Sequence preferences of DNA interstrand crosslinking agents: quantitation of interstrand crosslink locations in DNA duplex fragments containing multiple crosslinkable sites

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ABSTRACT

A general approach to the quantitative study of the sequence specificity of DNA interstrand crosslinking agents in synthetic duplex DNA fragments is described. In the first step, a DNA fragment previously treated with an interstrand crosslinking agent is subjected to denaturing PAGE. Not only does this distinguish crosslinked from native or monoadducted DNA, it is shown herein that isomeric crosslinked DNAs differing in position of the crosslink can in some cases be separated. In the second stage, the now fractionated crosslinked DNAs isolated from denaturing PAGE are subjected to fragmentation using iron(II)/EDTA. For those fractions which are structurally homogeneous, analysis of the resulting fragment distribution has previously been shown to reveal the crosslink position at nucleotide resolution. It is shown herein that in fractions which are structurally heterogeneous due to differences in position of crosslink, this analysis quantifies the relative extent of crosslinking at distinct sites. Using this method it is shown that reductively activated mitomycin C crosslinks the duplex sequences 5'-GCGC and 5'-TCGA with 3 ± 1 :1 relative efficiency.

INTRODUCTION

DNA interstrand crosslinking agents figure prominently among both drugs useful in cancer chemotherapy and environmental toxins and toxicants. In many cases, the sites on DNA which are covalently modified by crosslinking agents (e.g., guanine N7) are well established. In contrast, our knowledge of the sequence specificity of DNA crosslinkers has lagged behind for want of appropriate analytical techniques. The major challenge in this regard is the study of a minor component of a complex mixture: due to the inefficiency of most DNA crosslinking agents, the major products arise from monoalkylation, with interstrand, bifunctional alkylation representing only a minor fraction of the product mixture.

Several methods of evaluating the sequence specificity of DNA interstrand crosslinking agents are known. The ability of crosslinks to inhibit certain enzymatic reactions has been exploited to evaluate crosslinked DNA fragments.^{1,2} Psoralen crosslinking has been studied through inhibition of polymerase,³ exonuclease,^{4,5} and restriction endonuclease activities.⁶ The polymerase and exonuclease assays are particularly powerful, in that they allow the evaluation of numerous potential crosslink sites simultaneously; deficiencies include the limited resolution with which crosslink sites can be determined and the possibility that a given crosslink may not inhibit the enzyme. Denaturing polyacrylamide gel electrophoresis (PAGE) of duplex DNA fragments readily distinguishes single stranded DNA from interstrand crosslinked DNA, by virtue of size differences, and this approach has been applied to both psoralen⁶ and mitomycin⁷ sequence specificities. This approach clearly demonstrates the presence or absence of a crosslink, but provides no information regarding the site of crosslinking. When combined with other information, such as the target nucleotide for the agent studied, the technique is nevertheless quite powerful. Alternatively, in some cases hydrolysis of the sugar phosphate backbone of a crosslinked DNA affords a conjugate diagnostic for the presence of a crosslink. Quantitation of this conjugate is thus diagnostic for the extent of crosslinking.⁸ In those cases where the reactive nucleotide is present only once in each of the opposing strands, this method additionally defines the site of crosslinking.

We have recently reported a method for locating sites of interstrand crosslinks in DNA fragments at single nucleotide resolution.⁹ This method involves separation of singly end-radiolabeled, singly crosslinked DNA fragments from residual single stranded and monoalkylated single stranded DNA by denaturing PAGE, followed by random fragmentation using iron(II)/EDTA.¹⁰ Only fragmentation between the radiolabel and the crosslinked nucleotide affords fragments shorter than starting single strand, and thus the abundance of various fragment sizes, analyzed by single nucleotide-resolving, denaturing PAGE, reveals sites of crosslinking. Using this approach, we have determined that reductively activated mitomycin C crosslinks

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deoxyguanosine residues on opposite strands at the DNA duplex sequence 5'-CG.^{9,11} Using a closely related method, mechlorethamine, the simplest of the nitrogen mustards, was found to crosslink several synthetic DNA duplex fragments through opposing deoxyguanosine residues at the sequence 5'-GNC.¹² In addition to these 'core' preferences, the flanking sequence influences the reactivity of crosslinking drugs such as psoralens⁵ and mitomycins.^{7,8,11} Flanking sequence preferences are subtly expressed, modulating the overall reactivity of a core sequence. As such, quantitative methods will be necessary for the study of these effects.

