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Reactions of the UVRABC excision nuclease with DNA damaged by diamminedichloroplatinum(II)

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ABSTRACT

Mutants of Escherichia coli, which are blocked in excision repair (uvrA6, uvrB5, or uvrC34) are exceptionally sensitive to the antitumor drug cis-Pt(II)$(NH_3)_2Cl_2$ (cis-DDP) but not to the trans isomer. Plasmid DNA, damaged by either the cis or trans compound and treated with the UVRABC excision nuclease was cut as shown by conversion of supercoiled DNA to relaxed forms. All three protein products of the uvrA, uvrB, and uvrC genes were required for incision. End-labeled fragments damaged with cis-DDP and reacted with the UVRABC nuclease were cut at the 8th phosphodiester bond 5' and at the 4th phosphodiester bond 3' to adjacent GG's. DNA treated with trans-DDP was not cut appreciably at adjacent GG's by the repair enzyme as subsequent analysis of reaction products after enzyme digestion gave a pattern similar to those obtained with control untreated fragments. The results indicate that the UVRABC nuclease may promote cell survival by the removal of adjacent GG's which are crosslinked by cis-Pt(II)$(NH_3)_2Cl_2$.

INTRODUCTION

The compound cis-Pt(II)$(NH_3)_2Cl_2$ (cis-DDP) is an effective antitumor agent while the isomeric trans-DDP is not (1). Additionally, other platinum compounds have been tested for antitumor efficacy and it appears that the cis configuration is essential for these activities (2). The mechanism by which cis-DDP and other anticancer drugs are exceptionally lethal for neoplastic cells is unknown but it could be due to various types of DNA repair deficiencies in cancer cells or the fact that escape from normal regulatory control causes the replication machinery of such cells to be particularly vulnerable to certain types of DNA damage. Inhibition of DNA synthesis by cis-DDP has been reported in human amnion AV3 cells (3). Additionally, the cis compound is a more potent mutagen (4-8) than the trans isomer and induces filamentous growth in treated cells of E. coli (9,10). Both of these phenomena may be associated with the induction of SOS DNA repair by cis-DDP.

In conjunction with these effects, both cis-DDP and trans-DDP have been

shown to bind to DNA and to cause a variety of monofunctional adducts plus protein-DNA crosslinks and both inter- and intrastrand crosslinks in DNA (2,11). The specific lesion responsible for the effects of platinum drugs on biological systems is unknown, but it does not appear to be interstrand crosslinks (2,11). Stone et al., (12) have proposed that both compounds act preferentially with guanines but that only cis-DDP is stereochemically able to form intrastrand crosslinks between adjacent guanines. Thus, an intrastrand crosslink unique to the cis configuration may be the biologically important lesion (12-13). This hypothesis is supported by the data of Tullius and Lippard (14) and Royer-Pokora et al. (15). They observed that end-labeled fragments of known base sequence were digested differently at the position of adjacent guanines with exonuclease III when DNA was treated with the cis compound as compared to the trans compound.

Further substantiation of DNA as the target of cis-DDP action is the fact that strains of bacteria deficient in DNA repair are less able to survive exposure as measured by colony forming ability than wild-type cells (9,10). Our results confirm that proficiency in excision repair is requisite for survival in the presence of cis-DDP but not the trans isomer. Purified proteins coded by the uvr genes were used to reconstitute the UVRABC nuclease to study the first step in excision repair which is the incision reaction at lesions in DNA (16). Damage in plasmid DNA caused by either cis or trans-DDP was substrate for the UVRABC nuclease which cut plasmid DNA treated with either compound. Adducts caused by these compounds in end-labeled linear DNA were recognized differently by the nuclease as predominant incisions were detected at the 8th phosphodiester bond to the 5' and at the 4th phosphodiester bond to the 3' of adjacent GG's for cis-DDP while a specific cutting pattern by the UVRABC nuclease was not detected with DNA damaged by the trans-isomer.

MATERIALS AND METHODS

Bacteria and Plasmids

The E. coli K12 strains AB1157 (uvr$^+$), AB1886 (uvrA6), AB1885 (uvrB5) and AB1884 (uvrC34) were obtained from Barbara Bachmann (E. coli Genetic Stock Center at Yale University). Plasmid pBEU47 was generously supplied by John Clark (Univ. of Calif., Berkeley) and it contained a cloned allele of the recA gene (lexB30). This was a runaway replication plasmid and was amplified as described by Uhlin et al. (17). Plasmids pXF3 and pBR322 were purified as described by Maniatis et al. (18). pXF3 was supplied by Z. Livneh (Stanford University).

