Sequence Specificity of DNA–DNA Interstrand Cross-Link Formation by Cisplatin and Dinuclear Platinum Complexes†

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ABSTRACT: The sequence specificity of interstrand cross-links induced in DNA by mononuclear and dinuclear platinum complexes in a 49-base-pair DNA duplex has been determined directly. This new assay takes advantage of the fact that 3′ → 5′ exonuclease digestion of randomly platinated DNA produces a pool of fragments of different lengths. This treatment allows identification of the spectrum of adducts impeding the exonuclease scission. Interstrand cross-linked adducts produce fragments that may remain complementary in the proximity of the binding site. As a result, these fragments may act as primer templates for extension upon subsequent treatment with a DNA polymerase. This extension increases the size of the oligonucleotide fragments, which may be evidenced by a more slowly migrating band on a sequencing gel. Concomitantly, the original band corresponding to the digested cross-link decreases in intensity. Therefore, comparison of a sequencing gel after digestion only and after the "digestion–extension" treatment should show the disappearance, or diminished band intensity, of only those fragments with interstrand cross-links. This approach was applied to the analysis of DNA interstrand cross-links formed by *cis*-$[PtCl_2(NH_3)_2]$ (*cis*-DDP) and $[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]Cl_2$. *cis*-DDP was confirmed to form interstrand cross-links at d(GC) sequences but, interestingly, interstrand cross-links predominated in a sequence GCGG, with possible 1,3-intrastrand but no 1,2-intrastrand cross-links forming. The dinuclear compound formed 1,2, 1,3, and 1,4 DNA interstrand cross-links between guanines on opposite strands. In 1,3 and 1,4 cross-links, the guanines are separated by one and two base pairs, respectively, whereas a 1,2 cross-link is formed from guanines on neighboring base pairs. The applicability of the approach was extended by examining the interstrand cross-links formed by 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT), where the expected d(TA) sequence specificity was observed.

The cytotoxicity and anticancer activity of many clinically important drugs are believed to result from reaction with DNA by the formation of DNA–DNA interstrand cross-links (Kohn, 1983; Roberts, 1987; Roberts et al., 1988; Tomasz et al., 1987). Interstrand cross-linking agents also present possible applications as structural probes (Alul et al., 1992; Cimino et al., 1985) and in the development of antisense oligonucleotides (Gruff & Orgel, 1991; Chu & Orgel, 1990; Uhlmann & Peyman, 1990). Dinuclear bis(platinum) complexes are a class of highly efficient DNA interstrand cross-linkers, both with isolated DNA of various origins (Roberts et al., 1989; Farrell et al., 1990a) and in intact cells (Hoeschele et al., 1990). Diplatinum complexes with equivalent coordination spheres are represented by the general formula, $[\{PtCl_m(NH_3)_{3-m}\}_2(H_2N\text{-}R\text{-}NH_2)]^{2(2-m)+}$ (m = 0–3 and R is usually a linear alkane chain). The complexes show high activity in vitro and in vivo in both murine and human tumor cell lines resistant to cisplatin (*cis*-DDP[1]) (Kraker et al., 1992; Farrell et al., 1990b). The formation and structure of the (Pt,Pt) interstrand cross-links are important features in differentiating the biological activity of the dinuclear complexes from that of their mononuclear analogs. Repair of interstrand cross-links may proceed by a different pathway than DNA adducts, which affect only one strand (Van Houten, 1990). Interstrand cross-links represent only a minor portion (<5%) of the total *cis*-DDP–DNA adducts (Roberts & Friedlos, 1982; Eastman, 1985), but their presence contributes to the drug's cytotoxicity (Kohn, 1983; Roberts, 1987; Roberts et al., 1988). *cis*-DDP interstrand cross-links may also be involved in the inhibition of transcription (Lemaire et al., 1991).

It is of considerable importance to obtain information on the sequence preference of DNA interstrand cross-links. Since both mononuclear and dinuclear platinum complexes form many different types of adducts on DNA, the challenge of easily recognizing the interstrand cross-links arises. Several methods may be used for this purpose, including gel-denaturing purification methods combined with other treatments (Rahmouni & Leng, 1987; Weidner et al., 1989; Millard et al., 1991). This article reports on a rapid, simple, and efficient assay for examining the sequence specificity of DNA interstrand cross-links formed by *cis*-DDP and the dinuclear platinum complex, $[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]Cl_2$ (Figure 1). In this assay, no pretreatment of DNA adducts is required prior to the enzymatic reactions. *cis*-DDP was confirmed to form interstrand cross-links at d(GC) sequences (Lemaire et al., 1991). The dinuclear compound formed 1,2, 1,3, and 1,4 DNA interstrand cross-links. In 1,3 and 1,4 cross-links, the guanines are separated by one and two base pairs, respectively, whereas a 1,2 cross-link is formed from

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[1] Abbreviations: *cis*-DDP, *cis*-$[PtCl_2(NH_3)_2]$, and cisplatin, *cis*-diamminedichloroplatinum; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen.