We demonstrate herein that the iron(II)/EDTA protocol which was previously employed to locate cross-links in duplex DNA fragments, can be used for quantitation of sites of crosslinking in duplex DNA fragments in which the site of crosslinking varies from one molecule to the next. In the course of this work, we have discovered that the mobility in denaturing PAGE of singly crosslinked DNA fragments is a function of the location of the crosslink in the duplex. This phenomenon is useful in that it provides a new preparative and analytical technique for the evaluation of crosslinked DNAs. The combination of these two techniques constitutes a general approach to the quantitative evaluation at single nucleotide resolution of the sequence specificity of DNA interstrand crosslinking agents in DNA fragments: a radiolabeled, duplex DNA fragment is incubated with the crosslinking agent of interest, DPAGE is then used to the limits of its ability to separate isomeric crosslinked products (quantified by Cerenkov counting), and finally the products are independently studied by the quantitative iron(II)/EDTA fragmentation method. In this last step, isomeric cross-linked products differing in nucleotide connectivity but not resolved by the initial DPAGE step are quantified. The methods we describe herein have proven their general utility in this laboratory.

MATERIALS AND METHODS

Buffers were as follows: TE: 10 mM Tris-Cl (pH 7.6), 1 mM EDTA, TBE: 89 mM Tris-borate (pH 8.0), 89 mM boric acid, 2 mM EDTA. Ultrapure urea was from Bethesda Research Laboratories (BRL); electrophoretic purity acrylamide and bisacrylamide, from Bio-Rad; mitomycin C, Sigma or a kind gift from Bristol-Myers. All other chemicals were of reagent grade.

Preparation of radiolabeled DNA duplexes

Oligonucleotides were synthesized on a 1 μ mole scale (Applied Biosystems Model 380A), deprotected through overnight treatment with 1–2 mL concentrated ammonium hydroxide at 65°C, and lyophilized to dryness. Following dissolution in ultrapure water (Millipore Milli Q Water System), 10–15 OD per lane were purified through denaturing PAGE (20%, 25:1 acrylamide: bisacrylamide, 40% urea, 1.5 mm thick, 17 $\times$ 15 cm). Such preparative gels were prepared by dissolving 25.2 g urea in 30 mL stock acrylamide (38.5 g acrylamide, 1.5 g bisacrylamide, 60 mL H₂O; stored at 4°C) and 6 mL 10 $\times$ TBE. In order to catalyze polymerization, 300 μ L 20% ammonium persulfate (APS) and 40 μ L TEMED were added. One h after polymerization, electrophoresis was performed for 1 h prior to loading the samples, which were dissolved and loaded in 30 μ L H₂O and 15 μ L 98% deionized formamide, 10 mM EDTA. Gels were run at $\sim$ 40 mA, $\sim$ 400 V on a BRL Model V16-2 gel stand with a Bio-Rad Model 2000/200 power supply for

approximately 4 h. The gel was then transferred from the glass plate to Saran Wrap and placed on a fluor-impregnated silica gel coated glass plate. DNA was visualized through UV shadowing (Model UVG-11 lamp from Ultra-Violet Products, Inc.). Bands were excised with a razor blade, crushed, and soaked in 1 mL TE buffer overnight at 37°C. Following a 1 mL TE wash for 1 h, the resulting combined eluant was flushed through a pre-wet Sep-Pak C₁₈ cartridge (Waters). The DNA on the column was washed with 10 mL of 10 mM aqueous NH₄OAc followed by 2 mL water and eluted with 2 mL of 25% (v/v) acetonitrile/water. This solution was concentrated to dryness and stored at 4°C until use, at which time it was dissolved in ultrapure water to an appropriate concentration ($\sim$ 0.1 OD/ μ L). 1 OD self complementary DNA was 5'-end labeled with [γ -³²P]ATP (3000 Ci/mmol, from New England Nuclear [NEN]) using T4 polynucleotide kinase (Boehringer Mannheim) under standard conditions.¹⁷ For non-self complementary DNA's, 0.5 OD single strand was 5'-labeled prior to annealing with 0.5 OD of the complementary strand. 3'-End labeling of 1 OD duplex DNA was carried out with the Klenow fragment of DNA polymerase I (Boehringer Mannheim)/[α -³²P]-dNTP (3000 Ci/mmol, from NEN) under standard conditions.¹⁷ In all cases, labeling was followed by ethanol precipitation, using 3 M NaOAc, with an 85% ethanol wash, and lyophilization.

