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Interstrand cross-links are preferentially formed at the d(GC) sites in the reaction between *cis*-diamminedichloroplatinum(II) and DNA

(T7 and SP6 RNA polymerases/dimethyl sulfate)

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ABSTRACT **A DNA restriction fragment with convergent SP6 and T7 promoters has undergone reaction with *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and was then used as a template for RNA synthesis *in vitro*. The T7 and SP6 RNA polymerases generate fragments of defined sizes. Analysis of the RNA fragments shows that the polymerases are mainly blocked at the level of the d(GG) and d(AG) sites and to a lesser extent at the level of the d(GC) sites. The adducts at the d(GC) sites are more resistant to cyanide ion attack than those at the major sites and are identified as interstrand cross-links. The formation of an interstrand cross-link between the N-7 atoms of two guanine residues at the d(GC) sites was further confirmed by chemical modifications.**

cis-Diamminedichloroplatinum(II) (*cis*-DDP) is an antitumor agent of major clinical importance. Much evidence suggests that the cytotoxic action of the drug is related to its ability to react with DNA even though the mechanism of action is still unknown (for general reviews, see refs. 1–5 and references therein). Like many chemicals used in cancer chemotherapy, *cis*-DDP is a bifunctional agent that forms *in vivo* and *in vitro* intrastrand and interstrand cross-links on DNA. The major lesions are d(GG) and d(AG) 1,2-intrastrand cross-links, representing 65% and 25% of the total adducts, respectively (1–5). Although the interstrand cross-links represent a minor portion (<10% of total adducts), several experiments in tissue culture systems have correlated the DNA interstrand cross-linking reaction with cytotoxicity (for general reviews, see refs. 6 and 7).

DNA interstrand cross-linking occurs predominantly between two guanine N-7 atoms on opposite strands (2, 8, 9). A distance of ≈3 Å is required for the *cis*-DDP cross-linking reaction (10, 11). Thus, two adjacent guanine residues on the opposite strands, either in the 5′-CG-3′ or 5′-GC-3′ sequences, are the most probable reaction sites on DNA. However, in both cases, formation of the cross-link in B-DNA implies a large distortion of the double helix since the two N-7 atoms in d(CG) and d(GC) sequences are separated by about 9 and 7 Å (12), respectively. Recently, manipulation of three-dimensional molecular models inferred that the d(CG) sequence is more able to match the interstrand cross-linking requirement (2, 8).

In vitro studies have shown that DNA synthesis by DNA polymerases of different origins acting on *cis*-DDP-modified DNAs was arrested at the level of the adducts (1–5, 13–16). This finding has been extensively used to map the sites of platination on DNA. However, in these studies, the interstrand adducts were not detected because either the platinated DNA was single stranded or the assay was not sensitive enough.

We show here that the *in vitro* RNA synthesis by bacteriophage RNA polymerase acting on platinated DNA is blocked at the level of the adducts. The ability of the adducts to terminate transcription has been used to map the *cis*-DDP binding sites on a double-stranded DNA. Furthermore, analysis of the modification patterns shows that the interstrand cross-links are preferentially formed at the d(GC) sequences.

MATERIALS AND METHODS

T7 and SP6 RNA polymerases, pSP73 vector, ribonucleoside triphosphates, and RNasin ribonuclease inhibitor were bought from Promega. 3′-Deoxynucleoside triphosphates were bought from Bethesda Research Laboratories. Restriction enzymes, alkaline phosphatase, Klenow polymerase, T4 polynucleotide kinase, and T4 DNA ligase were from Boehringer Mannheim and from Bethesda Research Laboratories. All the radioactive products were from Amersham. Electrophoresis-grade acrylamide, bis(acrylamide), and sodium cyanide were from Merck.

Plasmid pSP73KB is a derivative of plasmid pSP73 and was constructed as follows: pSP73 DNA was digested with endonucleases *Bam*HI and *Kpn* I. The 3′ end of the *Kpn* I site was recessed by T4 DNA polymerase under conditions in which the enzyme acted as a 3′ → 5′ exonuclease. After filling in the *Bam*HI site, the plasmid was blunt end-ligated with T4 DNA ligase and transformed in *Escherichia coli* HB101. The cloning was confirmed by restriction nuclease mapping and DNA sequencing.

