMSF] was added to the pellet and stirred on ice for 15 min. The sample was spun at 16 000 x *g* for 30 min to pellet the chromatin. The chromatin was then brought to 1 mM $CaCl_2$ and warmed to room temperature. Approximately 3 units/mg chromatin of micrococcal nuclease (Sigma) was

added with successive trituration with broken pasteur pipets of orifices ~4, 3, 2, and 1 mm. Total digestion time was 30 min, at which time the solution was quite liquid and easily pipeted. Digestion was stopped with the addition of 0.5 M EDTA to a final concentration of 2 mM. The sample was centrifuged at 10 000 x *g* for 30 min. The soluble chromatin supernatant was then stirred on ice and CM-Sephadex C-25 was added (30 mg/mL solution) to remove histones H1 and H5. The solution was slowly adjusted to 50 mM NaCl by the slow addition of 4 M NaCl, stirred on ice for 60 min, and then centrifuged at 10 000 x *g* for 10 min. The pellet was discarded, and the supernatant was dialyzed in a slide-a-lyzer (Pierce) against 750 mL of buffer containing 0.02 M Tris (pH 7.8), 0.2 mM EDTA overnight at 4 °C. The dialyzed sample was centrifuged at 10 000 x *g* for 15 min. The supernatant was collected into a microcentrifuge tube, placed on ice, and adjusted to 1 mM $CaCl_2$. A test reaction was used to determine the optimal time for micrococcal nuclease (1 μL of a 1 mg/mL solution from Worthington) digestion to core particles with DNA length ~150 bp. Aliquots were removed from the test digestion, extracted with phenol/chloroform/isoamyl alcohol, ethanol precipitated, run on a 6% (37.5:1 acrylamide/bisacrylamide) denaturing polyacrylamide gel on a Mighty Small apparatus (Pharmacia Biotech), and visualized with ethidium bromide staining. Digestions were typically 25−35 min at 37 °C. Following digestion of the bulk of the sample, it was stirred with a "flea" in a microcentrifuge tube on ice and adjusted to 0.125 M NaCl by the slow addition of 4 M NaCl. Stirring was continued for 60 min and followed by centrifugation at 10 000 x *g* for 15 min to precipitate out unwanted H1, H5, and over- or under-digested cores. The sample was then dialyzed in a slide-a-lyzer overnight against buffer containing 0.01 M Tris (pH 7.2), 0.01 M cacodylate, and 0.2 mM EDTA, and stored in the refrigerator. Eighteen percent (20:1 acrylamide:bisacrylamide) SDS-polyacrylamide gel electrophoresis (PAGE) with a 5% stacking gel on 0.1−0.2 OD_{260} sample showed a clean product with four protein bands of approximately equal intensities with the same mobilities as a commercially obtained sample of a histone mixture containing histones H3, H4, H2A, and H2B (Boehringer Mannheim).

Isolation of DNA Fragment. *Eschericia coli* transformed with pXP-11 (*50*), containing the 5S RNA gene of *Xenopus borealis*, was kindly supplied by the Tullius laboratory (Johns Hopkins University). Plasmid purifications were performed 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 (*51*). 5′-End radiolabeling was performed after treatment with *Eco*RI/calf intestinal phosphatase and followed by digestion with *Rsa*I using standard conditions (*51*). Radiolabeled 154-mer was purified through 6% native PAGE (37.5:1 acrylamide:bis) followed by the crush-and-soak procedure (*51*).

Reconstitution. Lyophilized, radiolabeled DNA fragment was incubated with core particles (0.4−2 OD_{260}) in 1 M NaCl, TE [10 mM Tris (pH 7.5) and 1 mM EDTA]/PMSF (20 μL total volume) for 1 h on ice. Aliquots of TE/PMSF (or 40 mM cacodylate [pH 8] for mustard reactions) were added at 1 h intervals to decrease the concentration of NaCl in a stepwise manner to 0.8, 0.67, 0.1, and 0.05 M. Reconstitution of the radiolabeled fragment to core particles was monitored by 6% native PAGE (37.5:1, acrylamide/bis) containing 5% glycerol, run in 0.5x TBE (0.045 M Tris-borate and 0.001 M EDTA) at 4 °C. Adjustment of the radiolabeled DNA/histone ratio resulted in different proportions of reconstitution with the lower amount of histones (0.4 OD_{260}) leading to an approximately equal mixture of free and nucleosomal DNA and the higher amount (2 OD_{260}) leading to greater than >90% nucleosomal DNA.

Fe(II)EDTA Cleavage. Reactions were run as previously described by Dixon et al. (*52*). Stock solutions of 500 μM ferrous ammonium sulfate/1 mM EDTA, 100 mM H_2O_2, and 10 mM ascorbate were made immediately before use. For cleavage of free DNA, 5 μL of each of these reagents was added to 85 μL of radiolabeled DNA in TE buffer and reacted for 1 min. For cleavage of nucleosomal DNA, 10 μL of each reagent was added to 70 μL of radiolabeled reconstituted DNA in TE and reacted for 1 min. Reactions were stopped by the addition of thiourea to 0.2 mM and EDTA to 10 mM. Samples were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated prior to loading on a sequencing gel.