Media

Luria Broth (19) was used in cultivation of bacteria and in survival experiments. Solid media contained 1.5% agar.

Survival Assays

Bacteria were grown and treated with platinum compounds as described previously (4). The platinum compounds (250-500 μg/ml in 0.85% NaCl) were added to logarithmically growing cells at an A_{550} = 0.2. At various times after compound addition, the bacteria were suitably diluted with sterile 0.85% NaCl and plated.

Chemicals

Platinum compounds were supplied by Dr. B. Rosenberg. Restriction enzymes, polynucleotide kinase, Klenow fragment, bacterial alkaline phosphatase, and chemicals for electrophoresis were obtained from Bethesda Research Labs. [γ-^{32}P]ATP (>7000 Ci/mmol) used for 5' labeling and [α-^{32}P]ATP (2903 mCi/mM) for 3' end labeling were obtained from ICN and NEN, respectively.

End-Labeled Fragments

The Eco RI - Bam HI 1.8 kb DNA fragment containing the regulatory region and 826 bases of the *lexB30* gene was purified from plasmid pBEU47 according to the methods published by Sancar and Rupp (20-21). This fragment was digested with Hinf I. Also, plasmid DNA of pXF3 was linearized with Bam HI. These DNA molecules were end-labeled with ^{32}P at the 5' end or at the 3' end as described by Maniatis et al. (18). Fragments were digested further with restriction enzymes and specific fragments purified from 6% polyacrylamide gels. Fragments obtained from pBEU47 were Taq I - Eco RI (105 bp) fragment and Taq I - Hinf I (189 bp) fragment consisting of bases 531-825 of the *recA* allele. Fragments from pXF3 were the Bam HI - Sal I (275 bp) and Bam HI - Eco RI (377 bp) fragments consisting of bases 0 - 650 from the gene coding for tetracycline resistance. End-labeled fragments were sequenced according to the method of Maxam and Gilbert (22). Restriction assays followed the manufacturer's protocols.

Treatment of DNA with Platinum Drugs

Fragments of DNA (10-50 ng) end-labeled with ^{32}P were suspended in TEN 7.4 (10 mM tris-base, 1mM EDTA, 10 mM NaCl, pH 7.4). Platinum compounds were added to yield a final concentration of 0 to 50 μg/ml in a volume of 50 μl in an Eppendorf tube and the mixture incubated at 37°C for 1 or 2 hr. The reaction was stopped by the addition of 0.1 volume of 5 M NaCl. Platinum compounds were immediately removed by spot dialysis (23) on a VM

millipore filter (0.05 μm pore size) which was floated on the surface of 50 ml TEN 7.4 in a petri dish with stirring at 4°C for 1 hr. This procedure has been shown to remove greater than 95% of the unbound platinum and results in high recovery of DNA (24). The DNA was precipitated with ethanol, dried, and suspended in the buffer used in the UVRABC nuclease assay (16).

Plasmid DNA of pBR322 or pXF3 (0.15-0.3 μg/μl) was treated as DNA fragments with platinum isomers. However, in the case of pBR322, only spot dialysis was used to remove DDP.

UVRABC Excision Nuclease Reactions

Purification of the UVR proteins, reconstitution of the UVRABC excision nuclease, and enzyme reactions were as previously described by Sancar and Rupp (16) except for the following modifications. DNA fragments were digested for 1 hr at 37°C with purified reconstituted UVR nuclease in a volume of 25 μl. To release platinum adducts from the DNA fragments and to reverse protein-DNA crosslinks, an equal volume of 2 M thiourea was added after the enzyme reaction period and incubation was continued at 50°C for 2 hr (25). Carrier DNA (1 μg calf thymus DNA) was added and radioactive DNA was purified by two extractions with phenol, an extraction with ether, and precipitation with ethanol. Dried DNA fragments were suspended in loading buffer, denatured by heating 90 sec at 90°C, and subjected to electrophoresis on denaturing 8% polyacrylamide gels and/or buffer gradient 6% polyacrylamide gels (26).

For the UVR nuclease reactions with plasmid DNA, the reaction mixture was as described by Sancar and Rupp (16) and contained 100 mM KCl. Digestion with the UVRABC nuclease was for 30 min and reaction products were analyzed by subjecting them to electrophoresis on 0.7% agarose gels.

Determination of Platinum Bound to DNA

The amount of platinum bound to DNA was determined by atomic absorption spectrophotometry. A Perkin-Elmer Model 603, Series X07, atomic absorption spectrophotometer with graphite furnace was used.