The reactions between *cis*-DDP and linear DNAs were performed as described (17). The platinum contents of the samples were determined with an atomic absorption spectrophotometer by J. L. Butour (Toulouse). In some experiments, the bound *cis*-DDP residues were removed partly or totally from DNA by treatment with NaCN. Total removal was obtained by overnight incubation of the products in 0.2 M NaCN (basic pH) and at 45°C. Partial removal was obtained by incubation of the products in 0.2 M NaCN/20 mM Tris·HCl, adjusted to pH 8.3 by addition of HCl and at 37°C. In all these experiments, the concentration of cyanide ions was much larger than the concentration of platinum residues.

Transcription with SP6 or T7 RNA polymerases was performed according to the protocol recommended by Promega. For nucleotide sequence analysis, the reaction mixtures were supplemented with 3′-deoxynucleoside triphosphates according to the protocol of Axelrod and Kramer (18). The electrophoretic analysis of transcripts was done as described (18).

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Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); DMS, dimethyl sulfate.

RESULTS

Mapping of the Adducts. Cleavage of pSP73 DNA by the two endonucleases *Nde* I and *Hpa* I yielded a 221-base-pair (bp) fragment with convergent T7 and SP6 promoters. Thus, either strand can be used as a template for RNA synthesis *in vitro* (18, 19). As shown in Fig. 1, RNA synthesis by T7 or SP6 RNA polymerases using the restriction fragment platinated at r_b = 0.008 [where r_b is the drug/nucleotide ratio (mol/mol)] generates a population of RNA fragments of defined sizes. This indicates that RNA synthesis has been prematurely terminated. Sequence analysis of the fragments (Fig. 2) reveals that the main bands resulting from termination of RNA synthesis appear at the guanine residues (or half a nucleotide preceding these residues) in the $d(G)_n$ ($n \geq 2$) sequences and in all the d(AG) sequences but two (located at positions 71 and 95). This is consistent with the presence of major adducts at d(GG) and d(AG) sites, which prevent the polymerase from synthesizing further.

In addition to the intense bands, fainter bands are also present. Examples are the band at position 92, suggesting a d(GA) adduct, the bands between positions 126 and 195, suggesting interstrand adducts at d(GC) sites or intrastrand adducts in which the two platinated residues are separated by at least 1 nucleotide residue or even rare adducts. It seems unlikely that these faint bands correspond to monofunctional adducts since no arrest was observed with DNA modified by the monofunctional platinum derivative chlorodiethylenetriamineplatinum(II) (results not shown).

Resistance of Interstrand Adducts to Cyanide Ions. Under normal conditions, all the adducts are stable over a long period of time, whereas in the presence of cyanide ions, most of the bound platinum residues but not all of them are rapidly removed (20–22). Immunological analysis of platinated DNA after treatment with cyanide ions suggests a preferential removal of d(GG) and d(AG) adducts (23). The relative resistance of the interstrand adducts and of all the adducts to cyanide ions has been compared. The amount of total adducts was determined by titration of the bound platinum residues by atomic absorption spectrophotometry. The amount of interstrand adducts was deduced from gel electrophoresis experiments. In denaturing conditions, the platinated 221-bp restriction fragments with and without interstrand adducts are easily separated (Fig. 3). From the ratio of the intensities of the two bands, the amount of interstrand adducts was calculated, assuming one interstrand adduct per fragment. In favor of this assumption is the fact that similar kinetics were observed with samples platinated at lower r_b (data not shown). After 4 hr of incubation, ≈90% of the total

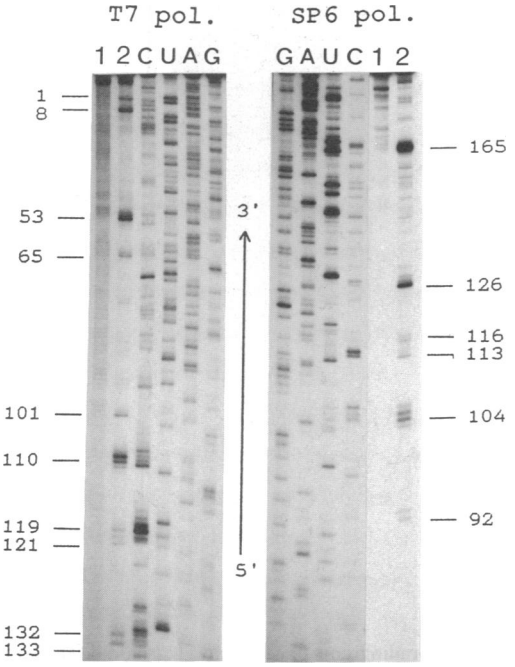

FIG. 1. Autoradiograms of 6% polyacrylamide/7 M urea sequencing gels showing inhibition of RNA synthesis on the *cis*-DDP-modified (*Nde* I/*Hpa* I) restriction fragments. (*Left*) Synthesis by T7 RNA polymerase. (*Right*) Synthesis by SP6 RNA polymerase. Lanes: 1, relative to the unplatinated samples; 2, to the platinated samples (the bands at the top of the gel corresponding to fully transcribed DNA are not shown); A, U, G, and C, relative to chain-terminated marker RNAs.