Mustard Cross-Linking Reactions. Fresh HN2 stock solutions (1 mM) were in 40 mM cacodylate (pH 8). Chlorambucil (CLB) and L-PAM stock solutions were 50 mM in 0.1 N HCl/DMSO. These stocks were then diluted with 250 mM cacodylate (pH 8) to 2.5 mM mustard prior to cross-linking reactions. Radiolabeled reconstituted DNA in 40 mM cacodylate (pH 8), 9 μg of calf thymus DNA, and mustard (final concentration = 50 μM for HN2; 250 μM for CLB and L-PAM) in a total volume of 100 μL were reacted for 2 h at 37 °C. After termination of the reaction by the addition of acetic acid to a final concentration of 50 mM and addition of 6x native loading dye (0.05% xylene cyanole in 50% glycerol), samples were subjected to 6% native PAGE run at 4 °C for purification of nucleosomal and free DNA by the crush-and-soak procedure (*51*).

Mitomycin C Cross-Linking Reactions. MC reactions were run as previously described (*53*). Briefly, MC was dissolved in 30% MeOH/H_2O to yield a 12.5 mM stock solution. Radiolabeled reconstituted DNA in TE buffer, 52.5 μg of calf thymus DNA, and MC (at 0.25 or 0.63 mM) in a total volume of 110 μL were incubated at 37 °C for 2 h, purged with argon for 15 min, and put on ice. The MC was reductively activated by three subsequent additions at 15-min intervals of 1 equivalent fresh sodium dithionite (SDT). After the addition of native loading dye, nucleosomal and free DNA were purified by 6% native PAGE as already described.

Separation of Cross-Link. Lyophilized cross-linker-treated free and nucleosomal samples were resuspended in 10 μL of 5 M aqueous urea/0.1% xylene cyanole and loaded onto a 6% 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 ~65 W and 37 °C to minimize breakdown of the heat-labile mustard adducts. Autoradiography revealed a family of bands of reduced mobility relative to single strands, corresponding to cross-links at different sites (*41*).

Piperidine Cleavage of Mustard-Alkylated DNA. Mustard cross-linked and monoalkylated DNA was recovered from formamide/urea gels following autoradiography by the crush-and-soak procedure (*51*). These samples were cleaved at

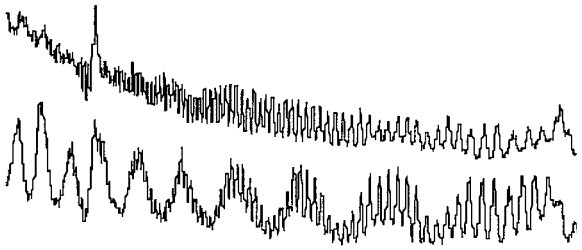


FIGURE 2: Two-dimensional scans of the phosphorimage of the hydroxyl radical cleavage pattern of 3′-radiolabeled *Eco*RI-*Rsa*I restriction fragment as both free DNA (top) and as nucleosomal DNA (bottom). The 10−11 bp periodicity of the bottom trace is evidence that the histone octamer occupies both a defined translational and rotational setting on the DNA.

sites of guanine N7 alkylation by heating at 90 °C in 10% aqueous piperidine for 15 min (*54*) and at putative sites of both guanine N7 and adenine N3 alkylation by heating in TE buffer at 85 °C for 25 min followed by ethanol precipitating and heating at 90 °C in 10% aqueous piperidine for 15 min (*36*). Samples were lyophilized, dissolved in 20 μL of water, lyophilized, dissolved again in 20 μL water, and lyophilized again. Under these conditions mock cross-linked samples showed virtually no degradation.

Cu(II)/SDT Cleavage of MC-Cross-linked DNA. MC-cross-linked DNA was recovered from formamide/urea gels following autoradiography by the crush-and-soak procedure (*51*). These samples were cleaved at sites of MC cross-linking by incubation in 9 μL of 50 mM Tris (pH 7.5)/100 μM $CuCl_2$ plus 1 μL of glycogen (20 mg/mL stock from Boehringer Mannheim) followed by addition of four 1-μL aliquots of SDT (5 mM stock) at 15-min intervals (*55*). Samples were then lyophilized for analysis on a sequencing gel.

Sequencing Gel Analysis. Samples were dissolved in 10 μL of 5 M urea/0.1% xylene cyanole and loaded onto an 8% denaturing gel (19:1, acrylamide/ bisacrylamide, 50% urea) run at ~65 W and 65 °C. After drying and phosphorimaging (Molecular Dynamics), bands were assigned through reference to Maxam−Gilbert G-lanes.

RESULTS

The 3′- or 5′-end radiolabeled 154-base pair restriction fragment of the 5S RNA gene of *Xenopus borealis* was incubated with purified chicken erythrocyte histones under conditions that maximized the formation of core particles. Hydroxyl radical has been reported to cleave at nucleosomal sites that face away from the histone octamer, resulting in a 10−11 periodicity (*24*). Likewise, the Fe(II)EDTA cleavage pattern of our reconstituted core particles demonstrated the periodicity indicative of a precise rotational and translational positioning (Figure 2).