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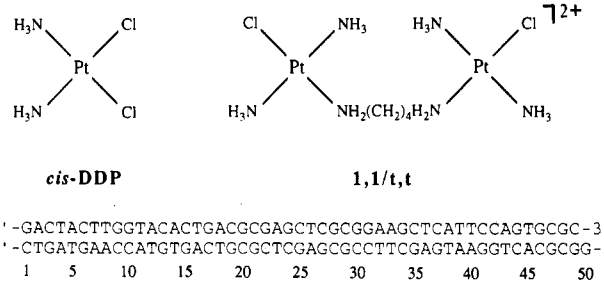

FIGURE 1: Structures of platinum compounds and the 49/50-bp duplex used to study the sequence specificity of interstrand cross-linking. In the text, A, G, C, and T refer to sequences on the top strand, while the terminology A′, G′, C′, and T′ is used for the sequence of the bottom strand (read from the 5′-end).

guanines on neighboring base pairs. The approach is expected to be generally applicable to DNA interstrand cross-links induced by other agents. This point was examined using a DNA duplex adducted with HMT, a psoralen that specifically cross-links d(TA) sequences (Gamper et al., 1984).

MATERIALS AND METHODS

Materials. The platinum complexes *cis*-$[PtCl_2(NH_3)_2]$ and $[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]Cl_2$ were prepared as previously described [see Dhara (1970) and Farrell et al. (1990), respectively]. The 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen was the kind gift of HRI Associates. Sequenase version 2.0 DNA polymerase was obtained from United States Biochemical. T4 DNA polymerase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. Deoxyribonucleotide triphosphates (dNTPs) were purchased from Promega. The [γ-^{32}P]ATP was bought from New England Nuclear Inc. (DuPont). Sodium cyanide was obtained from Fisher Scientific Company. All chemicals for electrophoresis were of the highest quality, purchased from Bethesda Research Laboratories.

The 49/50-bp oligodeoxynucleotides (Figure 1) were synthesized as two separate single strands on a DuPont automatic DNA synthesizer and purified by electrophoresis on 12% polyacrylamide gels under denaturing conditions (8 M urea). Either strand oligomer was 5′-terminally labeled with ^{32}P by standard techniques and then annealed to its complement. The duplex was purified on a 12% polyacrylamide native gel (Zou et al., 1993).

DNA Modification. Platination of DNA was carried out in the following manner. The duplex (5–10 ng/μL, radiolabeled on either strand) was incubated with platinum complexes (0.5 μM) in TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.4) at 37 °C. Total incubation times were 9 and 3 h for *cis*-DDP and the diplatinum agent, respectively. The reactions were terminated by the addition of 0.2 M NaCl. The unbound platinum complexes were removed by extensive dialysis against TEN buffer (10 mM Tris-HCl, 0.1 mM EDTA, and 50 mM NaCl, pH 7.4) at 4 °C overnight in a microdialysis system. The r_b (number of bound Pt per nucleotide) was estimated to be less than 0.01 on the basis of the data from the reactions with calf thymus DNA under the same conditions. The r_b was calculated as detailed previously (Zou et al., 1993).

Modification of the DNA fragment by HMT was performed by incubating the radiolabeled DNA (2 μg/mL) with HMT (20 μM) in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM NaCl, and 0.1 mM EDTA for 1 h at room temperature. The mixture was then irradiated in a cell culture dish by an ultraviolet source (0.45 mW/cm^2) at 360 nm for 10 min, followed by extensive dialysis against TE buffer at 4 °C. All experiments with HMT were carried out under yellow light or in the dark.

The sequencing of the 49/50-bp DNA fragments was performed by using standard Maxam–Gilbert procedures with a DuPont NEN sequencing kit.

Mapping of Interstrand Cross-Links. In our experiments, the modified 49/50-bp oligomer (1 ng/μL), 5′-terminally labeled on either strand, was digested by the 3′ → 5′ exonuclease activity (10 units) of T4 DNA polymerase in the absence of dNTPs at 37 °C for 5–10 min in 20 μL of pH 7.5 buffer containing 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $MgCl_2$. The digestion resulted in a series of fragments of different lengths. The solution was then immediately brought to 70 °C for 10 min to terminate the enzymatic reactions. Upon cooling, a mixture of dNTPs (0.5 mM) was added to the solution. An aliquot of the mixture was incubated with 13 units of Sequenase version 2.0 DNA polymerase at 37 °C for 20 min for extension. Another aliquot of equal volume was incubated with TE buffer and no Sequenase. To stop the polymerase reactions, the samples were quickly brought to 90 °C for 5 min. Fragments were identified by cosequencing the Maxam–Gilbert products (Maxam & Gilbert, 1980) of the 49/50-bp oligonucleotides.

Some alternative procedures were also examined. After the enzymatic digestion, an aliquot of the solution was mixed directly with dNTPs (0.5 mM) and incubated at 20 °C for an additional 20 min. Another aliquot of the same volume was incubated in the absence of dNTPs for comparison. The reactions were then terminated by heating at 90 °C for 5 min, followed by electrophoresis and sequencing.