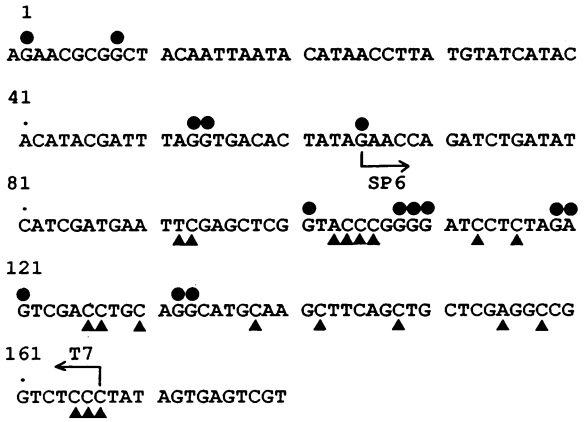

FIG. 2. Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by *cis*-DDP. Only the upper strand of the template is given. The arrows indicate the starts of the two polymerases. T7 RNA polymerase used as template the upper strand of DNA, and SP6 RNA polymerase used the lower strand. ● and ▲, Stop signals (from Fig. 1) for T7 RNA polymerase and SP6 RNA polymerase, respectively. Nucleotides 1 and 65 correspond, respectively, to nucleotides 2401 and 1 on the pSP73 nucleotide sequence map.

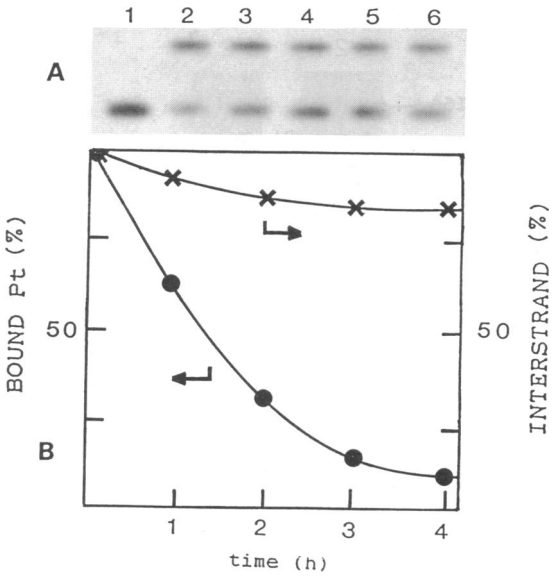

FIG. 3. Relative stability of the adducts in the presence of NaCN. (*A*) Autoradiogram of a denaturing 3% agarose gel of the unplatinated (*Nde* I/*Hpa* I) restriction fragment (lane 1) and of the corresponding platinated fragments after incubation in 0.2 M NaCN (pH 8.3) for 0, 1, 2, 3, and 4 hr (lanes 2–6, respectively). (*B*) Variation (%) versus time of the total amount of bound platinum residues (*Left*) (●) and of the amount of interstrand adducts (*Right*) (X). The DNA was modified by *cis*-DDP at r_b = 0.022; ≈55% of the fragments contained at least one interstrand adduct.

adducts and only ≈15% of the interstrand adducts are removed (Fig. 3).

Mapping of the Interstrand Adducts. The 221-bp restriction fragment was platinated at r_b = 0.022 and then incubated for 4 hr in 0.2 M NaCN (pH 8.3). The DNA fragments with and without interstrand cross-links were separated by gel electrophoresis in denaturing conditions. After neutralization of the gel, the two DNAs were eluted and then used as templates for RNA synthesis. In both cases (Fig. 4, lanes 2 and 3), faint bands reveal that a few intrastrand cross-links are not completely removed by cyanide ions [see sequences $d(G)_3$ (position 105), $d(G)_4$ (position 109), $d(G)_3$ (position 166)]. On the other hand, intense bands located at the d(GC) sites are only observed with the fragments having interstrand cross-links.