For the cross-linking studies, reconstitution was carried out with a lower ratio of histones to radiolabeled DNA to achieve an approximately 1:1 mixture of free and nucleosomal DNA. This mixture was then treated with mustard under conditions predetermined to yield approximately one modification per DNA molecule prior to separation of free and nucleosomal DNA by 6% native PAGE (Figure 3). The ratio of free to nucleosomal DNA did not change in the presence of cross-linker, indicating that the integrity of the core particle was not compromised. Moreover, this experimental strategy ensured that both samples were subjected to identical reaction conditions. Mustard-treated free and nucleosomal DNA was purified from the native gel and then subjected to 6% denaturing PAGE. Gels were temperature controlled to mimimize loss of alkylated product. Autoradiography demonstrated a family of bands of reduced mobility relative to single strands, corresponding to cross-links at different sites (Figure 4). The cross-linking profile was similar for the three mustards, indicating a shared sequence preference within this DNA for these agents, as previously reported (*41*). Moreover, the positions of these low-mobility cross-linked bands were similar for the free and nucleosomal sample for each of the three mustards examined, indicating that sequence preferences are unaltered by the histone octamer. Cross-linking of reconstituted 5′-end radiolabeled 5S DNA fragment gave similar results.

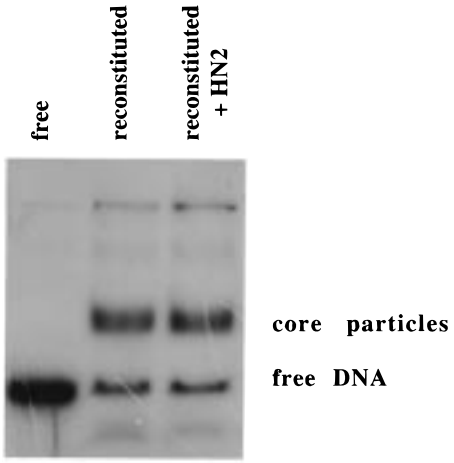


FIGURE 3: Native PAGE of reconstituted 3′-radiolabeled 5S DNA fragment. Lane 1, free DNA; Lane 2, control reconstituted DNA; Lane 3, reconstituted DNA incubated with 50 mM mechlorethamine. The two major bands in the reconstituted samples correspond to nucleosomal (top band) and free DNA (bottom band).

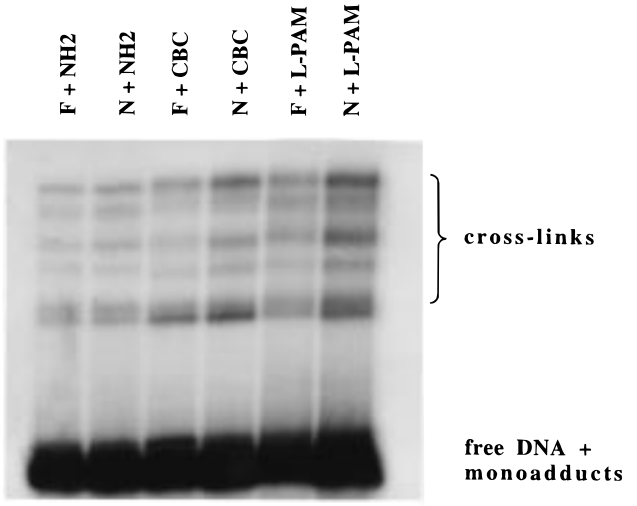


FIGURE 4: Denaturing PAGE analysis of mustard-treated free and nucleosomal 3′-radiolabeled DNA. Shown here is the autoradiograph of the formamide/urea-containing gel used to separate cross-linked from single-stranded 154-mer. Bands of lower mobility than single-strands correspond to interstrand cross-links. Lane 1, HN2 (50 μM)-treated free DNA; Lane 2, HN2 (50 μM)-treated nucleosomal DNA; Lane 3, CBC (250 μM)-treated free DNA; Lane 4, CBC (250 μM)-treated nucleosomal DNA; Lane 5, L-PAM (250 μM)-treated free DNA; Lane 6, L-PAM (250 μM)-treated nucleosomal DNA.