The bound platinum was removed in a reaction with 0.3 M NaCN (pH 11) at 37 °C overnight, while HMT-induced interstrand cross-links were reversed by irradiation with 250-nm UV light (1.5mW/cm^2) for 5 min. Samples were then dialyzed at 25 °C for 1.5 h against TE buffer with a membrane of 6000 MW cutoff limit. The final products were then subjected to electrophoresis on 12% polyacrylamide sequencing gels under denaturing conditions.

RESULTS

Interstrand Cross-Link Assay. The inhibition of 3′ → 5′ exonuclease activity of enzymes by DNA adducts may be used to monitor the sites of these adducts. The exonuclease activity is usually arrested 1–3 bp away from the adduct, and enzymatic digestion of the damaged DNA produces a pool of fragments of varying lengths due to inhibition by the array of adducts (Royer-Pokora et al., 1981; Tullius & Lippard, 1981; Fuchs et al., 1983; Malinge et al., 1987). The sequence specificity and differentiation of interstrand cross-links from other adducts may be obtained by the application of a new assay. The strategy of this assay is based on the following considerations: (i) DNA replication usually requires a primer with the sequence matching a locus of the template; (ii) the size of a DNA increases as the polymerization occurs in vitro; (iii) DNA remains as a duplex at or near interstrand cross-linking sites even under denaturing conditions; and (iv) upon degradation, monoadducted and intrastrand-adducted DNA fragments will not be complementary at 37 °C and thus are not primed for extension, whereas (v) the termination of exonuclease activity prior to an interstrand cross-linking site leaves a fragment with complementary base pairs at the cross-linking site. Thus, we reasoned that a cycle of DNA digestion and inhibition of exonuclease activity by Pt–DNA adducts followed by the extension of any primed templates could be used to measure interstrand cross-linked fragments.

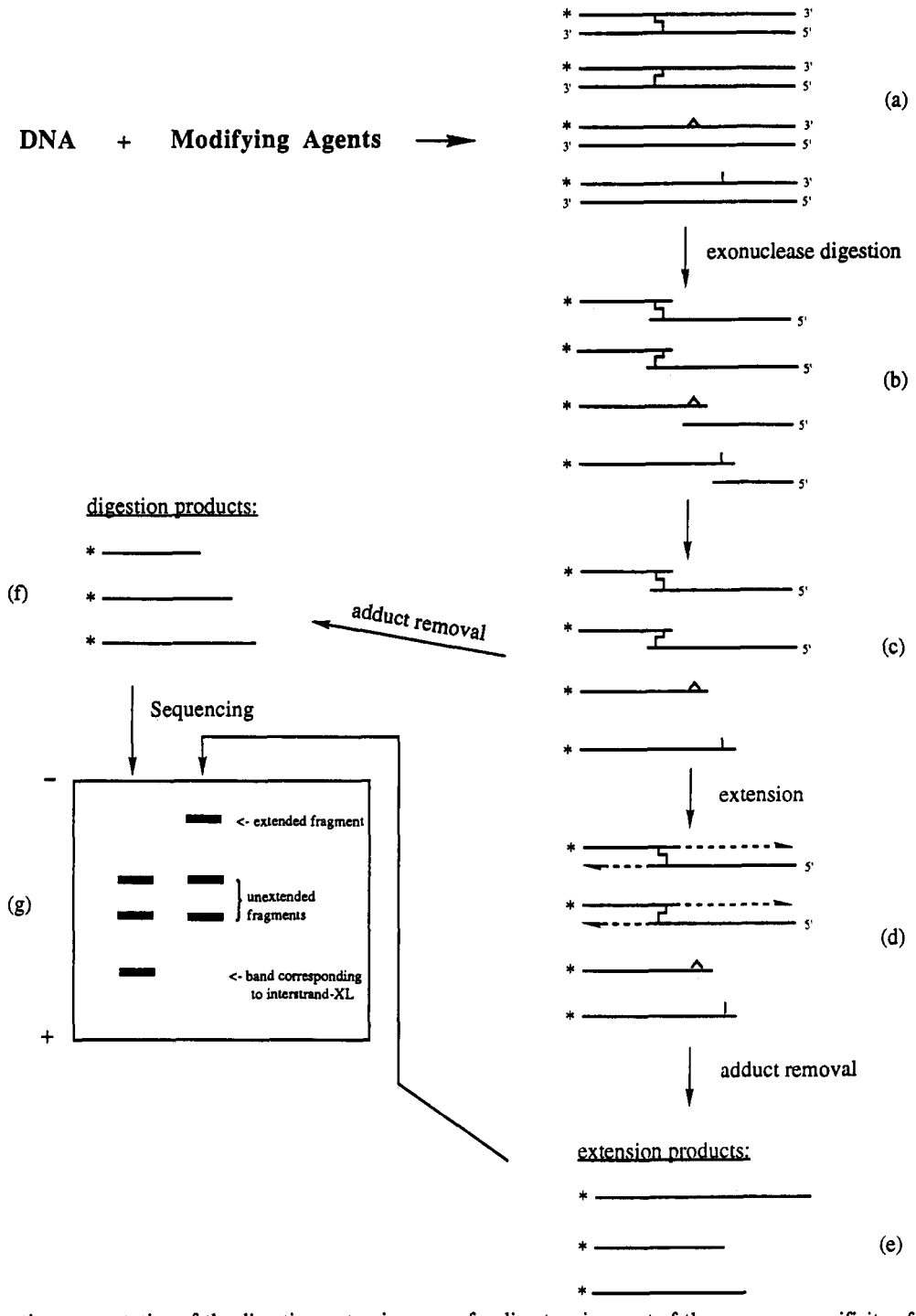

FIGURE 2: Schematic representation of the digestion–extension assay for direct assignment of the sequence specificity of interstrand cross-linking. See text for details.