Preparation of mitomycin C-crosslinked DNA duplexes

1 OD₂₆₀ radiolabeled duplex DNA (0.076 μ mol base pairs, for a final concentration of 0.7 mM) was dissolved in 100 μ L of 15 mM Tris (pH 7.5), and 10 μ L MC solution (20 mM MC in 33% aqueous methanol, stored at -4°C for less than 1 month; 0.2 μ mol, for a final concentration of 1.8 mM) was added to effect a 2.5:1 drug-to-base-pair ratio. Samples in septa-covered tubes were incubated at 37°C for 1 h. The samples were then deoxygenated by bubbling through with argon for 15 min, after which time they were put on ice. The MC was activated through 3 additions at 15 min intervals of 1 equivalent (6 μ L) of fresh sodium dithionite (Baker; 33 mM aqueous solution was prepared as follows: 0.87 g of solid Na₂S₂O₄ was in a septum-covered tube, was evacuated and flushed with argon 3 times. Within 1 h, 1.5 mL deoxygenated water, which had been bubbled through with argon for 25 min, was added to the solid via syringe through the septum. The solution was vortexed briefly and used within 1 min.) Fifteen min following the final addition, the sample was ethanol precipitated, washed with 85% ethanol, lyophilized, and dissolved in 10 μ L loading buffer (90% deionized formamide, 10 mM Tris (pH 7.5), 0.1% xylene cyanole, 0.1 mM EDTA). The samples were denatured at 90°C for 4 min and chilled on ice prior to 20% PAGE (19:1 acrylamide:bisacrylamide, 50% urea, 0.35 mm thick, 41 $\times$ 37 cm). Gels were prepared as follows: 19 g acrylamide, 1 g bisacrylamide, 50 g urea were dissolved in 10 mL of 10 $\times$ TBE and 20 mL of ultrapure water. The volume was brought to 100 mL, and 350 μ L 20% APS was added prior to filtering through Whatman #5 filter paper. To a 5 mL portion of this solution was added 4 μ L TEMED, and this material was used to pour a plug. To the remainder of the solution was added 24 μ L TEMED to induce polymerization, and the gel was poured. After 1 h, electrophoresis on a Hoeffer thermostated Poker Face gel stand was performed until the gel reached ca. 65°C. Samples were loaded using flat sequencing tips (Marsh Biomedical) on a Rainin P-100 Pipetman, and the gel was run at 65–80 Watts with a Bio-Rad Model 3000 XI power supply for $\sim$ 4 h. The gel was autoradiographed on Kodak XAR-5 film.

For cleavage chemistry, the crosslinked material, of roughly half the mobility of the corresponding single strand, was cut from the gel and isolated as described above.

Preparation of HMT-crosslinked DNA duplexes

1 OD₂₆₀ radiolabeled DNA, 19 μ M in duplex DNA, 12 μ M in 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT, from a 4 mM stock in ethanol), and containing 10 mM NaCl, 10 mM MgCl₂, 50 mM Tris (pH 8.0), 0.1 mM EDTA, total volume 200 μ L, was nutated for 2 hrs at 25°C and then irradiated in a silanized Pyrex test tube at 351 nm (100 mW, Spectra-Physics argon laser model 2025-05) for 5 min, followed by ethanol precipitation.

Iron(II)/EDTA-promoted fragmentation

Iron(II)/EDTA cleavage reactions were performed on 25,000 cpm (geiger counter) DNA with 50 μ M (NH₄)₂Fe(SO₄)₂, 100 μ M EDTA, 1 mM sodium ascorbate, 10 mM H₂O₂, 5 mM Tris (pH 7.5) in a total volume of 10 μ L for 1 min at 25°C. Each of these reagents, 1 μ L at 10 $\times$ the final concentration, was added to the walls of a microfuge tube containing 7 μ L of the DNA solution. The reaction was initiated by brief centrifugation. Reactions were stopped with thiourea (1 μ L of 0.01 M solution), followed by vortexing, lyophilization, dissolution in 3.5 μ L loading buffer, heat-denaturation (90°C, 4 min), chilling 0°C, and loading onto a 25% polyacrylamide gel, which was run as described above. The gel was dried (Bio-Rad Model 583) onto Whatman 3MM paper and autoradiographed overnight. Bands were identified by reference to a Maxam–Gilbert G-reaction¹⁸ on uncrosslinked duplex.

