Superhelical torsion controls DNA interstrand cross-linking by antitumor cis-diamminedichloroplatinum(II)

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ABSTRACT

Negatively supercoiled, relaxed and linearized forms of pSP73 DNA were modified in cell-free medium by cis-diamminedichloroplatinum(II) (cisplatin). The frequency of interstrand cross-links (ICLs) formed in these DNAs has been determined by: (i) immunochromatographic analysis; (ii) an assay employing NaCN as a probe of DNA ICLs of cisplatin; (iii) gel electrophoresis under denaturing conditions. At low levels of the modification of DNA (<1 Pt atom fixed per 500 bp) the number of ICLs formed by cisplatin was radically enhanced in supercoiled in comparison with linearized or relaxed DNA. At these low levels of modification, the frequency of ICLs in supercoiled DNA was enhanced with increasing level of negative supercoiling or with decreasing level of modification. In addition, the replication mapping of DNA ICLs of cisplatin was consistent with these lesions being preferentially formed in negatively supercoiled DNA between guanine residues in both the 5′-d(GC)-3′ and the 5′-d(CG)-3′ sites. Among the DNA adducts of cisplatin the ICL has the markedly greatest capability to unwind the double helix. We suggest that the formation of ICLs of cisplatin is thermodynamically more favored in negatively supercoiled DNA owing mainly to the relaxation of supercoils.

INTRODUCTION

A probable mechanism of anticancer activity of cis-diamminedichloroplatinum(II) (cisplatin) involves formation of platinum–DNA adducts which are capable of blocking DNA and RNA synthesis (for general reviews see 1–7). The formation of interstrand cross-links (ICLs) in DNA has been found in DNA from cisplatin-treated eukaryotic cells. Furthermore, DNA adducts of cisplatin have been extensively studied, in particular in linear DNA modified by this drug in cell-free medium to relatively high levels (8,9). The DNA adducts of cisplatin have been extensively studied, in particular in linear DNA modified by this drug in cell-free medium to relatively high levels (8,9). The DNA adducts of cisplatin have been recently shown (11) that cisplatin toxicity is influenced by DNA supercoiling in prokaryotic cells.

The formation of cisplatin adducts in DNA is altered by its conformation (3–5,7,23,40–44). The conformational changes induced by bifunctional adducts of cisplatin result in particular in bending and unwinding of the DNA duplex (4,5,23,26,40,42). In general, negative supercoiling tends to stabilize conformations in which there is unwinding of the DNA (35–37). Thus, it seems reasonable to assume that some bifunctional adducts of cisplatin could be more favored in negatively supercoiled DNA (in comparison with relaxed or linear DNAs) than others. In other words, it may be expected that the individual types of cisplatin adducts could appear in negatively supercoiled DNA with a different frequency and/or sequence specificity than in relaxed or linear DNAs or even new types of platinum adducts not allowed in linear DNA could appear in supercoiled DNA. These expectations are corroborated by recent observations demonstrating that monofunctional cisplatin adducts in negatively supercoiled DNA are converted to bifunctional lesions more readily than in relaxed DNA (45). Also, interestingly, a novel adduct of cisplatin in the sequence 5′-TACT-3′, capable of terminating DNA synthesis, has been found in DNA from cisplatin-treated eukaryotic cells. Nevertheless, DNA ICLs of cisplatin have also been considered lesions relevant to the biological effects of this drug (see for instance 12–16), but their relative efficacy remains unknown. For example, recent results (13,17) suggest that there is a selective increase in gene-specific ICL repair in cisplatin-resistant human ovarian cells. Thus, formation of DNA ICLs of cisplatin is challenging to understand, not only from a mechanistic view but also from the therapeutic one. Therefore, it is not surprising that the nature of the DNA ICLs of platinum complexes and the biological significance of these lesions are being intensively investigated at present (see for example 18–33).