Figure 2 shows the principal procedures of the new assay. Modified DNA fragments with one strand 5′-terminally labeled are first digested by 3′ → 5′ exonuclease activity, resulting in a pool of fragments of different lengths (Figure 2b-c). This treatment allows identification of the spectrum of adducts impeding the exonuclease scission. After an appropriate period of digestion, the samples are immediately heated to eliminate the exonuclease activity. Upon cooling to 37 °C, DNA polymerase and an excess of dNTPs are added to allow any primed fragments to be extended (Figure 2d). The DNA fragments containing only monofunctional and intrastrand adducts are most likely single-stranded at this stage. In contrast, those fragments with interstrand cross-links that remain complementary in the proximity of lesions (Figure 2c) can be extended by the action of a DNA polymerase (Figure 2d). As a result, this extension increases the size of the oligonucleotide fragments (Figure 2e), which may be evidenced by a more slowly migrating band on a sequencing gel (Figure 2g). Concomitantly, the original band corresponding to the digested cross-link decreases in intensity. Therefore, comparison of a sequencing gel both after digestion only and after the digestion–extension treatment should show the disappearance, or diminished intensity, of those interstrand cross-links with complementary ends. No changes should be apparent for fragments not due to cross-links, since these are not extendable.

Optimization of this scheme must take into account a number of factors. The exonuclease activity of T4 DNA polymerase is not completely stopped by Pt–DNA adducts (Malinge et al., 1987), and indeed, structurally different

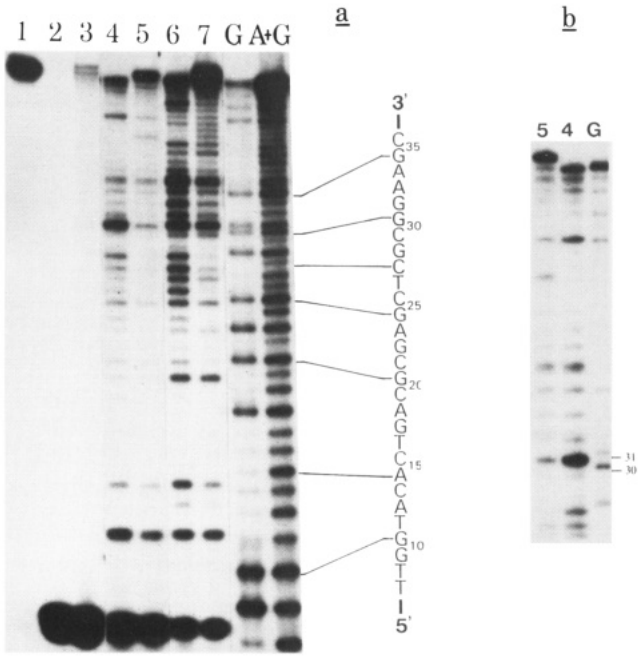

FIGURE 3: Mapping of interstrand cross-links of the top (49 base) strand labeled DNA duplex induced by platinum agents. See Materials and Methods for details. In panel a, lane 1 is DNA without any enzymatic treatment; lane 2 is unmodified DNA subjected to enzymatic treatment; lanes 4 and 6 represent the digestion patterns of DNA modified by *cis*-DDP and [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$, respectively, upon treatment with exonuclease; lane 3 represents the product of lane 2 incubated in the presence of dNTPs and DNA polymerase; and lanes 5 and 7 are the extension products of lanes 4 and 6, respectively, upon incubation in the presence of dNTPs and DNA polymerase. The G and G+A lanes were loaded with the Maxam–Gilbert sequencing products of G and A, respectively. Panel b shows the autoradiograph in the d($G_{28}C_{29}G_{30}G_{31}$) region obtained upon prolonged electrophoresis of the gel.

adducts inhibit exonuclease activity to different extents. The exonuclease activity is also, therefore, dependent on the concentration of polymerase used and the incubation time. In practice, an excess of T4 polymerase over substrate was used. In control experiments, no obvious effects on the digestion pattern were noted upon variation of the polymerase concentration (data not shown). The longer the sample was incubated in the presence of T4 polymerase, the more adducts that were removed. However, interstrand cross-linked fragments were still extendable as long as bands were observable, and no different digestion–extension *patterns* were seen with time. An r_b of equal to or less than 1 adduct per DNA molecule is desirable to avoid the "coformation" of interstrand cross-links and other types of lesions on the same fragment. For simplicity and rapidity, the same buffer should be used for both exonuclease and polymerase activities.