RESULTS

Effects of platinum compounds on excision repair-deficient mutants

cis-DDP but not the *trans* isomer is lethal to mutants of *E. coli* blocked in excision repair (Fig. 1). This sensitivity was noted by the fact that survival (colony-forming units) was reduced to 37% when *uvrA*, *uvrB* or *uvrC* mutants were grown for 1 hr in Luria Broth containing 30 μg/ml *cis*-DDP,

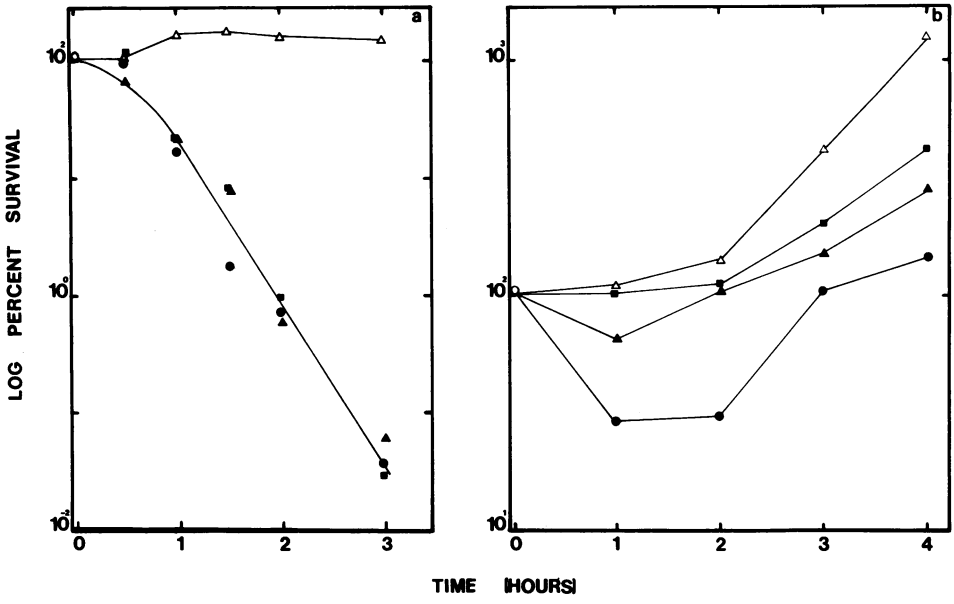

Fig. 1. Survival (colony-forming ability) of Escherichia coli strains AB1157 uvr$^+$ (△), AB1886 uvrA6 (●), AB1885 uvrB5 (■), and AB1884 uvrC34 (▲) for different times of exposure to (a) cis-DDP (30 μg/ml) and (b) trans-DDP (60 μg/ml). Drugs were added to logarithmically growing cells at A_{550} = 0.20.

whereas wild-type cells were not killed after 3 hr exposure. In experiments with wild-type cells, colony-forming units doubled 1 hr after drug addition and then remained at a plateau level for the next 2 hr. As has been published previously (9,10), both mutant and wild-type bacteria were inhibited in cell division by cis-DDP and formed filamentous cells (observed microscopically). Filamentous growth of E. coli is thought to be due to the induction of SOS repair after DNA damage by the platinum drug. One result of the induction of SOS repair is increased cellular levels of certain DNA repair enzymes such as the uvr gene products which function in excision repair (27).

In contrast to the toxicity noted with cis-DDP, a higher concentration of 60 μg trans-DDP per ml medium caused only a 30% reduction in the number of colony-forming units of uvr mutants after 1 hr; thereafter, the number of colony-forming units increased. Wild-type cells grew slightly better than mutants in the presence of the trans-isomer as indicated by their higher rate of increase in colony-forming units. Filamentation of wild-type

Table 1. Effect of platinum drugs on cell division and turbidity of Escherichia coli.[a]

	cis-DDP (30 μg/ml)			trans-DDP (60 μg/ml)		
	Absorbance$_{550}$ [a]		Filamentation[b]	Absorbance$_{550}$ [a]		Filamentation[b]
Strain	90 min	180 min	180 min	90 min	180 min	180 min
AB1157 uvr$^+$	0.68	1.0	++	0.41	0.78	-
AB1886 uvrA6	0.74	1.0	++	0.45	0.75	±
AB1885 uvrB5	0.76	1.0	++	0.44	0.79	±
AB1884 uvrC34	0.68	0.98	++	0.35	0.70	±

[a] Platinum drug was added to logarithmically growing bacteria at A_{550} = 0.20. Samples were taken periodically and A_{550} determined using a Bausch and Lomb Spectronic 20.