The formation of cisplatin adducts in DNA can be affected by its conformation (34). One of the important factors that influences the conformation of DNA is its global topology (35,36). DNA in vivo is generally negatively supercoiled in both eukaryotic and prokaryotic cells (for general reviews see 35–37). Whereas a significant portion of bacterial DNA supercoils is thought to be unrestrained inside the cell, the superhelicity of eukaryotic DNA is attributed to wrapping into nucleosomes. In addition, RNA polymerase transiently changes the local level of DNA supercoiling as it transcribes, also generating positive supercoiling. There are well-documented examples of the stabilization of alternative DNA conformations by supercoiling (35–38). Interestingly, it was recently shown (39) that cisplatin toxicity is influenced by DNA supercoiling in prokaryotic cells.

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cells, but not in linear DNA modified by this drug in cell-free medium (46).

Here we present experiments which were carried out in cell-free medium to investigate the effect of negative supercoiling on DNA interstrand cross-linking by cisplatin. We find that superhelical torsion can affect interstrand cross-linking efficiency of this anticancer drug in plasmid DNA.

**MATERIALS AND METHODS**

**Starting materials**

Cisplatin was synthesized and characterized by Lachema (Brno, Czech Republic). cis-[Pt(NH₃)₂(H₂O)Cl]⁺ was generated from cisplatin as described previously (18). Ultrapure acrylamide, bis(acrylamide) and urea were from Merck. The CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent DNA polymerase, the Klenow fragment of DNA polymerase I, restriction endonucleases and calf thymus DNA topoisomerase I were purchased from BioLabs. T4 polynucleotide kinase was from Boehringer. If not stated otherwise, the enzymes were used employing the buffers and other experimental conditions recommended by the manufacturer. The primer 5′-d(TAATACGACT- CACTATAGGG)-3′ was from BioVendor (Brno, Czech Republic). The radioactive products were from Amersham. Plasmid pSP73 (2464 bp) was prepared as described earlier (47). The sample of the native plasmid prepared in this laboratory contained >95% negatively supercoiled and <5% relaxed forms. Under the present experimental conditions, superhelical density ς of this naturally supercoiled DNA was −0.063, calculated on the basis of the data on DNA unwinding by cisplatin (48). Plasmid pSP73 DNA was γ-irradiated at a concentration of 40 µg/ml in 5 mM Tris–HCl, 0.5 mM EDTA, pH 7.8, using a cobalt source; other conditions were as described earlier (49,50). The nicked DNA samples were subjected to electrophoresis on 1.5% agarose gels run at 25°C in the dark with TBE buffer with the voltage set at 30 V. The gels were then stained with ethidium bromide and were examined on Polaroid 667 film using a transilluminator.

**Preparation of DNA molecules of varying superhelical density**

DNA topoisomers were prepared by relaxation of plasmid pSP73 in the presence of ethidium bromide or netropsin (45,51). Twelve micrograms of plasmid were incubated in the dark with 24 U DNA topoisomerase I in 72 mM KCl, 35 mM Tris–HCl, pH 8.0, 5 mM MgCl₂, 5 mM dithiothreitol, 2 mM spermidine, 5% glycerol in a final volume of 300 µl also containing different amounts of ethidium bromide (0–30 mM) or netropsin (15–60 mM). The mixtures were incubated for 90 min at 37°C. The reactions were terminated by adjusting the concentration of EDTA to 10 mM. The mean linking number (ΔLk) for each negatively supercoiled DNA preparation was determined with respect to relaxed DNA using a series of chloroquine gels (52). The superhelical density, ς, was calculated (53) as 10.4 × ΔLk/N, where N is the number of base pairs in the plasmid (2464 for pSP73).

**Platination reactions**

Supercoiled, relaxed or linearized forms of pSP73 DNA were modified by cisplatin in 10 mM NaClO₃ at 37°C in the dark for 48 h if not stated otherwise. The ςₜ values in these samples were determined by flameless atomic absorption spectrophotometry (FAAS) using a Unicam 939 AA spectrometer with graphite furnace.

**Immunochemical analysis**

Antibodies designated AB_inter were elicited against poly(dG–dc)poly(dG–dc) modified by cisplatin at ςₜ = 0.08 in 10 mM NaClO₃ for 48 h at 37°C (28,53). It was shown in the same way as in the recent paper of Malinge et al. (28) that these antibodies did not recognize platinum residues, DNA intrastrand or monofunctional adducts of cisplatin and that recognition is specific for DNA ICLs of cisplatin. The procedures for immunoenzymatic analysis of the antibodies and enzyme-linked immunosorbent assay (ELISA) were also as described recently (55,56).