Densitometry

Densitometry (Hoeffer GS-300, interfaced to an IBM PC) data were smoothed and plotted using Spectra Calc (Galactic Industries Corporation, Salem, NH). Quantitation of multiple sites through iron(II)/EDTA cleavage chemistry was achieved as follows. In the psoralen crosslinked DNA, a single scan through the center of a lane was recorded and integrated. Correction for differential loading of lanes *i* and *j* was achieved by multiplying the integrated peak area, *A*, of each peak in lane *j* by a normalization factor equal to the average value of A'_n/A_n where *n* is a given fragment size, for all fragments derived from cleavage 1 residue or more to the radiolabeled side of the first anticipated site of crosslinking (e.g., residues G3–A6 in Figure 1). A similar procedure was used for mitomycin C crosslinked DNA except that for each lane a set of scans was taken, starting at one side and moving 2 mm/scan through the lane. A set of scans was typically four linear scans. The total peak area of each band was assumed to be the sum of the corresponding peak areas from each linear scan.

Scintillation counting

The DNA's III and IV were 3'-radiolabeled, MC crosslinked, and analyzed through denaturing 25% PAGE, as described above. The bands corresponding to crosslinked and native DNA were excised and placed into a 1.5 mL eppendorf tube, which was then placed into a 20 mL glass scintillation vial. The samples were counted in a Packard 2000 CA Tri Carb liquid scintillation analyzer with a window setting from 1 to 1000. The samples were counted for 5 min.

RESULTS

Analysis of a duplex containing two psoralen-crosslinkable sites

We chose as an initial goal to demonstrate that the iron(II)/EDTA method could be used to define the relative extent of crosslinking at two distinct sites in an interstrand crosslinked oligonucleotide duplex. A derivative of psoralen was chosen as the crosslinking agent in these experiments because the crosslinking sequence specificity of psoralens is already well studied.

We have previously demonstrated the use of iron(II)/EDTA-promoted cleavage chemistry to pinpoint the site of crosslinking in DNA duplexes containing a single crosslinked site.⁹ Single hit, random cleavage to the radiolabeled (*) 3'-side of the crosslink in the DNA A shown schematically below yields short radiolabeled fragments. Cleavage at any other site on either strand gives a much larger radiolabeled fragment comprised of both strands covalently linked by the crosslink. Electrophoretic separation of the fragment mixture provides a discontinuity diagnostic for the crosslink site.

We anticipated that this same approach would prove useful in the quantitative analysis of crosslinked DNA duplex fragments in which the site of crosslinking varies from one molecule to the next. Consider for example a mixture of 30-residue DNA's A and B, below. Single cleavage of both A and B can yield radiolabeled fragments 0 (phosphate) to 9 nucleotides in length. Single cleavage of only B can produce radiolabeled fragments 10 to 19 nucleotides in length. Neither affords radiolabeled fragments of length 20 to 30. This analysis predicts that the yield of fragments 10 to 19 in length will be lower than for fragments 0–9 in length, and that this decrease will, as described below, correspond to the relative abundance of A and B in the original sample.

The feasibility of this crosslinking assay for quantitation of multiple-site containing DNA's was demonstrated using duplex I (Table I), containing two roughly equivalent 4'-(hydroxymethyl)-4,5',8-trimethylpsoralen (HMT) crosslinkable sites. HMT is known to bridge covalently thymine residues of a 5'-d(TA) sequence upon photoactivation.^{4,6,19} The radiolabeled duplex was photoreacted with a limiting amount of HMT to ensure that for the most part one crosslinking event occurred per duplex. Isolation of crosslinked material through PAGE was followed by iron(II)/EDTA fragmentation, PAGE, and autoradiography to yield the densitometer scan in Figure 1A. As anticipated, the cleavage pattern of the isolated crosslinked material showed that relative to the native sample, the yield of fragments 7 to 11 nucleotides long was diminished relative to fragments 2 to 6 nucleotides long. This is exactly the result expected from a population of molecules of which some are crosslinked at T7 and the remainder at T12. The ratios of corresponding peak areas from a one-dimensional scan of the crosslinked and native samples were calculated. From this (Figure 1B), it was not only obvious that fragment yields dropped