[b] DDP-induced filamentation at 3 hrs: (++), filaments approximately 5 times longer than control cells; (±), approximately half of the population slightly elongated; and (-), cells equal in size to control cells.

bacteria did not occur but uvr mutants appeared elongated during the period 1-2 hr after the addition of trans-DDP. Turbidity measurements (Table 1) indicated that mass was increasing in all cultures in the presence of both isomers. Since these treatments utilized concentrations of the trans derivative twice that of the cis compound, there were resultant decreased rates of increases in cell mass.

Cutting of damaged DNA by the UVR nuclease

Atomic absorption spectrophotometry was used to determine the amount of platinum bound to plasmid DNA. Under the conditions of treatment, trans-DDP

Table 2. Mole ratio of cis or trans DDP bound per base pair of pBR322 DNA.

DDP Treatment Concentration (μg/ml)	r_b[b] cis	trans
1	0.30 ± 0.05	1.3 ± 0.1
5	1.0 ± 0.4	3.4 ± 0.1
10	1.7 ± 0.2	4.8 ± 0.1
25	3.7 ± 0.2	7.9 ± 0.1
50	7.3 ± 0.1	12.8 ± 0.3

[a] Values determined using AAS on 10 μl samples of DNA treated with cis or trans DDP at 37°C for 1 hr after removal of unbound platinum. Values are the mean of 4 samples and their standard deviation.

[b] r_b = mmoles DDP/moles of base pair. Moles of DDP in 10 μl samples of DNA treated with cis or trans DDP determined by extrapolating AAS Absorbance reading from a standard plot of Absorbance (λ = 266 nm) vs. Pt concentration for H_2PtCl_6 standards. Concentration of DNA before treatment was measured spectrophotometrically at an Absorbance of 260 nm and after treatment on agarose gels stained with ethidium bromide in comparison to standards of known concentration.

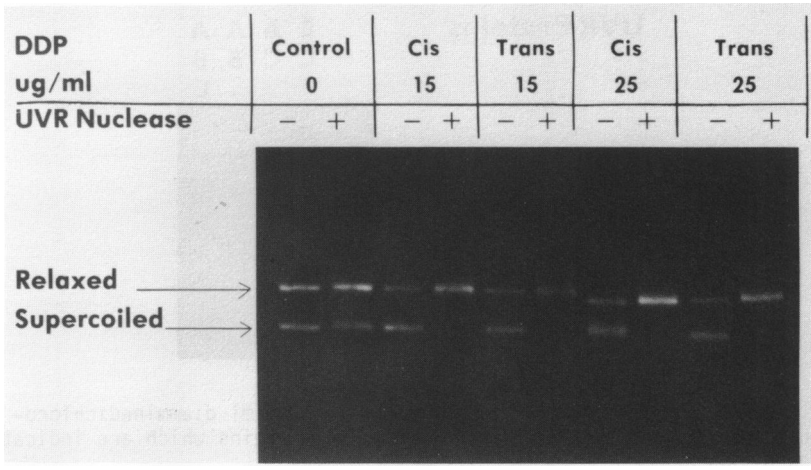

Fig. 2. Agarose gel of pBR322 DNA treated with diamminedichloroplatinum(II) before and after reaction with UVRABC nuclease. Control DNA and DNA that was reacted with the UVRABC nuclease are shown in adjacent lanes. Drug concentrations used to treat DNA are shown above adjacent lanes containing the DNA; (-) indicates lack of digestion and (+) indicates that the DNA was digested by the UVRABC nuclease.

appeared to react more readily with pBR322 DNA than did the cis-derivative (Table 2); there were greater mole ratios of platinum bound per mole base pair (r_b values) for equal treatment mole ratios of input platinum atoms per DNA base pair. From these determinations, the number of platinum adducts per pBR322 DNA molecule ranged from 1 to 30 for cis-DDP treatment and from 5 to 60 with trans-DDP.

The platinum adducts were substrates for the UVRABC nuclease as shown by the fact that supercoiled DNA treated with either cis- or trans-DDP was almost totally converted to the relaxed form upon digestion with the reconstituted enzyme (Fig. 2). All 3 protein products of the uvrA, uvrB, and uvrC genes were needed to cut pXF3 DNA damaged with 10 μg cis-DDP/ml (Fig. 3). When any one of the proteins were omitted, there was only a small amount of conversion of supercoiled DNA to relaxed form, probably as a result of a low level contamination of the subunit preparations with a non-specific endonuclease or topoisomerase 1.