**Sodium cyanide assay**

These experiments were performed in the same way as described in a recent paper (47). Briefly, 6.4 µg plasmid pSP73 containing cisplatin adducts and linearized by EcoRI before or after the platination reaction (vide infra) were treated with 0.2 M NaCN, pH 8.3, for 4 h at 37°C in the dark (the stock solution of 1 M NaCN was prepared in 0.1 M Tris–HCl and the pH was adjusted to 8.3 with 12 M HCl). The final volume of this reaction was 0.5 ml. After 4 h the samples were precipitated with ethanol, the pellets dissolved in 0.1 M HNO₃ and analyzed for platinum content by FAAS.

**Interstrand cross-link assay**

If not stated otherwise, cisplatin at varying concentrations was incubated with 2 µg supercoiled pSP73 DNA or with this plasmid linearized by EcoRI. The samples were then precipitated with ethanol and the circular DNA already modified by cisplatin was subsequently linearized by EcoRI. The linear duplexes were then analyzed for DNA ICLs in the same way as described in several recent papers (18,25,47,57,58). The linear duplexes were first 3′-end-labeled by means of the Klenow fragment of DNA polymerase I and [α-³²P]dATP. The samples were deproteinized with phenol, precipitated with ethanol and the pellet dissolved in 18 µl 30 mM NaOH, 1 mM EDTA, 6.6% sucrose, 0.04% bromphenol blue. The number of ICLs was analyzed by electrophoresis under denaturing conditions (18,47) on alkaline agarose gels (1%). After electrophoresis was completed the bands corresponding to single-stranded DNA and interstrand cross-linked duplex were cut out and their radioactivity quantified on a LKB Wallac 1410 Beta spectrometer (Finland).

**Sequence specificity of cisplatin–DNA adducts**

Replication mapping of lesions induced by cisplatin in plasmid pSP73 DNA was conducted in the way described in detail in a recent paper (58). Control or platinated double-stranded DNAs were used as the templates. The platinated templates were prepared by modification by cisplatin at ςₜ = 0.005 of either supercoiled pSP73 DNA (ςₜ = −0.05) or pSP73 DNA linearized (before the platination reaction) by NdeI. Supercooled control (non-platinated) or platinated supercoiled DNAs were cleaved by the two endonucleases NdeI and HpaII, while DNA platinated in linearized form was, after the platination reaction, also cleaved by HpaII. The resulting (NdeI/HpaII) fragments were deproteinized and the platinated fragments treated with 0.2 M NaCN in 20 mM
Tris–HCl, pH 8.3, for 4 h at 37°C, if not stated otherwise. The 221 bp fragments with and without ICLs were purified using 2% denaturing agarose gels and used in the mapping experiments (18). The 221 bp fragments used in the mapping experiments were also subjected to restriction analysis using BglII, ClaI or HindIII. The cleavage products of the (Ndel/HpaI) fragment were analyzed by electrophoresis on non-denaturing 15% polyacrylamide [mono:bis (acrylamide) ratio 29:1] gels running at 25°C in the dark with TBE buffer with the voltage set at 45 V. The gels were then stained with ethidium bromide and were examined on Polaroid 667 film with a transilluminator.

**RESULTS**

The initial experiments of the present work were carried out to compare the numbers of ICLs formed by cisplatin in supercoiled and linear DNAs. In these experiments, we used plasmid pSP73 (2464 bp, native supercoil density σ = −0.063) which was modified by cisplatin in two ways. One series of the samples was prepared by modifying the plasmid only after it had been linearized by EcoRI (EcoRI cuts only once within plasmid pSP73). The samples of the other series were prepared by modification of the supercoiled plasmid by cisplatin and only after the platination reaction was complete was the plasmid linearized by EcoRI. Thus, we prepared two types of linear DNA molecules of the same length and nucleotide sequence modified by cisplatin which could be differently affected by this drug if different DNA topology during the platination reaction plays a role. The two samples were analyzed for ICLs by different techniques in order to reveal these differences. It was also verified by means of FAAS that the amount of cisplatin coordinated to the base residues in DNA was independent of the DNA topology during the platination reaction in a broad range of r1 values (0.0001–0.08) (r1 is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA) and at any reaction time. Importantly, the modification was complete (i.e. all platinum was bound) at these r1 values within 48 h.