Table I. DNA duplex fragments used in these studies^a

Nucleotide Sequence	Descriptor
5'GAACGCGCTACGCTACGGCC3' 3'G*CGCGATGCGATGCCGG5'	I
5'(*)AACCTATAATTGATATTGCGCTATAATA(*A)3' 3'(G*)GATATTAAGCTATAACGCGATATTATTA5'	II
5'AATATAATTGCAATTAT*A3' 3'TATTAAGCTTAATATAA5'	III
5'AATATAATTGCGCAATTAT*A3' 3'TATTAACGCGTAAATATAA5'	IV
5'*AATATAATACGTATTAT3' 3'TATTATGCATAATATAA5'	V
5'*AATATACGATATCGTAT3' 3'TATGCTATAGCATATAA5'	VI
5'*AATATCGAATATTCGAT3' 3'TAGCTTATAAGCTATAA5'	VII
5'(*)AATATCGAATATTCAT3' 3'TAGCTTATAAGTTATAA(*)5'	VIII

^aAsterisk (*) indicates position of ³²P-radiolabel. Residues shown in parentheses were present in selected experiments (see text). Unless otherwise specified, hydroxyl groups were present at 3'- and 5'-termini.

following T7 and T12 in the crosslinked sample, but also that reactivity toward iron(II)/EDTA cleavage at the HMT-crosslinked thymidines was higher than that in the native duplex. While the direction of this effect (increase or decrease in fragmentation efficiency) was not predicted, aberrant reactivity of residues near the crosslink site is not surprising given the structural changes involved. If residues A6, T7, A11 and T12 are for this reason excluded from the analysis, and the average of the ratios for cleavage at G3–G5 is normalized to 1.0 (Figure 1B), then an average relative yield of G8–G10 of 52% is obtained. This is consistent with a 1:1 mixture of crosslinks at T7 and T12 in the original DNA duplex.

Iron(II)/EDTA cleavage quantitation of a duplex containing two MC-crosslinkable sites

The iron(II)/EDTA method was then used to study the impact of flanking sequence on an interstrand crosslinking reaction of reductively activated mitomycin C (MC). Duplex II (Table I), contains the two MC sites 5'-TCGA and 5'-GCGC. Crosslinked material was subjected to iron(II)/EDTA cleavage, as was native DNA. Following PAGE, quantitative densitometric analysis was performed. For the 5'-end labeled case normalization of the ratio (crosslinked/native) of fragments derived from cleavage at A9–T11 to 100% results in a calculated average fragment yield of 79% for A14–T18, indicating 79% crosslinking of the 5'-GCGC site and 21% of the 5'-TCGA site (Figures 2A, 2B). For the 3'-end labeled sample (Figure 2C, 2D), normalization of T23–T25 to 100% results in a calculated average yield of 32% of A14–C20 corresponding to 68% and 32% crosslinking, respectively, at these same two sites (Figures 2C, 2D). Through this analysis it appears that 5'-GCGC is preferred by MC for crosslinking over 5'-TCGA by a 1 ± 1:1 ratio. The results are in agreement with Borowy-Borowski *et al.*⁸ who found a 2:1 ratio of reactivity in related duplexes.

As an independent evaluation of the relative crosslinking efficiencies of these two sequences, we compared the crosslinking of duplexes III and IV (Table I). Under the conditions we have chosen for crosslinking, the 5'-TCGA (III) and 5'-GCGC (IV) containing oligomers crosslinked with 0.22% and 0.51%

Figure 1. (A) Partial fragmentation patterns for radiolabeled (*) = ³²P) native and HMT-crosslinked DNA duplex I using iron(II)/EDTA. Lettering indicates residue cleaved. The splitting of G5 presumably results from coelectrophoresis of some unidentified, non-radioactive material. The autoradiogram of this band is a 'doughnut,' which scanned in one dimension yields two peaks. (B) Ratio of fragment abundance in HMT-crosslinked and native DNA duplex I as a function of cleavage site.

efficiencies, or 2.3:1. These results concur with those previously discussed. Although these efficiencies are dramatically lower than those reported by Borowy-Borowski *et al.*,⁸ they are comparable to those found by Teng *et al.*⁷ It is unknown why these efficiencies vary, as similar crosslinking conditions are employed.