When pBR322 (data not shown) or pXF3 was reacted with DDP and the unbound platinum compound removed by dialysis and alcohol precipitation, a small fraction of the plasmid molecules migrated more slowly on 1% agarose gels than monomeric forms (Fig. 4A-B). In pXF3 DNA, there were 4 visible

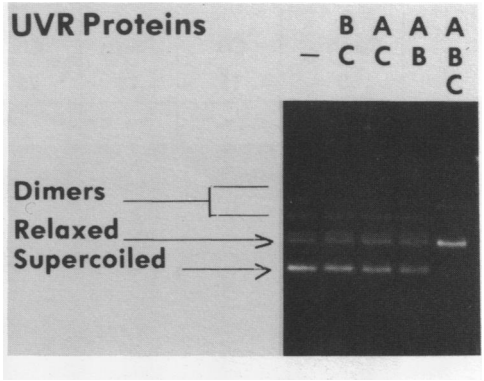

Fig. 3. Agarose gel of pXF3 DNA treated with 10 μg/ml diamminedichloroplatinum(II) and reacted with the purified UVR proteins which are indicated above each lane.

bands which were not present on gels of untreated DNA and which were slower migrating compared to supercoiled and relaxed monomers. Reversal of the platinum crosslinks from DDP treated plasmids with thiourea caused a concomitant loss of the slow migrating forms, as the DNA when subjected to electrophoresis on agarose gels was composed of only form I and form II monomers (Fig. 4A). The arrows in Fig. 4A-C identify the positions on agarose gels of one such slow migrating form which migrated just above the relaxed monomer and which was converted by thiourea treatment to molecules having the electrophoretic mobility characteristic of supercoiled monomers. All four of the slow migrating forms appear to be made up of combinations of relaxed and supercoiled monomers. This is shown in Fig. 4C which is a gel of resolved trans-DDP treated plasmids which has been incubated in 1 M thiourea to reverse platinum crosslinks and then the DNA subjected to electrophoresis in a second direction. Perhaps these molecules were polymeric molecules which resulted upon ethanol precipitation of DNA molecules containing monofunctional adducts. A second bifunctional reaction could result in interhelical crosslinks between monomeric forms. DNA containing adducts caused by cis-DDP gave similar results as that obtained for trans-DDP (data not shown).

Reactions of DDP-treated pXF3 preparations with the UVRABC nuclease resulted in the appearance of two bands of incised molecules which were visualized on agarose gels, the monomeric form II pXF3 and one higher molecular weight form migrating at about the position of the undigested slowest migrating molecules (Fig. 5). Cutting by the repair enzyme occurred