Platinum Complexes. Digestion of platinated DNA fragments by the 3′ → 5′ exonuclease activity of T4 DNA polymerase reveals a series of stop sites due to enzyme inhibition by the adducts (Figure 3a). The inhibition pattern was similar to that observed previously (Farrell et al., 1990). The exonuclease may stop up to two nucleotides to the 3′ direction of the bound platinum, depending on the type of lesion (Malinge et al., 1987). Enzymatically digested fragments migrate 1–1.5 nucleotides more slowly than the corresponding Maxam–Gilbert products due to the absence of a phosphate group, in comparison to the corresponding chemically treated fragments (data not shown). Note that the migration of the exonuclease fragments with respect to the Maxam–Gilbert sequencing lanes is partially dependent on how far away the lesion sites are from the DNA labeled end.

Intrastrand Cross-Links. The principal adduct of *cis*-DDP on DNA is usually the intrastrand cross-link between adjacent guanines (Sherman & Lippard, 1987; Reedijk et al., 1987). Model studies have shown that the dinucleotide d(GpG) may also form (Pt,Pt) intrastrand cross-links with the dinuclear complex (Bloemink et al., 1992). The intense stop site comigrating with d(T_{11}) (lanes 4 and 6, corresponding to termination at d(A_{12})) is therefore assigned to the expected d(G_9G_{10}) intrastrand cross-link. These bands are not extendable within the limit of experimental error (see lanes 5 and 7) and may be used as an internal control for subsequent analysis.

Interstrand Cross-Links. Upon the addition of excess dNTPs, the recessed DNA fragments with interstrand cross-links were extended preferentially by DNA polymerase activity (lanes 5 and 7). The sequence specificity of these interstrand cross-links can be obtained by comparing the pattern of bands in lanes 4 and 6 (digestion only) and those in lanes 5 and 7 (digestion followed by extension), respectively. The appearance of a new intense band at the top of extension lanes 5 and 7 represents a 50-bp oligomer of the same length as the template "bottom" strand. In addition, the intense bands at the bottom of the gel correspond to the digested fragments without any lesions (lanes 2–7) and can be used to correct the quantitative differences in the samples loaded between the comparable lanes. The complex $[PtCl(NH_3)_3]Cl$, capable only of monofunctional binding, showed no extension under similar conditions (data not shown).

cis-DDP. For *cis*-DDP, the results of the digestion and digestion–extension treatments were analyzed in detail for the "top" strand of Figure 1. An example of how this assay may be used is demonstrated for the bands within the d($C_{27}G_{28}C_{29}G_{30}G_{31}$) sequence (lanes 4 and 5, Figure 3a). If an intrastrand cross-link were to appear in this sequence at $G_{30}G_{31}$, then a nonextendable band would be expected at A_{33}. The absence of such a band suggests that no intrastrand cross-links occur at this GG sequence. Alternatively, a 1,3-intrastrand cross-link could be formed between G_{28} and G_{30}. This would produce a nonextendable band at G_{31}; such a band is seen, suggesting (Figure 3b) that a few intrastrand cross-links do form in this sequence. However, the majority of stop sites resulting from binding within this sequence are extendable, suggesting that the majority of lesions are interstrand cross-links. The specific cross-links possible are a d(GC) adduct between G_{28} and G'_{29} and a d(CG) adduct between G'_{29} and G_{30}. (Note that A, G, C, and T refer to sequences on the top strand, while the terminology A′, G′, C′, and T′ is used for the sequence of the bottom strand, both numbered from the 5′-end of the top strand.) It is difficult to distinguish between the two possibilities with the present sequence. The d(GC) cross-link has been considered more likely (Lemaire et al., 1991; Hopkins et al., 1991). It is possible that the d(CG) adduct is less resistant to the CN^- treatment required to observe *cis*-DDP interstrand cross-links (Schwartz et al., 1990). Nevertheless, the results show that, surprisingly, *cis*-DDP prefers to form a 1,2-interstrand cross-link rather than the alternative 1,2-d(GG) or 1,3-d(GG) intrastrand cross-links within the $G_{28}C_{29}G_{30}G_{31}$ sequence.

We note that, in previous experiments using this sequence but with shorter incubation times (1 h), the vast majority of *cis*-DDP was bound to the G_9G_{10} site (Farrell et al., 1990). We consider that the relative lack of intrastrand cross-linking for *cis*-DDP is an artifact of both the specific sequence used and the experimental conditions used for binding. These results are consistent with the well-documented findings that the susceptibility of any particular GG site to *cis*-DDP is