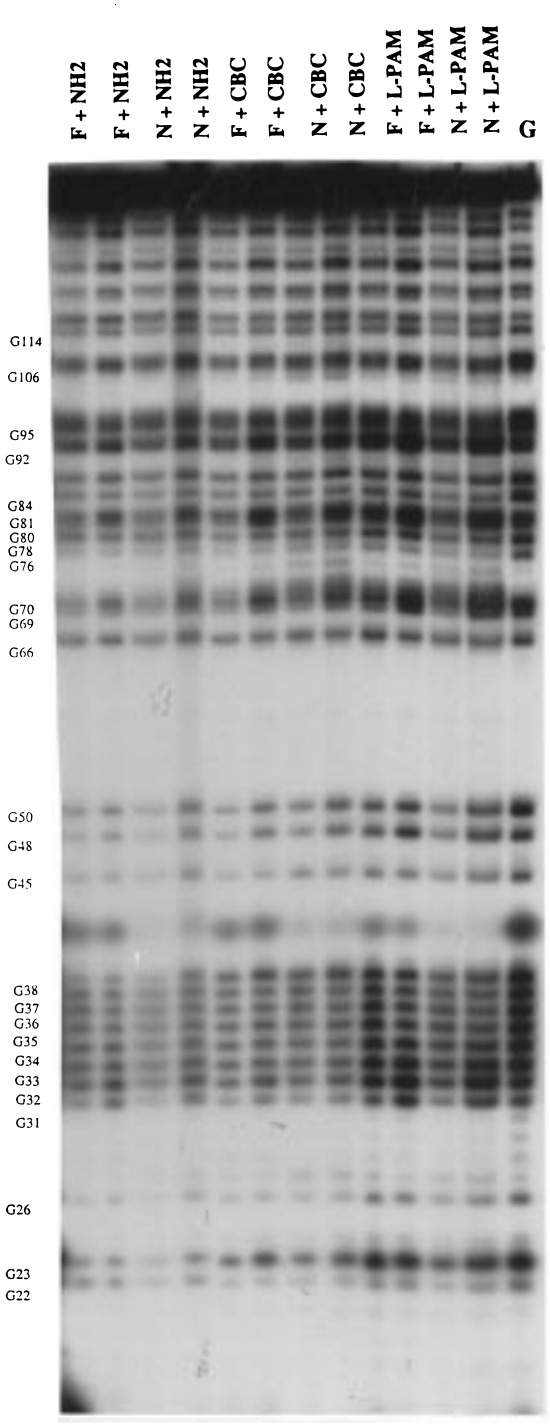


FIGURE 5: Denaturing PAGE analysis of piperidine-cleaved monoalkylated 154-mer. Shown here is the autoradiograph of the DNA sequencing gel used to separate the cleavage products of 3′-end radiolabeled 154-mer. "G only" lanes were treated with piperidine; "G + A" lanes were heated prior to piperidine treatment as described in *Materials and Methods*. The numbers to the side denote positions of deoxyguanosine residues on the bottom strand, radiolabeled in this experiment. Lane 1, HN2-treated free DNA, G only; Lane 2, HN2-treated free DNA, G + A; Lane 3, HN2-treated nucleosomal DNA, G only; Lane 4, HN2-treated nucleosomal DNA, G + A; Lane 5, CBC-treated free DNA, G only; Lane 6, CBC-treated free DNA, G + A; Lane 7, CBC-treated nucleosomal DNA, G only; Lane 8, CBC-treated nucleosomal DNA, G + A; Lane 9, L-PAM-treated free DNA, G only; Lane 10, L-PAM-treated free DNA, G + A; Lane 11, L-PAM-treated nucleosomal DNA, G only; Lane 12, L-PAM-treated nucleosomal DNA, G + A; Lane G, Maxam−Gilbert G-reaction.

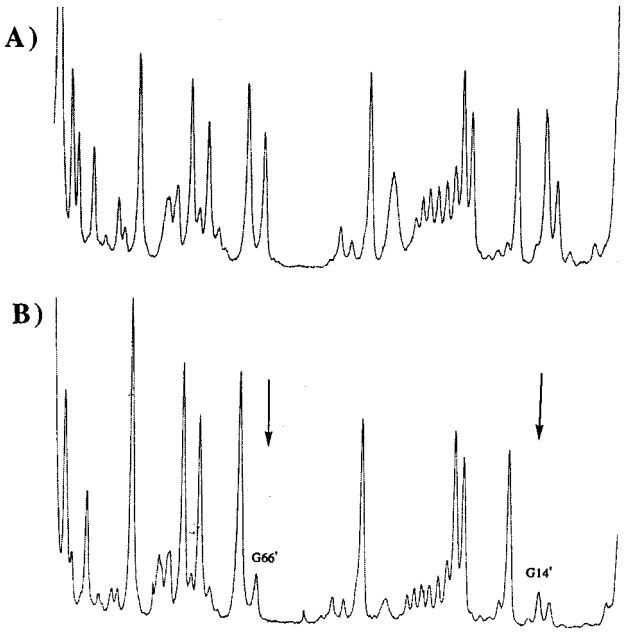


FIGURE 6: Denaturing PAGE analysis of piperidine-cleaved cross-linked 154-mer. Two-dimensional scans of the phosphorimage of the piperidine-cleavage products of 3′-end radiolabeled mustard cross-linked 154-mer. A, HN2-cross-linked free DNA; B, HN2-cross-linked nucleosomal DNA. Sites that differ in intensity are marked with an arrow. Numbering denotes residue position in bottom strand.

To verify the similar mustard sites in free and nucleosomal DNA, both cross-linked and monoalkylated DNA (from the single-stranded region of the denaturing gel) were isolated from the denaturing gel and piperidine-cleaved at sites of N7 alkylation (*54*). The resulting fragment mixtures and a reference Maxam−Gilbert G-reaction were analyzed by 8% denaturing PAGE followed by phosphorimaging.