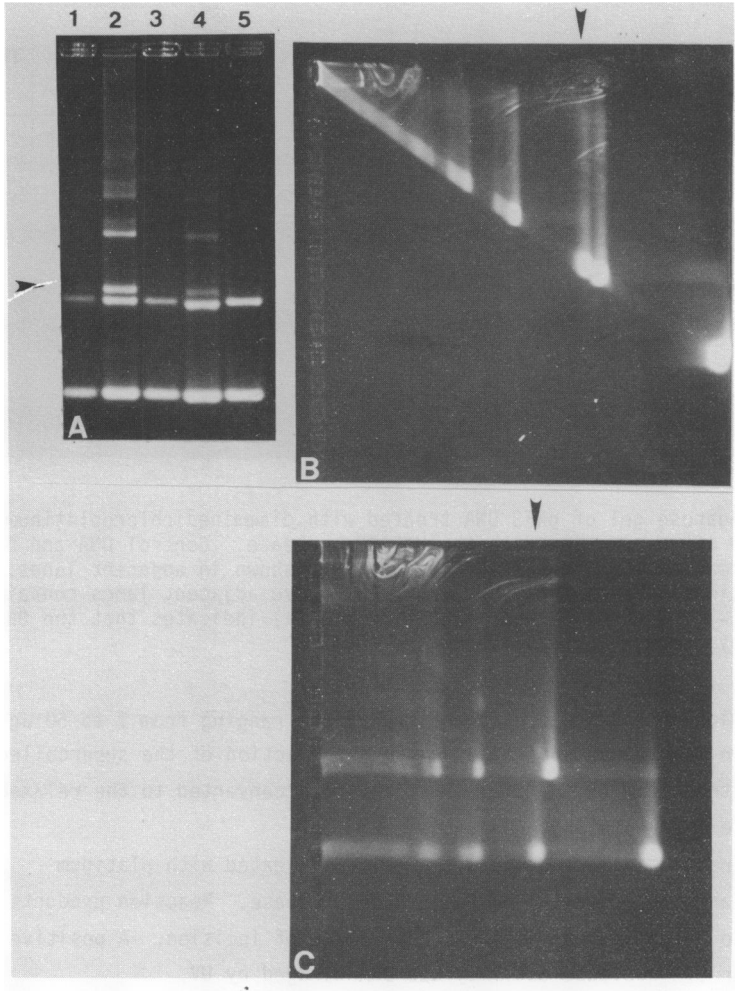

Fig. 4. Agarose gels of pXF3 DNA treated with diamminedichloroplatinum(II) before and after reversal of platinum crosslinks with thiourea. Lanes of gel A contain (5) control DNA without DDP treatment, (4) DNA treated with 10 μg/ml cis-DDP for 1 hr, (3) DNA treated with cis-DDP and the platinum crosslinks reversed by incubation in 1 M thiourea for 3 hr at 50°C, (2) DNA treated with 10 μg/ml trans-DDP for 1 hr, (1) DNA treated with trans-DDP and then the platinum crosslinks reversed by reaction with thiourea. (B) DNA treated with trans-DDP was subjected to electrophoresis and then the gel was turned 90 degrees and the plasmid molecules subjected to electrophoresis in this second direction. (C) DNA is treated as in B but with the exception that the gel was incubated for 16 hr in electrophoresis buffer containing 1 M thiourea at 37°C before subjecting the DNA to electrophoresis in the second direction. The arrow points to the position of one form of platinated DNA migrating slightly slower than relaxed monomers of pXF3.

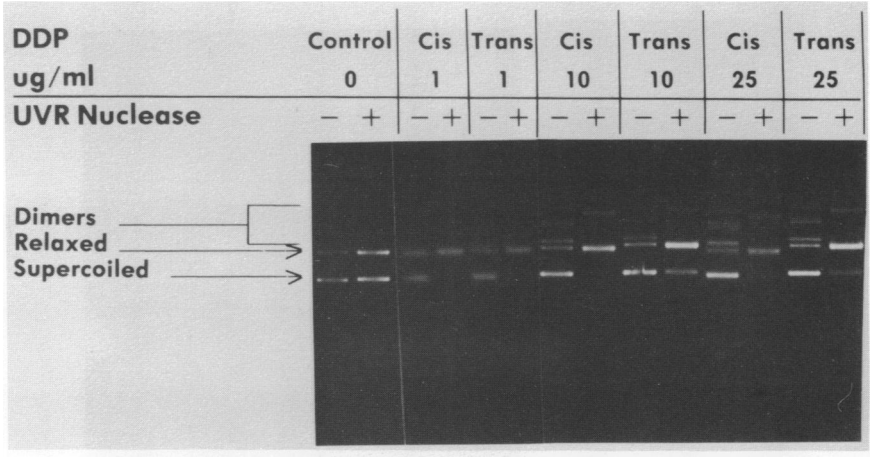

Fig. 5. Agarose gel of pXF3 DNA treated with diamminedichloroplatinum(II) before and after reaction with the UVRABC nuclease. Control DNA and DNA that was reacted with the UVRABC nuclease are shown in adjacent lanes. Drug concentrations used to treat DNA are shown above adjacent lanes containing the DNA; (-) indicates lack of digestion and (+) indicates that the DNA was digested by the UVRABC nuclease.

when plasmids were treated with concentrations ranging from 1 to 50 μg DDP/ml. In some digests of pXF3, a greater fraction of the supercoiled molecules treated with the cis-isomer appeared converted to the relaxed form II molecule than with the trans-isomer.

DNA fragments end-labeled with ^{32}P were treated with platinum compounds and then digested by the UVRABC nuclease. Reaction products were analyzed to determine base sequences at sites of incision. A positive control for UVR nuclease activity was DNA damaged by UV.