**Immunochromical analysis**

We prepared and characterized antibodies which bind selectively to ICLs formed by cisplatin in DNA (AB\textsubscript{inter}) in the same way as in the work of Malinge et al. (28). These antibodies do not recognize platinum residues, intrastrand cross-links or monofunctional DNA adducts of cisplatin. We used AB\textsubscript{inter} to show whether linearized pSP73 DNA, which was modified by cisplatin either in the linear or native supercoiled form, competitively inhibits binding of these antibodies to their immunogen in an ELISA. The competitive inhibition was evaluated in terms of the inhibitor concentration at 50% inhibition of the antibodies (IC\textsubscript{50}). As shown in Figure 1, AB\textsubscript{inter} recognized the DNA modified by cisplatin in the supercoiled form better than in the linearized form. The difference in recognition was increased with decreasing level of modification. The results of this analysis (Fig. 1) are consistent with the idea and support the hypothesis that at relatively low levels of modification by cisplatin (r1 < 0.001) ICLs are formed in supercoiled DNA with a higher frequency (the percentage fraction of platination events resulting in ICLs) than in linear DNA.

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**Table 1.** Stability of the adducts formed by cisplatin in supercoiled or linearized pSP73 plasmid in the presence of NaCN\textsuperscript{a}

<table>
<thead>
<tr>
<th>r\textsubscript{b} × 10\textsuperscript{4}</th>
<th>Pt content\textsuperscript{b} (pmol)</th>
<th>SC\textsubscript{NaCN}\textsuperscript{c}</th>
<th>LIN\textsubscript{NaCN}\textsuperscript{d}</th>
<th>Fraction of stable Pt adducts\textsuperscript{e} (%)</th>
<th>SC\textsubscript{NaCN}\textsuperscript{f}</th>
<th>LIN\textsubscript{NaCN}\textsuperscript{f}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>40</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>28</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>1.3 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>20.0</td>
<td>2.4 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}pH 8.3, 37°C; for other details see text.
\textsuperscript{b}r\textsubscript{b} before NaCN treatment.
\textsuperscript{c}The amount of DNA modified by cisplatin was 6.4 µg.
\textsuperscript{d}The fraction of cisplatin adducts resistant to the NaCN treatment.
\textsuperscript{e}Platinum content before NaCN treatment.
\textsuperscript{f}DNA was modified in supercoiled form, linearized and treated with NaCN. The values represent mean ± SEM of four determinations from two independent experiments.

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**Figure 1.** Competitive inhibition in an ELISA of AB\textsubscript{inter} binding to plasmid pSP73 linearized by EcoRI and containing cisplatin adducts. Δ. The competitor was DNA linearized before incubation with cisplatin with r\textsubscript{b} values indicated in the graph. □. The competitor was naturally supercoiled DNA linearized after incubation with cisplatin with r\textsubscript{b} values indicated in the graph. IC\textsubscript{50} is defined as the competitor concentration at 50% inhibition; relative IC\textsubscript{50} is an IC\textsubscript{50} value divided by the IC\textsubscript{50} obtained for DNA modified by cisplatin in linear form at r\textsubscript{b} = 0.001. Data points measured in triplicate varied on average ±3% from the mean.
NaCN treatment

Most of the cisplatin molecules bound to DNA can be removed by the action of cyanide ions. It has been shown (47) that at pH 8.3 and 37°C treatment with NaCN at a concentration of 0.2 M for 4 h can effectively remove ~90% of 1,2-intrastrand cross-links of cisplatin from double-helical DNA, whereas ICLs are more stable under these conditions (only ~15% of all ICLs are removed). The samples of linearized plasmid containing adducts of cisplatin formed when the DNA was either in supercoiled or in linearized form were treated with 0.2 M NaCN at pH 8.3 and 37°C for 4 h in the dark. After this treatment, the content of platinum was determined in these samples by FAAS (Table 1). More than 90% of the platinum was removed from DNA modified in the linear form. In contrast, significantly less platinum was removed from DNA modified in the supercoiled form (and subsequently linearized) and the fraction of platinum not extracted with CN− increased with decreasing level of the initial modification (ρn). These results are consistent with the immunochemical investigations (Fig. 1), i.e. they also indicate that: (i) more ICLs were formed in negatively supercoiled than in linear DNA; (ii) at relatively low levels of platination (ρn < 0.001) the frequency of ICLs formed by cisplatin in supercoiled DNA was enhanced with decreasing level of DNA modification.