For the monoalkylated samples, the major radioactive products corresponded to cleavage at deoxyguanosine residues with some traces of cleavage at deoxyadenosine residues in the CBC and L-PAM samples (Figure 5). Previous reports have suggested the N3 position of adenine as a secondary alkylation site for aromatic mustards, preferred in runs of adenines (*56, 57*). There are few sites containing adjacent adenine residues in our sequence, but we did observe monoalkylation by CBC and L-PAM at the single AA site in the labeled strand (A71 and A72). Other molecules containing adenine adducts may have degraded because of spontaneous depurination and subsequent cleavage during one of the previous electrophoresis steps. Two-dimensional scans verified similar sites of guanine monoalkylation not only in the nucleosomal and free DNA samples for each mustard but also for the three different mustards. However, some small differences in intensity were apparent. These subtle differences could not be correlated to either rotational or translational positioning.

Piperidine cleavage of the cross-linked samples suggested N7 of deoxyguanosine as the predominant site for cross-linking by all three mustards in both free and nucleosomal DNA: virtually no HN2-cross-linked DNA remained intact and only traces of L-PAM- and CBC-cross-linked DNA remained intact. Independent analysis of individual cross-linked bands verified that the least mobile band was comprised of cross-links at G64-G66′, G68-G70′, G79-G81′,

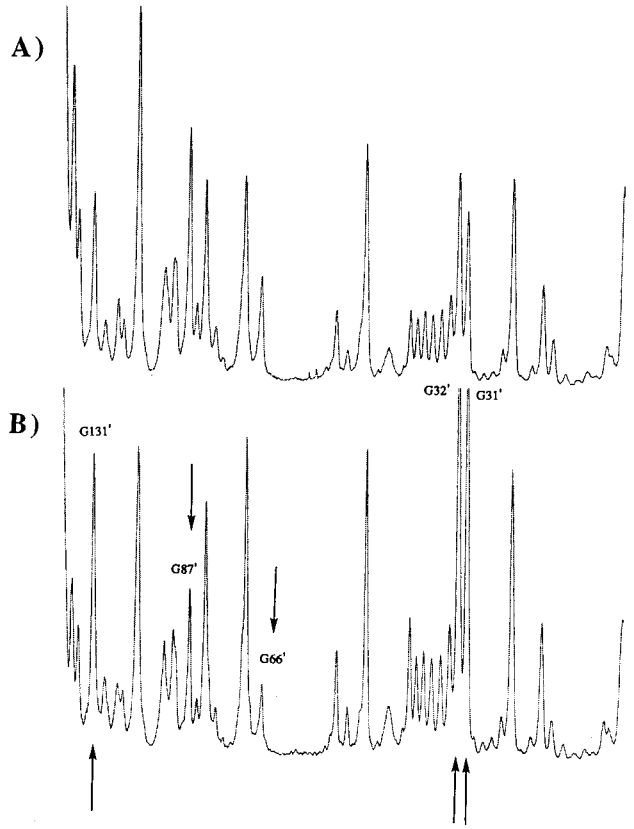


FIGURE 7: Denaturing PAGE analysis of piperidine-cleaved cross-linked 154-mer. Two-dimensional scans of the phosphorimage of the piperidine-cleavage products of 3′-end radiolabeled mustard cross-linked 154-mer. A, CBC-cross-linked free DNA; B, CBC-cross-linked nucleosomal DNA. Sites that differ in intensity are marked with an arrow. Numbering denotes residue position in bottom strand.

G85-G87′, and G86-G88′ (residue in the top strand linked to residue in the bottom strand), which are the most centrally cross-linked isomers (*41*). The most mobile band was comprised of cross-links at G139-G141′, G143-G145′, G144-G146′, and G12-G14′ (residue in the top strand linked to residue in the bottom strand), which are the isomers containing cross-links at the ends of the DNA (*41*).

An overlay of the cleavage patterns from phosphorimagery of total mustard-cross-linked free and nucleosomal 3′-end-radiolabeled DNA shows that all 5′-GNC sequences were cross-linked in both samples with only some small (two- to three- fold) differences in intensity (Figures 6−8). HN2 showed only decreases in intensity (at G14′ and G66′- the cross-linked residues in the bottom, labeled strand) in the nucleosomal sample relative to free DNA (Figure 6), whereas CBC showed both decreases (at G66′ and G87′) and increases (G31′, G32′, and G131′) in the nucleosomal sample (Figure 7). L-PAM showed minimal differences between the two samples (Figure 8). Again, these differences in intensity did not appear to correlate with either the rotational or the translational position of the alkylated residue.

MC cross-linking was also performed on a reconstituted sample of radiolabeled 154-mer. Free and nucleosomal DNA were purified by 6% native PAGE, and the resulting samples were subjected to 6% denaturing PAGE. Again, a family of low mobility bands, presumably corresponding to different cross-linked isomers, was visible after autoradiography. In this case, there were obvious differences between the free and nucleosomal samples (Figure 9). Two-dimensional scans were made from this gel, and the data were normalized to