The 221-bp restriction fragment contains several d(CG) sites and none of them appears to be platinated. It cannot be excluded that interstrand cross-links were formed at the d(CG) sites but were not detected because of their instability in the presence of cyanide ions. However, our experiments are done with DNA having one interstrand cross-link per fragment and the NaCN treatment removes only 15% of these adducts. Therefore, the possible formation of interstrand adducts at the d(CG) sites should be a negligible event.

Our data show that in the reaction between DNA and *cis*-DDP, interstrand adducts are preferentially formed at the d(GC) sites. Although the bands at positions 142 and 148 [sequence d(AGCT)] are more intense than the bands at positions 130, 138, and 151 [sequences d(TGCA) and d(TGCT), respectively], analysis of several other platinated fragments seems necessary to firmly establish the role of the neighboring bases on the formation of the interstrand cross-links.

Chemical Reactivity of an Interstrand Adduct. Since it is not yet known how the two RNA polymerases are stopped by the adducts, it seemed to us important to reveal the interstrand adducts by another approach. We have studied the chemical reactivity of the adduct (position 97; Fig. 4) within the d(CGAGC) sequence with dimethyl sulfate (DMS) and formic acid, respectively. DMS is not expected to react with the platinated guanine residues (24), the N-7 being no longer accessible. It is known that platination decreases the acid-catalyzed depurination (25). A 43-bp (*Bgl* II/*Pst* I) restriction fragment with a unique interstrand adduct was purified by taking advantage of the fact that the adduct is located within the recognition site of the endonuclease *Sst* I. To make sure that the inhibition of the endonuclease was not due to intrastrand adducts still present after a cyanide reversal experiment within the d(CCCGGGG) sequence, the starting material was pSP73KB DNA, a derivative of pSP73 DNA in which the d(CCCGGGG) sequence has been deleted. The DNA was digested with the endonuclease *Bgl* II, platinated at r_b = 0.025, and then incubated in 0.2 M NaCN (pH 8.3) for 4 hr at 37°C. The DNA was cleaved with the endonucleases *Pst* I and *Sst* I and then the 43-bp fragment with the interstrand adduct was purified by gel electrophoresis in denaturing conditions. The fragment was 3′ or 5′ ^{32}P-end-labeled at the *Bgl* II site and then treated with DMS or formic acid (26). The results relative to the upper strand are shown in Fig. 5. After reaction with formic acid or with DMS and then treatment with piperidine, all the purine residues (lane 1) or all the guanine residues (lane 2) on the 5′ side of the *G residue within the d(CGA*GC) sequence are detected. The *G residue (not reactive) and the residues (reactive) on the 3′ side of the *G residue are not detected because the two strands of DNA are cross-linked. It has been verified that after reaction with DMS and then removal of the platinum residues by overnight incubation at 45°C in 0.2 M sodium

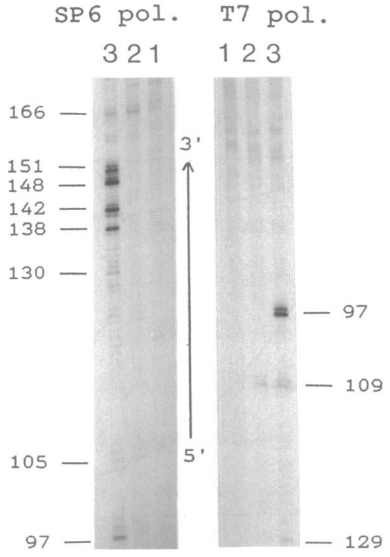

FIG. 4. Autoradiograms of 6% polyacrylamide/7 M urea sequencing gels showing inhibition of RNA synthesis on *cis*-DDP-modified (*Nde* I/*Hpa* I) fragments. After reaction with *cis*-DDP, the platinated DNA was incubated for 4 hr in 0.2 M NaCN/20 mM Tris·HCl, pH 8.3. The fragments with and without interstrand adducts were separated on a denaturing 3% agarose gel. Autoradiograms present synthesis by SP6 RNA polymerase (*Left*) and T7 RNA polymerase (*Right*). Lanes: 1, relative to the unplatinated DNA; 2, relative to the DNA without interstrand adducts; 3, relative to the DNA with interstrand adducts.