DNA interstrand cross-linking

A method for precise and quantitative determination of interstrand cross-linking by cisplatin in DNA has been previously described (18,25,47,57,58). Upon electrophoresis under denaturing conditions, 3′-end-labeled strands of linearized plasmid pSP73 containing no ICLs migrate as a 2464 base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand cross-linked fragments were noticed if cisplatin was used to modify DNA in both the linearized and supercoiled form (and subsequently linearized) and the fraction of platinum not extracted with CN− increased with decreasing level of the initial modification (ρn). The bands corresponding to more slowly migrating interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand cross-linked fragments were noticed if cisplatin was used to modify DNA in both the linearized and supercoiled forms at ρn as low as 1 × 10−4 (Fig. 2A and B). The intensity of the more slowly migrating band increased with growing level of modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The frequency of ICLs was determined in these samples by FAAS (Table 1). More than 90% of 1,2-intrastrand cross-links of cisplatin from double-helical DNA, whereas ICLs are more stable under these conditions (only ~15% of all ICLs are removed). The samples of linearized plasmid containing adducts of cisplatin formed when the DNA was either in supercoiled or in linearized form were treated with 0.2 M NaCN at pH 8.3 and 37°C for 4 h in the dark. After this treatment, the content of platinum was determined in these samples by FAAS (Table 1). More than 90% of the platinum was removed from DNA modified in the linear form. In contrast, significantly less platinum was removed from DNA modified in the supercoiled form (and subsequently linearized) and the fraction of platinum not extracted with CN− increased with decreasing level of the initial modification (ρn). These results are consistent with the immunochemical investigations (Fig. 1), i.e. they also indicate that: (i) more ICLs were formed in negatively supercoiled than in linear DNA; (ii) at relatively low levels of platination (ρn < 0.001) the frequency of ICLs formed by cisplatin in supercoiled DNA was enhanced with decreasing level of DNA modification.

The kinetics of interstrand cross-linking in naturally supercoiled and linearized DNA by cisplatin at 37°C was also examined. DNA was mixed with cis-[Pt(NH3)2(H2O)Cl]2+ at ρn = 0.001 in 10 mM NaClO4. Samples were withdrawn from this mixture at various time intervals and immediately cooled to ~70°C. The rates of ICL formation were estimated from gel electrophoresis experiments as described above. The platinum binding studies confirmed that all cis-[Pt(NH3)2(H2O)Cl]2+ molecules were bound to supercoiled or linearized DNAs in < 20 min (47). As shown in Figure 2D, interstrand cross-linking by cisplatin in both samples of DNA was complete within 48 h. The dependence of the frequency of ICLs on time reached a plateau within 48 h of the cross-linking reaction. The half-time of the interstrand cross-linking (t1/2) in linearized DNA was ~4–8 h, whereas the kinetics of ICL formation in supercoiled DNA was faster (t1/2 ~ 2 h). The maximum frequency of ICLs attained after 48 h was ~6% for interstrand cross-linking in linearized DNA, whereas ~12% of ICLs were formed in supercoiled DNA under identical conditions. Plasmid pSP73 was also used to prepare DNAs with different superhelical density. Negatively supercoiled plasmid DNAs with different ΔΛk were modified by cisplatin to ρn = 0.0001 for 48 h, linearized by EcoRI, 3′-end-labeled and analyzed on denaturing agarose gel for ICLs as described above. As shown in Figure 3, the yield of ICLs was enhanced with increasing level of global negative supercoiling. The number of ICLs formed in relaxed DNA was almost identical to that formed under identical conditions in linearized DNA (cf. Figs 2 and 3).

All topoisomers modified by cisplatin at ρn = 0.001 and 0.01 were also analyzed in % native agarose gels. No new, more slowly migrating bands were observed, which would indicate the formation of cross-links between DNA strands belonging to different closed circular duplexes.