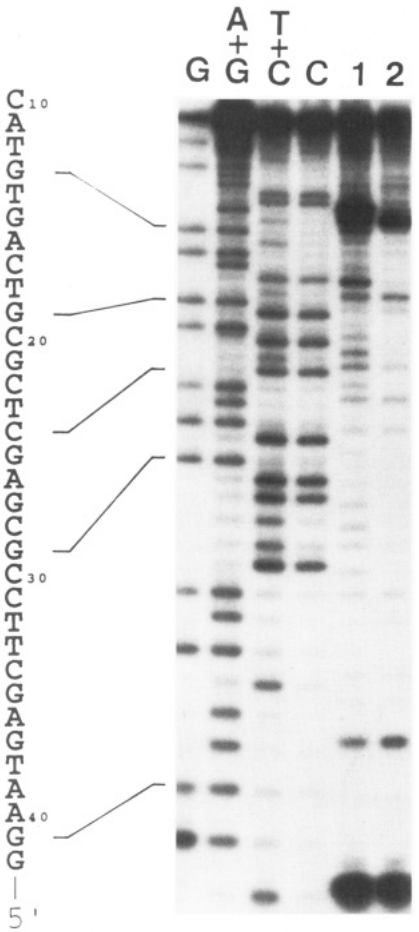

FIGURE 4: Mapping of interstrand cross-links of the bottom strand labeled DNA duplex induced by [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$. See Materials and Methods for details. Lane 1 refers to the digestion pattern of the platinated DNA fragment digested, whereas lane 2 refers to the digestion–extension pattern upon incubation with dNTPs and DNA polymerase. Lanes G, G+A, T+C, and C are the same as in Figure 3 for G, A, T, and C, respectively.

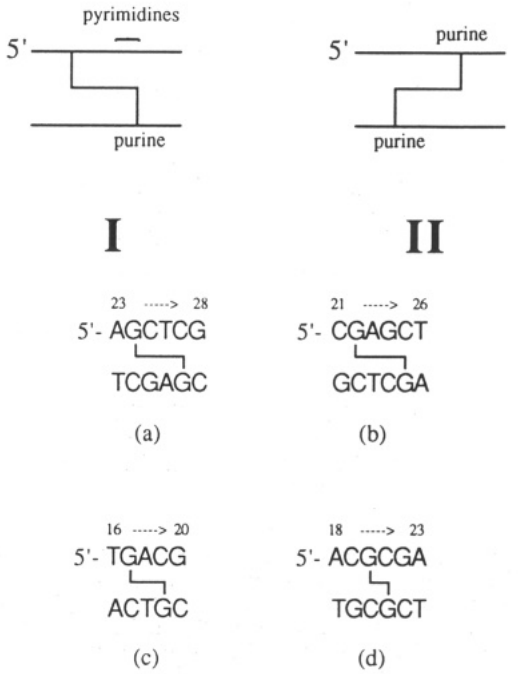

FIGURE 5: Possible DNA–DNA interstrand cross-links formed by the dinuclear complex [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$. Type I is oriented in the 5′ → 5′ direction, and type II is the 3′ → 3′ direction, where 5′ and 3′ represent the phosphate and hydroxyl ends of the double-stranded DNA, respectively. Sequences a–d represent sites of 1,2-, 1,3-, and 1,4-interstrand cross-links deduced by analysis of Figures 3 and 4. Numbering of the oligonucleotides is the same as in Figure 1.

dependent on local sequence and conformation (Hemminki & Thilly, 1988; Marrot & Leng, 1989; Tullius & Lippard, 1981).

Diplatinum Complexes. A comparison of lanes 6 and 7 (in Figure 3a) reveals several interesting features. Upon extension, the clearest examples of bands that decrease in intensity include those corresponding to binding in the C_{19}–G_{22} and G_{25}–G_{28} regions. The latter sequences previously have been identified as a potentially rich source of interstrand cross-links for dinuclear complexes (Farrell et al., 1990). While regions of interstrand cross-linking are thus readily identified, the exact description of their structure is somewhat more complicated. *cis*-DDP, and indeed most alkylating agents, gives rise to only 1,2-interstrand cross-links. In contrast, molecular models and previous studies (Farrell et al., 1990; Gruff & Orgel, 1991) suggest that both the length and the flexibility of the 1,1/t,t dinuclear compound allow the targeting of much larger DNA sequences for cross-link formation. A dinuclear complex must first bind to DNA through only one end of the dinuclear unit. Thus, the kinetic preferences will be similar to those of a mononuclear species, but the *array* of adducts will become different upon the binding of the second Pt (Wu et al., 1993). It is therefore likely that 5′ → 5′ binding to guanine bases will be more favored for interstrand cross-linking, considering that the 5′-dG reacts faster with *cis*-DDP than the 3′-dG base in short $d(G_n)$ sequences (Lempers et al., 1991; van der Veer et al., 1987). We thus consider only this possibility in the assignment of cross-linking sites (type 1, Figure 5).