Reaction products were analyzed on DNA sequencing gels and autoradiograms are shown in Figures 6-8. Lanes containing Maxam-Gilbert reactions (22) are labeled with the specific bases cut. The Taq I - Eco RI DNA fragment is rich in pyrimidines and thus after UV irradiation is cut at many sites by the repair nuclease (Fig. 6, lane 5). Incision sites were at the 8th phosphodiester bond to the 5' side of pyrimidine dimers as published previously (16). The DNA sequence is given to the right of the autoradiogram. In this DNA fragment, there was one run of 3 G's located in the middle of the fragment which was visualized on the gel. Two intense bands were observed on gels of DNA treated with 10 μg or 50 μg cis-DDP per ml (lane 4 and 3, respectively). These bands were 7 bases 5' to the run of

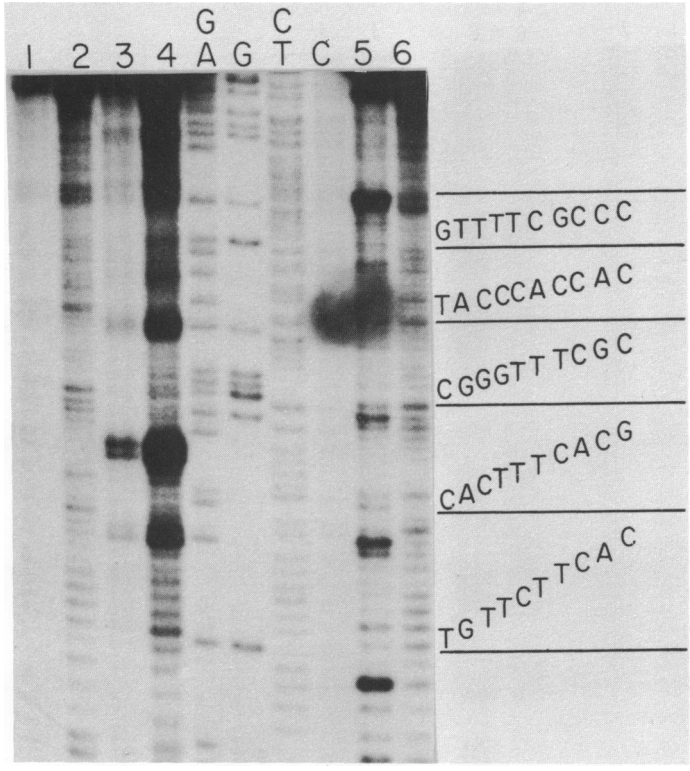

Fig. 6. Cutting of (Taq I - Eco RI)$_{105}$ DNA fragments, from pBEU47 by the UVRABC nuclease. The fragment was labeled at the 5' end. Lanes containing Maxam-Gilbert reactions are lettered according to the bases modified and specifically cut for each reaction. Undamaged DNA (lane 6), DNA treated with 1 μg/ml cis-DDP (lane 1), 10 μg/ml cis-DDP (lane 4), 50 μg/ml cis-DDP (lane 3), 25 μg/ml trans-DDP (lane 2) and 100 J/M^2 UV (lane 5) was reacted with the repair nuclease. Reaction products were submitted to electrophoresis on an 8% polyacrylamide denaturing gel.

3 G's and did not occur in undamaged control DNA (lane 6), in DNA damaged with UV (lane 5), nor in DNA treated with 25 μg/ml trans-DDP (lane 2). Less intense bands at other sites were also apparent in lane 4. These bands were visualized due to overexposure of the film and when the film was properly exposed only two bands were visible as in lane 3.

The Bam HI - Sal 1 (275 bp) fragment had 17 sites in its base sequence which consisted of adjacent GG's. Fragments treated with 10 μg/ml cis-DDP for 2 hr and reacted with the UVRABC nuclease were cut at the eighth phosphodiester bond 5' to the adjacent GG's for all but one site (Fig. 7,