There is a theoretical possibility that cisplatin could also form ICLs in supercoiled DNA between nucleotide residues separated along the DNA contour by a longer segment of double-stranded DNA. The two distant binding sites in linear or relaxed DNAs could be brought close together in supercoiled DNA so that they could be cross-linked by cisplatin. This could take place because
DNA in this topological configuration appears as a compact molecule in which the DNA helix has wound about itself. If such long-range ICLs were formed in supercoiled DNA, they would result in loops in DNA molecules closed by the interstrand adducts of cisplatin. It is reasonable to expect that the formation of such covalently closed loops would prevent complete relaxation of the supertwisted DNA readily attainable in the unmodified plasmid and induced, for instance, by DNA nicking. Thus, the introduction of a nick into supercoiled DNA containing long-range ICLs between formally distant DNA binding sites should result in formation of more compact DNA molecules in comparison with those of fully relaxed circular DNA which was not modified. It is also reasonable to expect that these more compact nicked DNA molecules (containing long-range ICLs) would migrate more rapidly in a non-denaturing agarose gel than entirely relaxed DNA molecules without long-range ICLs.

In order to test this eventuality, the negatively supercoiled plasmid pSP73 (σ = −0.05) was modified by cisplatin for 48 h at \( r_\gamma = 0.008 \) and subsequently γ-irradiated at a dose of 45 Gy under conditions described previously (50). This dose was a threshold one just sufficient to relax all supertwisted molecules in the sample of unmodified supercoiled DNA. The average size of loops which could be closed by eventual ICLs formed between formally distant sites in pSP73 DNA (σ = −0.05) can be approximately deduced from the fact that there was −1 negative turn for every 200 bp in this plasmid molecule. The irradiation of both modified and non-platinated supercoiled DNAs resulted in a complete disappearance of the band in the non-denaturing 1.5% agarose gel corresponding to the supercoiled DNA and in the occurrence of a new, radically more slowly migrating band corresponding to relaxed DNA (Fig. 4, lane 5). Importantly, all molecules of supercoiled DNA interstrand cross-linked by cisplatin were converted by γ-irradiation into nicked DNA which co-migrated in the agarose gel with relaxed unmodified DNA (cf. lanes 3 and 5 in Fig. 4). Thus, these results are consistent with the view that ICLs of cisplatin in negatively supercoiled DNA are not extensively formed between formally distant sites which could be brought close together when DNA becomes supercoiled.

**Sequence preference of DNA interstrand cross-linking**

It has been shown (47) that in linear DNA cisplatin preferentially forms ICLs between guanine residues in 5'-GC-3' base pairs. The results of the present paper (Figs 1–3 and Table 1) indicate that ICLs of cisplatin can be formed in negatively supercoiled DNA with a higher frequency than in linear or relaxed DNAs. It could be suggested, as one possible explanation of this phenomenon, that cisplatin forms ICLs in negatively supercoiled DNA at more sites and/or different sequences than in linear DNA. In order to test this hypothesis, we prepared a 221 bp (NdeI/HpaI) restriction fragment which was isolated from DNA modified by cisplatin either in the supercoiled (σ = −0.05) or the linear form. These fragments modified by cisplatin (at \( r_\gamma = 0.005 \)) were subsequently incubated in 0.2 M NaCN, pH 8.3, to remove ~90% of the intrastrand cross-links, leaving ~85% ICLs (47; Table 1). The DNA fragments containing ICLs were then separated from those containing no ICLs.