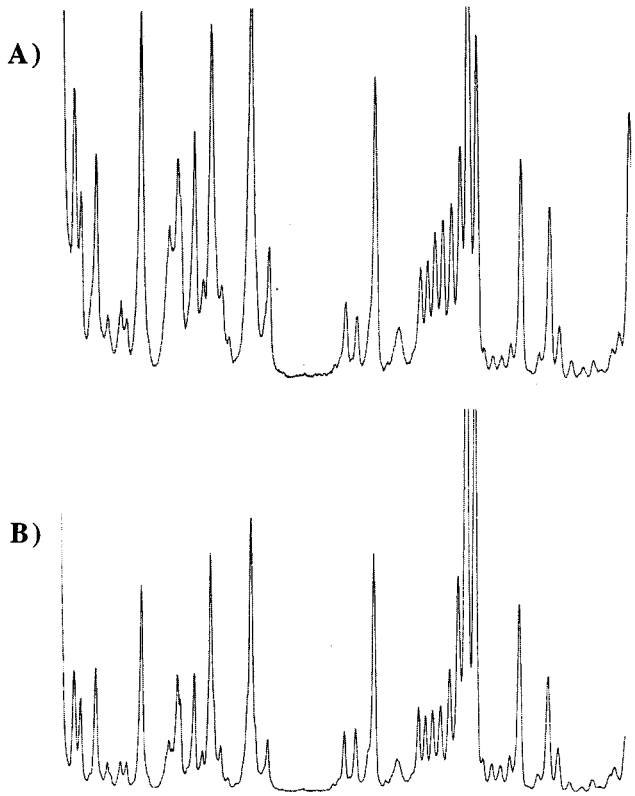


FIGURE 8: Denaturing PAGE analysis of piperidine-cleaved cross-linked 154-mer. Two-dimensional scans of the phosphorimage of the piperidine-cleavage products of 3′-end radiolabeled mustard cross-linked 154-mer. A, L-PAM-cross-linked free DNA; B, L-PAM-cross-linked nucleosomal DNA.

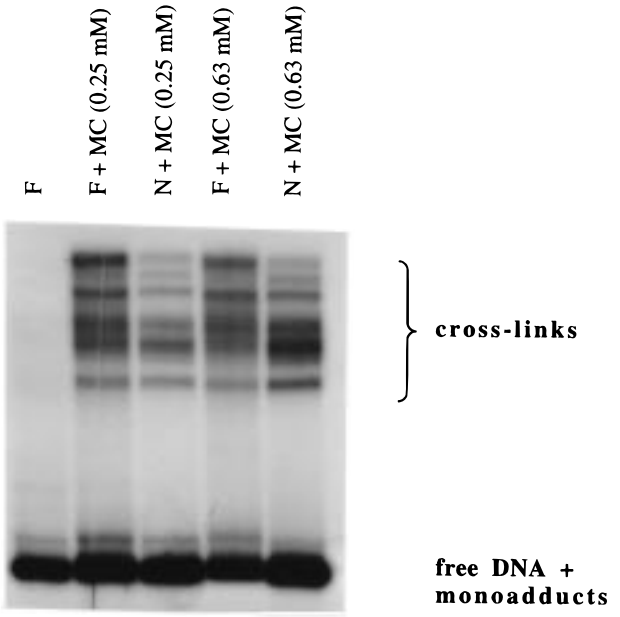


FIGURE 9: Denaturing PAGE analysis of MC-treated free and nucleosomal DNA. Shown here is the autoradiograph of the formamide/urea-containing gel used to separate cross-linked from single-stranded 3′-end radiolabeled 154-mer. Bands of lower mobility than single-strands correspond to interstrand cross-links. Lane 1, control free DNA (no MC); Lane 2, MC-treated free DNA (0.25 mM); Lane 3, MC-treated nucleosomal DNA (0.25 mM); Lane 4, MC-treated free DNA (0.63 mM); Lane 5, MC-treated nucleosomal DNA (0.63 mM).

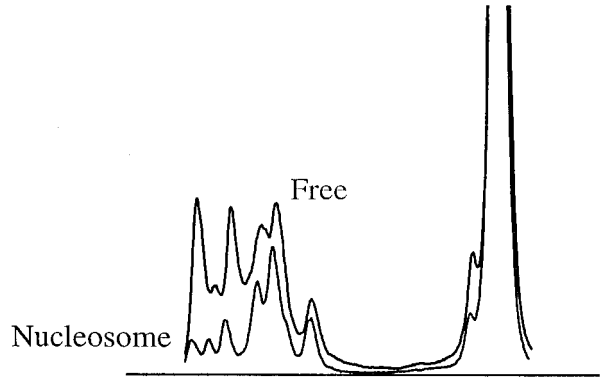


FIGURE 10: Two-dimensional scans of the phosphorimage of gel lanes 2 (top trace) and 3 (bottom trace) shown in Figure 9.

account for any differences in the counts of radioactivity loaded. The resulting overlay showed a dramatic reduction in the lowest mobility cross-linked bands, whereas the higher mobility bands were approximately equal in intensity (Figure 10). Because there are no direct chemical cleavage methods to confirm directly the sites of MC cross-linking, purified cross-linked DNA was subjected to Cu(II)/sodium dithionite treatment, which was previously demonstrated to cleave DNA with low efficiency at the site of cross-linking (*55*). This analysis was consistent with the assignment of the lowest mobility bands corresponding to centrally cross-linked isomers (data not shown).