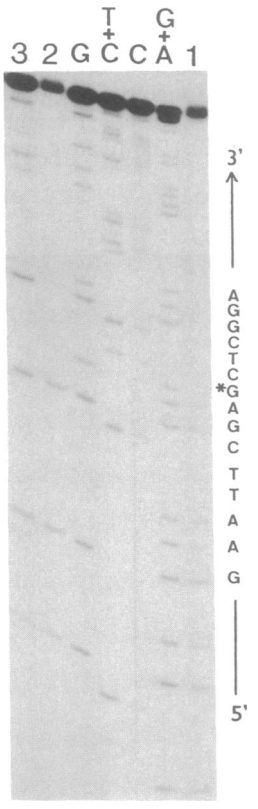

FIG. 5. Autoradiograms of a 15% polyacrylamide/7 M urea gel of the products of the reaction between DMS or formic acid and the platinated (*Bgl* II/*Pst* I) restriction fragments from pSP73KB DNA. The fragments are 5′ ^{32}P-end-labeled at the *Bgl* II site. Lanes: 1, fragments generated by the reaction with formic acid and then treatment with piperidine; 2, fragments generated by the reaction with DMS and then treatment with piperidine; 3, same fragments as in lane 2 but the bound platinum residues have been removed by cyanide ions; G, G+A, C, and T+C, Maxam–Gilbert-specific reactions for the unplatinated (*Bgl* II/*Pst* I) restriction fragments.

cyanide (pH 10) all the guanine residues except the *G residue (lane 3) are revealed. Similar results were obtained with the lower strand (data not shown), which confirms formation of the interstrand adduct at the d(GC) site within the d(CGAGC) sequence.

DISCUSSION

The present study shows that the adducts formed in the reaction between *cis*-DDP and DNA inhibit RNA synthesis *in vitro*. We used this premature termination of transcription to characterize the preferred sites for interstrand cross-link formation.

Analysis of the RNA fragments generated by SP6 and T7 RNA polymerases using platinated double-stranded DNA as template suggests that *cis*-DDP reacts predominantly at d(GG) and d(AG) sites and to a lesser extent at other sites, in agreement with previous results (1–5). All the potential d(GG) sites and all the potential d(AG) sites but two are reactive with *cis*-DDP. The T7 RNA polymerase is not stopped at the d(AG) site within the sequence d(CGAGC) (position 97). We previously reported (27, 28) that the d(AG) adducts within the sequences d(YGAGC) (where Y is pyrimidine), if formed, were not detected by the 3′ → 5′ exonuclease activity of T4 DNA polymerase. Taken together, these results suggest that within the d(YGAGC) sequences, the formation of the d(AG) adducts is unlikely.

Termination of RNA synthesis was also observed at the d(GC) sites and could be due to formation of either interstrand adducts or intrastrand adducts in which the two cross-linked nucleotide residues are separated by at least 1 nucleotide residue. A direct use of the transcription assay was unable to distinguish between the two possibilities. We then found a way to isolate DNA fragments containing mostly interstrand cross-links. Our finding is that the interstrand cross-links are more resistant to cyanide attack than the major intrastrand adducts. The difference in kinetics can be due to a different protective effect of the exocyclic oxygens of the platinated bases (29, 30), presumably because the adducts do not have the same conformation and/or the local conformation of DNA is different (22, 31).

After platination, the DNA is first incubated with NaCN, and then the fragments with and without interstrand adducts are isolated by gel electrophoresis in denaturing conditions. Comparison between the patterns of RNA fragments obtained using templates with and without interstrand adducts shows two main points: (*i*) Most of the major intrastrand adducts have been removed by cyanide ions; the more stable are those within the $d(G)_n$ ($n \geq 2$) sequences. (*ii*) Intense termination bands are only observed in the case of the template with interstrand adducts. The arrests are always located at the level of the d(GC) sites and never at the level of the d(CG) sites. Thus, we conclude that interstrand cross-links are preferentially formed at the d(GC) sites.

This has been confirmed by a chemical study of the interstrand adduct within the d(CGAGC) sequence. The guanine residues at the d(GC) site are not reactive with DMS, which suggests that platination occurs at the N-7 position of these residues. Moreover, in acidic medium these residues are more stable than the other purine residues. It is known that platination decreases the acid-catalyzed depurination (25). Thus, both the chemical and the enzymatic approaches lead to the same conclusion.

It is generally argued that the mechanism of *cis*-DDP action is related to inhibition of DNA synthesis (1–5). This has been recently reevaluated (14, 32, 33). Sorenson and Eastman (32, 33) have proposed that arrest of cells in G_2 phase after incubation with *cis*-DDP is due to inability of the cells to transcribe genes required for passage into mitosis. Recently, it has been shown that transcription elongation by *E. coli* and wheat germ RNA polymerases, using a platinated DNA model as a template, is strongly inhibited by the adducts (34). These findings along with the results presented here support an *in vivo* action of *cis*-DDP at the transcriptional level.

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