It has been suggested in this work (Fig. 4) that extensive formation of long-range ICLs of cisplatin due to negative supercoiling is unlikely. In order to further support this suggestion, specifically for the (NdeI/HpaI) fragment containing the ICLs formed by cisplatin in negatively supercoiled DNA and used in the mapping experiments, this fragment was further cleaved by other restriction enzymes, namely BglIII, ClaI or HindIII. All these endonucleases cut only once within the (NdeI/HpaI) fragment. Cleavage of the unmodified fragment by BglIII, ClaI or HindIII yields 89 with 132, 102 with 119 or 158 with 63 bp fragments respectively. The expected average size of loops closed by long-range ICLs formed in pSP73 DNA (σ = −0.05) was estimated to be −200 bp. Digestion of the (NdeI/HpaI) fragment containing a long-range ICL between sites separated by −200 bp by any of BglIII, ClaI or HindIII should thus produce markedly shorter fragments, which would, however, remain cross-linked.
Figure 5. Electrophoresis in a non-denaturing 15% polyacrylamide gel of the (NdeI/HpaI) restriction fragment from plasmid pSP73. Lane 1, unmodified (control) fragment; lane 2, the fragment containing the ICLs formed by cisplatin in supercoiled plasmid (\( \sigma = -0.05 \)) at \( r_b = 0.005 \) (for further details see text); lane 3, the same sample as in lane 1 cleaved by Clal; lane 4, the same sample as in lane 2 cleaved by Clal.

The electrophoretic mobility of these cross-linked fragments in 15% non-denaturing polyacrylamide gels should be approximately identical to that of the uncleaved (NdeI/HpaI) fragment and it should be radically lower than that of the fragments obtained by the same cleavage of the non-platinated (control) (NdeI/HpaI) fragment. The (NdeI/HpaI) fragments, control (non-platinated) sample and the sample used in the mapping experiments containing the ICL formed in negatively supercoiled DNA (from which the fragments containing no ICL were removed, \( \text{vide supra} \) were cleaved under identical conditions by either BglII, Clal or HindIII. The products of digestion of the interstrand cross-linked fragment were analyzed by electrophoresis in 15% non-denaturing polyacrylamide gels. They co-migrated with the fragments obtained by digestion of the control, non-platinated (NdeI/HpaI) fragment, i.e. they migrated markedly faster than the undigested (NdeI/HpaI) fragments (shown for digestion by Clal in Fig. 5). Also, importantly, digestion of the interstrand cross-linked fragment was complete, leaving no material which would co-migrate with the undigested 221 bp fragment.

Replication mapping involved extension by VentR DNA polymerase at the 3'-'end of the radioactively 5'-'end-labeled primer up to the nucleotide residue involved in the metal adduct on the template strand (1,58). Using thermal cycling (58), this process was repeated many times in order to amplify the signal. The products of this linear amplification were then examined on DNA sequencing gels and the sequence specificity of cisplatin adduct formation was determined.

No intense bands indicating premature termination of DNA synthesis were observed if the templates containing no ICL were used. On the other hand, DNA synthesis on the templates containing ICLs produced DNA fragments migrating as intense bands, which corresponded to termination sites of DNA synthesis exclusively at guanine residues. One termination site at the guanine residue at position 32 in a 5'-d(GC)-3' sequence was only observed in the case of the template isolated from DNA platinated in the linear form (Fig. 6A, lane ICL linear, and Fig. 6B).

Importantly, the identical result (i.e. only a single termination site at the same position) was obtained if ICLs formed by cisplatin in the same (linearized) (NdeI/HpaI) fragment were mapped by

Figure 6. (A) Autoradiogram of a 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by Ventq DNA polymerase on the cisplatin-modified (NdeI/HpaI) restriction fragment of plasmid pSP73. The gel contained the linear amplification products of the fragment which was obtained from the plasmid modified by cisplatin at \( r_b = 0.005 \) either in negatively supercoiled (\( \sigma = -0.05 \)) (lane ICL super) or in linear (DNA linearized by NdeI; lane ICL linear) form. After reaction with cisplatin the platinated DNAs were incubated for 4 h in 0.2 M NaCN with 20 mM Tris–HCl, pH 8.3, and the fragments with ICLs purified using a denaturing agarose gel. Lane Control, unmodified template; lanes T, C, A and G, chain terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand). The numbers correspond to the nucleotide sequence numbering of (B). (B) A diagram showing the portion of the base sequence of plasmid pSP73 used to monitor inhibition of DNA synthesis on the template containing ICLs of cisplatin. The bold sequence designates the 5'-end-labeled primer and the star indicates its 5'-end-labeling. The arrow indicates the start site of the Ventq DNA polymerase and the direction of synthesis. and represent stop signals from (A) lanes ICL super and ICL linear respectively. The numbering of the nucleotides in this scheme corresponds to the numbering of the nucleotides in the pSP73 nucleotide sequence map.
transcription assay using T7 RNA polymerase (47). In contrast, several termination sites at guanine residues in both 5'-d(GC)-3' (positions 32 and 83) and 5'-d(CG)-3' (positions 36 and 2447) sequences were observed for DNA synthesis on the template containing ICLs formed when DNA was in the supercoiled form (Fig. 6A, lane ICL super, and Fig. 6B).