DISCUSSION

Previously we reported the sites of mustard cross-linking in the *Eco*RI/*Rsa*I restriction fragment of the *Xenopus borealis* 5S RNA gene (*41*). These studies confirmed the 5′-GNC cross-linking preference (found in DNA oligomers) of HN2, CBC, and L-PAM in this restriction fragment, thereby validating the use of short DNA oligomers as good model systems for studying mustard reactions. We have now extended our studies to the uniquely positioned core particle formed by this fragment, thus allowing a direct comparison of the cross-linking sites in free DNA with those in the same DNA sequence complexed with the histone octamer. We again tested the three mustards HN2, CBC, and L-PAM, as well as reductively activated MC.

Previous studies of mustards with nucleosomal DNA were limited to examining total alkylation products. Hartley et al. (*35*) treated human cells with HN2 and examined the resulting alkylation sites of a purified 296 base pair fragment of alpha DNA. They reported a similar sequence preference in intact cells to that of the free DNA, concluding that the nuclear environment does not impact the pattern of mustard reactivity. Smith et al. (*36*) used a fragment of the 5S rRNA gene of *Xenopus laevis* to form defined sequence nucleosomes. In this system L-PAM induced only slight changes in intensity of alkylation relative to the same sequence of free DNA, most dramatic at the nucleosomal dyad. These small differences were attributed to the reduction of thermal motions of nucleosomal DNA required to accommodate L-PAM binding.

Although monoalkylation does not induce major conformational changes in DNA, mustard interstrand cross-linking results in substantial structural changes (*46*) that may not be tolerated by highly constrained nucleosomal DNA. We therefore examined mustard cross-linking of a fragment of the 5S RNA gene of *Xenopus borealis* as both nucleosomal and free DNA. The 154-mer used in our study forms a uniquely positioned core particle with 16 putative sites for mustard cross-linking (Figure 1). These sites are distributed randomly throughout the sequence and are therefore present in a variety of rotational positions. For example, G68, G79, G129, and G139 are at sites that undergo minimal cleavage by hydroxyl radical (indicating sites that face the octamer and contain a compressed minor groove [*26*]). On the other hand, G12, G43, G64, G85, G105, G115, and G143 are present at sites that undergo maximal cleavage by hydroxyl radical (indicating sites that face away from the histone core and contain a compressed major groove [*26*]).

Following isolation of nucleosomal and free mustard-treated DNA, denaturing PAGE was performed to separate the different cross-linked isomers. The patterns of cross-links visible on these gels were remarkably similar not only for all three mustards examined but also for nucleosomal and free DNA, suggesting that mustard access to the major groove is not greatly impaired due to the presence of the histone core. Moreover, the DNA within a core particle must be flexible enough to undergo the conformational changes required for mustard cross-linking. Some small differences in intensity of particular cross-linked bands were obvious after phosphorimagery and comparison between free and nucleosomal samples. For example, in the HN2 samples, G14′ and G66′ were reduced about three-fold in the nucleosomal sample. Although both of these sites would be expected to contain a compressed major groove relative to free DNA, other regions also facing away from the histone octamer did not show reduced HN2 cross-linking. In the CBC nucleosomal sample, there were two sites of decreased intensity (G66′, G87′) and three sites of increased intensity (G31′, G32′, G131′) relative to free DNA. Again, the two inhibited sites would be expected to contain a compressed major groove, but not all such sites were inhibited. Likewise, the sites of enhanced cross-linking would be expected to contain a wider major groove, but not all sites with a wide major groove were enhanced. Furthermore, these differences were again small (two-to-three-fold). Interestingly, the site inhibited in both the HN2 and CBC nucleosomal samples (at G66′) corresponds to a "hot spot" for cleavage by calicheamicin γ_1^I, which targets the minor groove (*50*). This site is located approximately -1 helical turn from the dyad axis. The site located approximately $+1$ helical turn from the dyad axis (G87′) was also inhibited in the CBC sample.

It seemed possible that during the isolation of our nucleosomal DNA from the native gels, the integrity of the core particles might have been compromised such that monoadducts could convert to cross-links. It was therefore vital to verify that the patterns of monoalkylation did not change in our free and nucleosomal DNA samples. Indeed, there was no obvious loss of monoalkylation sites indicating conversion to cross-links in the nucleosomal sample. Because monoalkylation sites and frequencies were so similar for free and nucleosomal samples, we are confident 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 distortion required for mustard cross-linking, consistent with a dynamic rather than a stagnant model of chromatin.