In order to obtain complete sequence information about the (Pt,Pt) interstrand cross-links, the digestion and digestion–extension experiments were also carried out on the complementary 50-bp bottom strand (Figure 4). A detailed description of some of the interstrand cross-links may be obtained by comparison of the results for both strands of the duplex. In the digestion pattern (Figure 3, lane 6), there are five bands within the G_{24}–G_{28} sequence. Thus, some stop sites are observed in regions of pyrimidine sequences, typically at $d(C_{25}T_{26}C_{27})$, as indicated by the extendable bands running at $d(T_{26})$ and $d(C_{27})$. Since purine bases are much more readily attacked by Pt complexes than pyrimidines, a plausible and reasonable explanation for these stop sites is that the complementary purine residues on the bottom strand were cross-linked to adjacent purines 5′ to them on the top strand. In this manner, exonuclease activity could be partially or totally inhibited at the pyrimidines complementary to the modified purines (type I, Figure 5). The alternative cross-link (type II, Figure 5) would not show such a stop site, as the exonuclease, proceeding from the 3′-end, would first encounter the purine base of the top strand. An extendable pyrimidine band may therefore be ascribed to a purine cross-linking site on the opposite strand. Since the platinated purine downstream of the pyrimidine site is also expected to inhibit exonuclease digestion, cross-links spanning more than two adjacent base pairs may produce more than one stop site on the same strand. This consideration clearly complicates matters, but careful analysis allows the identification of specific cross-links.

Thus, the extendable band running with G_{28} of the top strand (Figure 3) corresponds to Pt binding to a purine residue on the complementary (bottom) strand (Figure 4). Examination of the appropriate complementary $d(C'_{28}G'_{27}A'_{26})$ sequence on the bottom strand shows G'_{27} to be a reasonable binding site. The interstrand cross-link can only be toward the 5′-end

of the top strand (type I, Figure 5), and therefore the binding site should be within the sequence d($A_{23}G_{24}C_{25}T_{26}$). An extendable stop site on the bottom strand (Figure 4) is observable at C'_{22}. This stop site likewise is attributable to G_{24} binding on the top strand. A reasonable interpretation of these data is the presence of a 1,4-interstrand cross-link between G_{24} and G'_{27} (Figure 5a).

Similar analyses have been carried out for the extendable stop sites at C_{25} and C'_{22}, as well as those at C_{19} and C'_{17}, indicating the presence of both 1,4- and 1,3-interstrand cross-links (Figure 5b,c). Finally, a band on the top strand migrating with A_{23} may be attributed to binding at G'_{21} (Figure 3). This stop site disappears on extension and is therefore assigned to an interstrand cross-link. The complementary sequence either side of this binding site is 5′-C_{19}-G_{20}-C_{21}-G_{22}-A_{23}. Thus, potential cross-linking sites are G_{20} and G_{22}. An extendable band migrating with T'_{18} may be attributable to G_{20} binding (Figure 4). A 1,2-interstrand cross-link between G_{20} and G'_{21} is therefore indicated (Figure 5d). Note that, in this case, only stop sites directly attributable to purine binding need be considered.

HMT. This clinically important compound produces, upon the absorption of near-UV (320–360 nm) light, interstrand cross-linked adducts with thymines primarily at d(TA) sequences (Gamper et al., 1984). In the present study, the DNA fragment was 5′-terminally labeled on the bottom strand, which has two d(TA) sequences. Upon the absorption of near-UV light (360 nm), two orientational interstrand cross-linked isomers of the oligomers (one with the HMT furan side on the top strand and another on the bottom strand) form in equal amounts (Cimino et al., 1985; Van Houten et al., 1986). Upon exonuclease digestion and photoreversal with 250-nm light, three fragments (the furan side and pyrone side monoadducts and the adduct-free oligomer) from the labeled strand may be identified by gel elctrophoresis (Van Houten et al., 1986). As shown in lane 1 of Figure 6, the 3′ → 5′ exonuclease activity of T4 polymerase results in major stop sites represented by a triplet set of bands at the two d(TA) sequences. These bands will also owe some of their intensity to monoadducts unconverted to interstrand cross-links by photolysis (Straub et al., 1981; Kanne et al., 1982). These products are indistinguishable from those of the same type obtained from the photoreversal of interstrand cross-links.

Lane 2 in Figure 6 shows the results from the digestion–extension treatment. In comparison to the bands in lane 1, the disappearance of the adduct-free band in lane 2 indicates that the corresponding adducted DNA fragments are extendable and that interstrand cross-links form at the d(TA) sequences. This result is consistent with the fact that the adduct-free products are produced only by DNA–psoralen interstrand cross-linked fragments. As a consequence of DNA extension, a new band is found at the top of lane 2, which represents the 50-mer with a monoadduct and is thus shifted about one nucleotide up from the position for the nondamaged 50-mer. Of the triplet bands, the behavior of the furan side monoadducts indicates that most have been converted to interstrand cross-links by reaction with a thymine on the complementary strand. In contrast, pyrone side monoadducts are less readily converted, and thus little extension is seen (Cimino et al., 1985).