**DISCUSSION**

The results of this work demonstrate that DNA ICLs of cisplatin can be formed at low levels of modification (\( n_\text{f} < 0.001 \)) in cell-free medium with a considerably higher frequency in negatively supercoiled DNA in comparison with relaxed or linear DNAs. The frequency of ICLs in supercoiled DNA increases with decreasing level of platination. At low levels of modification at 37°C, corresponding to \( n_\text{f} \sim 10^{-4} \), after a modification reaction lasting 48 h the frequency of ICLs of cisplatin in naturally supercoiled plasmid DNA was 30–40% of all adducts and approximately three times higher than in linear DNA (Fig. 2C).

In addition, the kinetics of ICL formation in supercoiled DNA are markedly faster than in linear DNA, which suggests that negative supercoiling makes formation of ICLs of cisplatin easier.

Major structural reorganizations of B-DNA due to bifunctional lesions of cisplatin involve bending and unwinding (4,5,23,26,40,42). There is no radical difference in bending induced by intrastrand cross-links and ICLs [32–35° for intrastrand adducts (59,60) and 20–45° for ICLs (23,28)]. In contrast, the unwinding induced by ICLs of cisplatin is \( \sim 90° \) (23,28), which is noticeably more than that induced by intrastrand cross-links (13 or 23° for 1,2,1.3-intrastrand adducts respectively; 40). It has been shown that the free energy of the negatively supercoiled molecule can drive reactions resulting in unwinding of the DNA double helix (36). Thus, a higher frequency of ICLs of cisplatin in negatively supercoiled DNA is consistent with the fact that among reactions of cisplatin with DNA that which results in ICLs requires the greatest extent of DNA unwinding.

A change in the topology of DNA could affect not only the frequency of formation of the individual types of cisplatin adducts, but also their preferences for particular base sequences in DNA. The analysis of binding sites revealed (Fig. 6) that ICLs were preferentially formed by cisplatin in both negatively supercoiled and linear DNA at guanine residues and that these residues were always contained in a GC base pair flanked by another GC pair. This result is consistent with the view that ICLs formed by cisplatin in negatively supercoiled DNA are preferentially formed between guanine residues in neighboring base pairs. In addition, the results of the mapping studies (Fig. 6) can also be interpreted to mean that in negatively supercoiled DNA ICLs are formed by cisplatin between guanine residues in sequences containing both 5′-d(GC)-3′ and 5′-d(CG)-3′ sites. This is in contrast to cisplatin modification of linear DNA, which results in ICLs between neighboring guanine residues only in 5′-d(GC)-3′ sites (47).

The results of replication mapping studies (Fig. 6B) suggest that in negatively supercoiled DNA ICLs of cisplatin are not formed at all 5′-d(GC)-3′ and 5′-d(CG)-3′ sites. This non-random distribution of DNA binding sites at which cisplatin can form ICLs has not been investigated in detail and will be addressed in future work. At present we only speculate that this non-random distribution might be a consequence of the fact that the formation of ICLs of cisplatin is affected by the base sequences flanking the 5′-d(GC)-3′ and 5′-d(CG)-3′ sites. In addition, \( \sim 15% \) of ICLs were removed from the template DNA fragment during treatment with NaCN, which might also contribute to the non-random distribution of termination sites due to ICLs. Thus, our results do not exclude the possibility that a small fraction of ICLs of cisplatin can also be formed at other sites in negatively supercoiled DNA than at those described in this work.

DNA topology is a significant factor in a number of genetic functions. Further investigation of the effect of DNA topology on the formation of adducts of cisplatin is warranted to define their role in the mechanism of toxicity of this drug. In addition, the fact that the rate of formation of DNA ICLs of cisplatin is dependent on the negative superhelical density could provide a basis of a new assay for probing unrestrained tension in the winding of the DNA double helix both in vitro and in living cells (53,61,62).

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