Impact of mitomycin C crosslink location on gel mobility of a 17-mer duplex

In the course of these studies, we noted that samples of crosslinked DNA's which were heterogeneous with respect to crosslink location yielded several distinct bands in denaturing PA-

Figure 2. (A) Partial fragmentation patterns for 3'-end labeled (* = ^{32}P) native and MC-crosslinked DNA duplex II using iron(II)/EDTA. Lettering indicates residue cleaved. (B) Ratio of fragment abundance in MC-crosslinked and native DNA duplex II as a function of cleavage site. (C) Partial fragmentation patterns for 5'-end labeled (* = ^{32}P) native and MC-crosslinked DNA duplex II using iron(II)/EDTA. Lettering indicates residue cleaved. (D) Ratio of fragment abundance in MC-crosslinked and native DNA duplex II as a function of cleavage site.

Figure 3. Denaturing PAGE migration pattern of MC-crosslinked DNA duplexes V, VI, and VII.

Figure 4. Partial fragmentation pattern of the isolated upper band (see Figure 3) of native and MC-crosslinked duplex VII using iron(II)/EDTA. Lettering indicates residue cleaved. Doubling of bands is presumably due to phosphoglycolate esters.²³

GE. To unequivocally establish that this mobility difference reflected distinct covalent connectivity rather than distinct conformations of a single substance as well as to evaluate the impact of variation of the crosslink site on mobility, self-complementary DNA's V, VI, and VII were exposed to reductively activated mitomycin C. DNA V contains only a single 5'-CG sequence for crosslinking by reductively activated mitomycin C.^{7,9,11,20} Duplexes VI and VII each have two such sequences, but they are symmetry related, except for the 5'-terminal phosphate present (in most molecules) on only one strand of the duplex. Although duplexes VI and VII contain two MC-crosslinked sites, the low efficiency of MC-crosslinking under the conditions employed here ensures that only one site will be crosslinked per duplex. The crosslinked DNA's were evaluated by denaturing PAGE (Figure 3): V gave a single low mobility band; VI and VII each afforded two major bands in a ca. 1:1 ratio.

The identities of the two distinct crosslinked DNA's present in VII were explored in two experiments which established that these bands represent singly dG-to-dG crosslinked DNA's in which the proximity of the 5'-phosphorylated terminus was distal (lower band) or proximal (upper band) to the crosslink. This was shown directly by iron(II)/EDTA fragmentation of the isolated upper band (Figure 4), and indirectly by preparation of MC-

Figure 5. Denaturing PAGE migration pattern of MC-crosslinked duplexes VII, VII (5' radiolabel on upper strand), and VII (5' radiolabel on lower strand).

crosslinked duplex VIII in two radiolabeled forms, 5'-phosphate on the upper or lower strand. For VIII, 5'-terminal phosphorylation of the terminus proximal (upper strand) and distal (lower strand) to the single crosslink site yielded products of denaturing PAGE mobility nearly identical to the upper and lower bands, respectively, derived from VII (Figure 5).

For these duplexes, the major determinant of mobility as a function of MC-crosslink site is distance from the center: the mobility of the crosslinked DNA's increased as the crosslink was moved farther from the center and toward either end of the duplex. Consistent with this, a 5'-end phosphorylated 34-mer, which can be thought of as two 17-mers linked 3'-to-5' by a single phosphate, was more mobile than MC-crosslinked VII (data not shown). The proximity of the crosslink to a phosphate group at a 5'-terminus had a smaller, but significant, impact on mobility: moving the crosslink site a given number of bases away from the phosphorylated terminus resulted in a greater enhancement in mobility than moving the crosslink the same number of bases toward the phosphorylated terminus.

DISCUSSION

Analysis by fragmentation

We report herein a general method for the quantification of crosslink location in DNA fragments involving iron(II)/EDTA cleavage of crosslinked and native DNA samples. Quantification of the resulting fragment abundances as a function of fragment length yields the relative efficiencies of crosslinking at various sites. As a trivial test of this method, we have demonstrated that two distinct 5'-CTAC sites in a duplex fragment are equally efficiently photochemically crosslinked by a psoralen derivative. In a second example, we have determined the relative efficiency with which reductively activated mitomycin C crosslinks two distinct 5'-CG sequences, 5'-GCGC and 5'-TCGA, in a single DNA duplex. In agreement with the results of others,⁸ we find the former to be preferred by $3 \pm 1:1$. This same result was obtained when these sequences were contained in distinct DNA's and the relative efficiency of crosslinking was monitored by direct quantitation of the crosslinked products by Cerenkov counting.