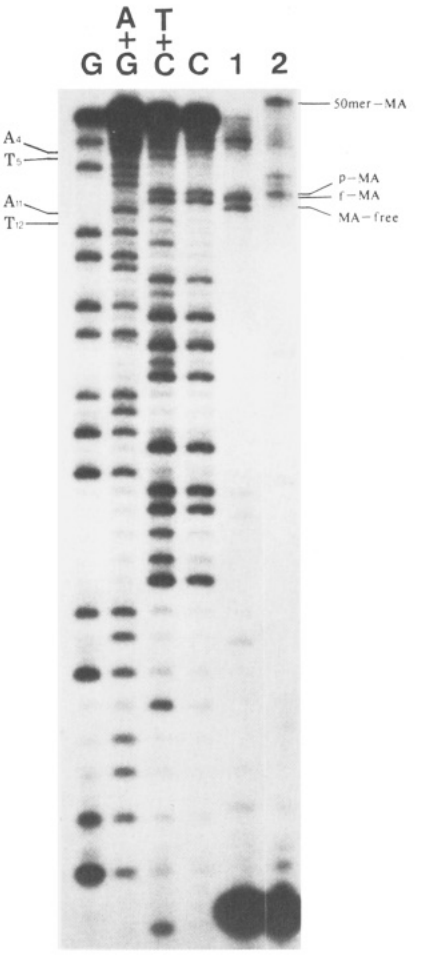

FIGURE 6: Sequence specificity of DNA interstrand cross-links formed with HMT. See Materials and Methods for details. Lane 1 is the digestion pattern upon treatment of HMT-adducted DNA with exonuclease, while lane 2 represents the products of lane 1 upon incubation with dNTPs and DNA polymerase. Lanes G, G+A, T+C, and C have the usual meanings.

DISCUSSION

DNA damage by interstrand cross-linking has been correlated with the cytotoxicity of many carcinogens and anticancer drugs (Pratt & Ruddon, 1979; Kohn, 1983; Roberts et al., 1987, 1988). The detailed mechanistic explanation of this correlation requires knowledge of the sequence specificity of the interstrand cross-links. However, most DNA-targeting drugs and agents produce more than one type of DNA adduct, making it difficult to study the effects of a specific lesion on random sequence DNA. This is especially true in the case of mononuclear platinum complexes, where interstrand cross-link adducts represent only a minor portion of the total adducts induced. The sequence specificity of interstrand cross-link formation has been assayed previously by a number of chemical methods. The approach for alkylating agents (Weidner et al., 1989; Millard et al., 1990) combines gel-purification procedures with Fe(II)/EDTA treatment and determines sequence specificity at single-nucleotide resolution of homogeneous interstrand cross-linking on defined sequences of oligonucleotides. For platinum compounds, the differences in chemical stability to NaCN of the various types of Pt–DNA adducts may be exploited to obtain information specifically on interstrand cross-links (Schwartz et al., 1990; Rahmouni & Leng, 1987; Lemaire et al., 1991).

In general, gel separation procedures are good for relatively high-yield formation of interstrand cross-links on DNA of defined sequences, but they require a long period of preparation during which time the DNA adducts may not be chemically stable. In some cases, separation of DNA adducts with similar structures may become difficult. The experiments presented here were developed to provide a simple and rapid approach to obtain information on the sequence specificity of interstrand cross-links without the necessity of pretreatment. The strategy

includes digestion of adducted DNA by exonuclease activity followed by extension of only the interstrand cross-linked fragments. The identification of extendable bands, through comparison of sequencing gels from digestion only and from digestion–extension, allows the assignment of sequence preferences of interstrand cross-links, even in the presence of other adducts.

The present experiments confirmed the formation of d(GC) interstrand cross-links with *cis*-DDP (Rahmouni & Leng, 1987; Lemaire et al., 1991). Most interestingly, an interstrand cross-link has been identified within the $d(G_{28}C_{29}G_{30}G_{31})$ sequence. The interstrand cross-link therefore formed in preference to the expected $d(G_{30}G_{31})$ 1,2-intrastrand cross-link. A regulatory sequence of d(GGGCGG) in tumor virus SV 40 DNA has previously been reported to be a very strong target of *cis*-DDP (Gralla et al., 1987; Buchanan & Gralla, 1990). The present results raise the possibility of interstrand cross-link formation in such GC boxes. In the present case of $d(G_{28}C_{29}G_{30}G_{31})$, the interstrand cross-link may include one of the guanosine nucleosides of the possible d(GG) *intrastrand* cross-link. The assignment of *cis*-DDP adducts at sequences related to d(GCGG) may require more caution. The structural explanation for our observation is under more detailed investigation.

The ability of a dinuclear diplatinum complex to target larger sequences of DNA and the formation of long-range interstrand cross-links have been confirmed in this study. The enhanced interstrand cross-link formation of the dinuclear complex is immediately apparent. Although specific sequences are more difficult to assign, an array of 1,2, 1,3, and 1,4 cross-links is identifiable, by consideration of the guanine binding sites (the most favored of the four bases) on both strands of a DNA duplex. Our analysis and interpretation indicate that 5′ → 5′ cross-links (type I, Figure 5) are favored over other possibilities.

In summary, the assay described here may be used to map the sequence specificity of interstrand cross-links on random sequence DNA. The assay affords the features of (1) rapidity, (2) simplicity, (3) efficiency, and (4) potential wide application. Experimental conditions may be optimized for individual purposes, and in conjunction with other approaches, the detection of sequence preferences of interstrand cross-linking agents may be possible from DNA damaged in vitro or in vivo.

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