There are 10 putative CG sites for MC in the *Xenopus borealis* restriction fragment (Figure 1): those at sites C23, C84, C114, and C124 are near regions of maximal hydroxyl radical cleavage; those at sites C78 and C108 are near regions of minimal hydroxyl radical cleavage (*26*). We subjected a sample of reconstituted 154-mer to MC under the necessary reductive conditions for cross-linking. Nucleosomal and free DNA were then purified by native PAGE. Again, a family of cross-linked bands was visible following denaturing PAGE and autoradiography. Quantitative phosphorimagery provided verification that the intensities of different isomers varied considerably between the free and nucleosomal samples, with the three bands of the lowest gel mobility substantially reduced (five-to-ten-fold) in the latter. Previously we reported that for free mustard-cross-linked 154-mer, the least mobile band on denaturing PAGE corresponds to centrally cross-linked material (*41*). Gel mobility then increases as the position of the cross-link moves toward either end of the DNA. The position of a MC cross-link in short DNA oligomers has also been demonstrated to affect mobility in denaturing gels, with centrally cross-linked isomers having the lowest mobility (*53*). Cu(II)/SDT-induced cleavage of purified discrete MC cross-linked bands was also suggestive of this assignment for the 154-mer. These findings are consistent with the hypothesis that the sites of reduced MC cross-linking are in fact at the dyad of the nucleosome, despite the lack of direct confirmation through piperidine cleavage as for the mustard adducts. Direct confirmation of reduced sites of MC cross-linking may be possible through an RNA polymerase stop assay such as that used to map sites of interstrand cross-linking by cisplatin (*58*). We have modified the 5S DNA fragment to contain convergent SP6 and T7 promoter sites flanking the nucleosomal positioning sequence and will use this modified DNA as a transcription template in both the cross-linked and native state. Sites of transcription termination should provide independent confirmation of MC cross-linking sites in this DNA.

Indirect evidence (from MC-cross-linked oligomers and mustard-cross-linked 154-mer) and Cu(II)/SDT cleavage studies suggest that it is cross-linking reactions at sites C78, C84, and C108 that are reduced in the nucleosomal sample, with C78 showing the most reduction. One of these sites has a wider minor groove (C84) and the other two have a narrowed minor groove relative to free DNA according to previous hydroxyl radical cleavage studies (*26*). It is interesting that the second band of these three (presumably corresponding to cross-linking at C84) is reduced less than the other two in the nucleosomal sample. Therefore, both the translational and the rotational positions of the site appear to correlate with a change in MC cross-linking. C84 appears to be in a translationally unfavorable position (near the dyad) but in a rotationally favorable position (facing away from the histone core). C108 is in a less translationally unfavorable position (further from the dyad) but in a rotationally unfavorable position (facing toward the histone core). The previously reported greatly reduced cross-linking efficiency of nucleosomal versus free DNA of *N*-methylmitomycin A (*34*) may be consistent with these results.

It is suggestive that despite the structural changes induced by mustard cross-linking, there were relatively modest differences in the attack of free and nucleosomal DNA and no common regions of either suppression or enhancement for the three mustards examined. MC showed more profound differences in its cross-linking, with sites at the nucleosomal dyad inhibited relative to those at either end as compared to free DNA. Monoalkylation events have been previously thought to correlate with the bulkiness of the agent binding. Relatively large molecules such as aflatoxin B1, which targets the major groove, and the diol epoxide metabolite of benzo[*a*]pyrene (BPDE), which targets the minor groove, demonstrate a two-to-three-fold reduction in binding to the center of the nucleosome relative to free DNA (*59*−*61*). This protection diminishes at the ends of the nucleosomes. On the other hand, the relatively small dimethyl sulfate shows a similar pattern and extent of alkylation for free and nucleosomal DNA (*62*, *59*). Our results indicate that adding a second alkylation event to form an interstrand cross-link does not greatly affect this general pattern. The relatively small mustards behave like dimethyl sulfate in that they react similarly with nucleosomal and free DNA, whereas the larger MC demonstrates reduced binding at the nucleosomal dyad. None of the monoalkylators demonstrate the 10−11 bp periodicity shared by cleavage by hydroxyl radical (*24*, *26*) and some enediynes (*50*, *63*), as well as the formation of UV-induced cyclobutane pyrimidine photodimers (*64*, *65*). For example, the degree of alkylation by BPDE does not correlate with the rotational positioning of the alkylated guanine residue (*61*); that is, sites facing both toward and away from the histone octamer are remarkably available to even bulky alkylators. This result also seems to be true for mustard-induced interstrand cross-linking. MC cross-linking seems to be further modulated by additional factors to account for the effects of rotational as well as translational positioning.

Recent work suggests that nucleosomes transiently expose DNA, thereby providing access to regulatory proteins (*66*). Such a dynamic structure might therefore show little difference in reactivity relative to free DNA toward reagents that react slowly on the time scale of exposure. For example, the rate-determining step in nitrogen mustard cross-linking is the relatively slow ($k \sim 0.04$ min^{-1}) cyclization to the aziridinium ion (*67*). On the other hand, under our MC reaction conditions (formation of a precovalent complex followed by reductive activation through incremental additions of sodium dithionite), the drug is rapidly converted to its active form (*68*) and may consequently react quickly on the time scale of nucleosome exposure. This could account for the observed rotational dependence of MC cross-linking. The exact contributions of such parameters as steric effects, nucleophilicities of reactants, and reaction kinetics in dictating the outcome of nucleosomal modification remain to be defined in further studies.

In conclusion, we have found that the histone octamer is a relatively minor inhibitor of DNA interstrand cross-linking reactions. However, recent evidence suggests that the linker histones may play a larger role than previously suspected in gene regulation (*69*−*71*). Our system examined only the core particle and did not include linker DNA, linker histones, or any additional constraints that may be imposed by higher order chromatin structure. Therefore, further studies will be necessary to understand DNA cross-linking in vivo.

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