Mobility of crosslinked DNA fragments in denaturing PAGE is a function of crosslink location

We have also recently evaluated by denaturing PAGE the products resulting from the reactions of a variety of DNA interstrand crosslinking reagents with numerous synthetic DNA duplex fragments. Incubations in which a highly sequence specific crosslinking agent (HMT, mitomycin C)⁹ was provided a single crosslinkable site invariably provided a low mobility (crosslinked)

product which migrated as a single, narrow band. Incubation of DNA duplex fragments with substances which it was ultimately concluded were less sequence specific (pyrrolizidine alkaloids, bis(acetoxymethyl)pyrroles,¹³ mechlorethamine,¹² nitrous acid¹⁵) or sequence specific agents (HMT, mitomycin C) which were tested against DNA duplexes which bore several distinct crosslinkable sites¹¹ provided instead diffuse and/or several low mobility (crosslinked) bands. Isolation of individual bands and analysis by iron(II)/EDTA fragmentation suggested that these crosslinked products differed with respect to the location of the crosslink. While the physical origin of the effect was unknown, its utility in the analysis of crosslinking reactions was obvious. Furthermore, although not critical to the utility of the technique, there appeared to be a correlation between mobility of MC-crosslinked DNA and the location of the crosslink relative to the center of the fragment: the least mobile bands were centrally crosslinked and the most mobile bands were terminally crosslinked.¹³

This idea was tested in a family of DNA duplexes of identical length, but in which 5'-CG sites were progressively displaced from the center of the duplex towards the ends. These DNAs were crosslinked with reductively activated mitomycin C. Most importantly, it was demonstrated that the mobilities of the crosslinked duplexes were distinct. Interestingly, the mobility of the crosslinked products increased as the crosslink was moved closer to either end. Proximity of the crosslink to the phosphorylated terminus of a singly-end phosphorylated duplex had a less important, but significant, impact on mobility, mobility being enhanced relatively more on moving away from rather than toward that terminus. Whether these relationships of electrophoretic mobility to crosslink location are retained with other DNAs and crosslinking agents remains to be seen.

This relationship of crosslink location and denaturing PAGE mobility likely accounts for some previously reported data involving reductively activated mitomycin C crosslinking of DNA duplex fragments. Teng *et al.*⁷ have reported denaturing PAGE data on DNA fragments bearing 1, 2, 3, and 5 mitomycin-crosslinkable sites (5'-CG). The range of mobilities reported for the crosslinked products is clearly proportional to the number of potential crosslink sites (Figure 2a in reference 7). A related phenomenon has been reported by Cech.²¹ In that case, the bandwidth ratio of lightly psoralen-crosslinked (1–10 crosslinks/duplex) and uncrosslinked duplex restriction fragments in alkaline agarose gels was found to be greater than 1. Although this ratio, which was assumed to reflect heterogeneity of crosslink location, was greatest when a few crosslinks were present (2–4/kBP), even a single crosslink caused band widening. In contrast to the present method employed denaturing PAGE on short DNA fragments (17–21 nucleotides), alkaline agarose electrophoresis of large (0.6–1.35 kBP) restriction fragments offers insufficient resolution to be preparatively useful. Roberts *et al.*²² have very recently reported the observation that DNA duplexes crosslinked with bis(platinum) complexes display a range of mobilities in denaturing PAGE. Their proposal that this was due to heterogeneity of crosslink location is fully consistent with the results herein.

CONCLUSION

The results reported herein have important implications for the quantitative study of sequence specificity of DNA interstrand crosslinking agents using short, synthetic DNA duplexes: DPA-

GE serves first as an analytical technique, distinguishing not only crosslinked from single stranded or monoadducted DNA, but as described herein providing through the distinct mobilities of isomeric crosslinked products a crude evaluation of the degree of sequence specificity of the crosslinking agent. In the second step, the eluate from individual bands is subjected to iron(II)/EDTA fragmentation. The nucleotide connectivity of structurally homogeneous fractions is thus revealed. Crosslinked isomers differing in nucleotide connectivity but with electrophoretic mobilities insufficiently different to allow separation are likewise revealed and quantified as demonstrated herein. These complementary techniques have made possible very rapid progress in the elucidation of crosslinking sequence preferences of a wide variety of agents.^{9,11–16}

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