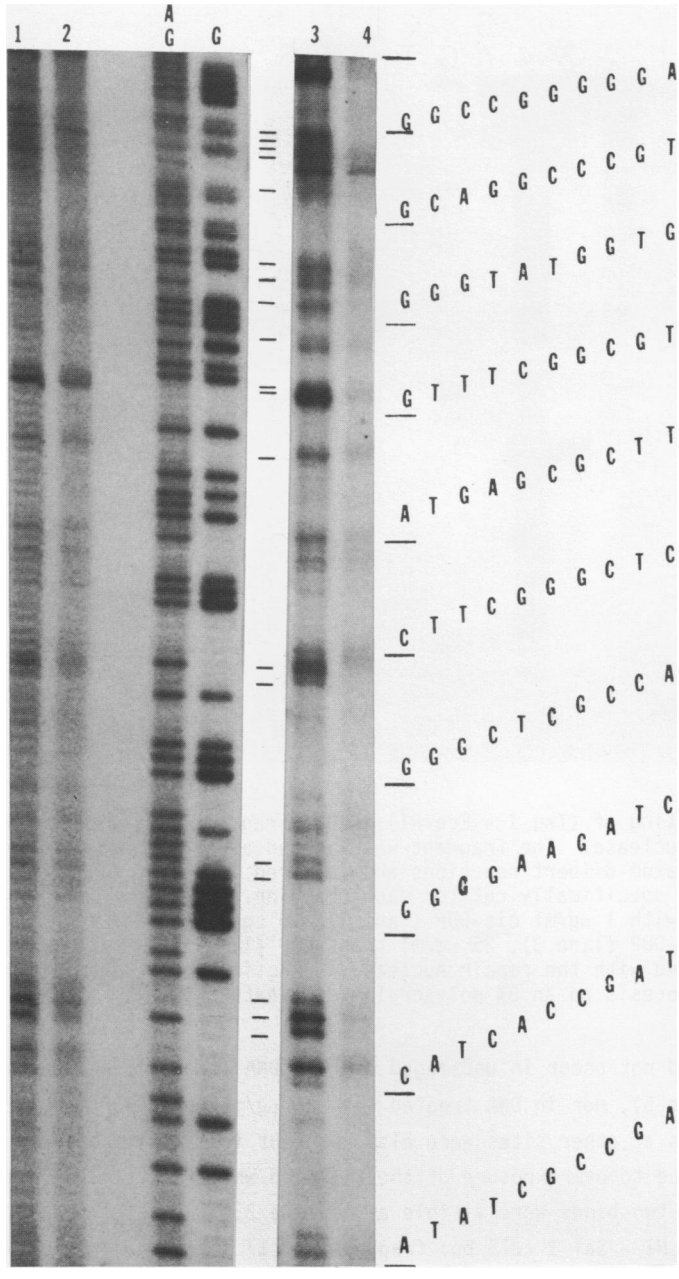

Fig. 7. Reaction products obtained by digestion of DDP-damaged (Bam HI - Sal I)$_{275}$ DNA fragments with the UVRABC nuclease. The DNA fragments were labeled at the 5' end. DNA treated with 0 DDP (lane 4), 10 μg/ml

trans-DDP for 1 hr (lane 1), 10 μg/ml trans-DDP for 2 hr (lane 2) and 10 μg/ml cis-DDP for 2 hr (lane 3) were digested with the UVRABC nuclease and the reaction products submitted to electrophoresis on a buffer gradient polyacrylamide gel. Lanes containing Maxam Gilbert reactions are labeled with the base(s) specifically cut. Bars indicate sites of incisions which appeared more intense in DDP-treated fragments than in untreated control DNA.

lane 3). The DNA sequence is given to the right of the autoradiogram which shows the reaction products after being subjected to electrophoresis. Bands due to incision reaction which occurred in treated DNA but not in control untreated DNA are indicated by bars to the left of lane 3. The seventh bar from the top of the autoradiogram designates a band 7 bases 5' to an AG sequence, while the other incision sites are 7 bases 5' to GG sequences. Less intense cutting bands were observed within and at the 5' and 3' side of the GAGCG sequence at position 493 in pXF3 (18). No specific cutting pattern different from control untreated DNA (lane 4) was observed in DNA treated with 10 μg/ml trans-DDP for 1 or 2 hrs (lanes 1 and 2 respectively). Similar results to these were obtained with the Taq I - Hinf 1 fragment which contains 7 sites with adjacent GG's. Reaction products of DNA treated with 10 μg/ml cis-DDP for 1 h gave bands on gels indicating cuts at the 5' of each GG (data not shown).

To study incision reactions of the nuclease at the 3' side of platinum adducts, excision assays were done on the Bam HI - Sal I DNA fragments labeled at the 3' end and treated with 10 μg/ml DDP for 2 hr (Fig. 8). Incisions were observed at the 4th phosphodiester bond to the 3' side of all adjacent GG's for DNA treated with cis-DDP but not for DNA treated with the trans isomer. No predominant cutting bands were observed 3' to AG or GXG sequences which were also in the region visualized on gels.

DISCUSSION

cis-DDP but not trans-DDP is a potent anticancer agent which induces filamentous growth of E. coli (9,10). The number of colony forming units increased when bacteria were grown in broth containing trans-DDP indicating that bacteria were able to undergo cell division in the presence of trans-DDP. In contrast, cis-DDP caused inhibition of cell division due to its ability to induce SOS DNA repair and colony forming units did not increase as cells became elongated.

Survival experiments of bacteria in broth containing DDP showed that proficiency in excision repair was needed for survival in the presence of

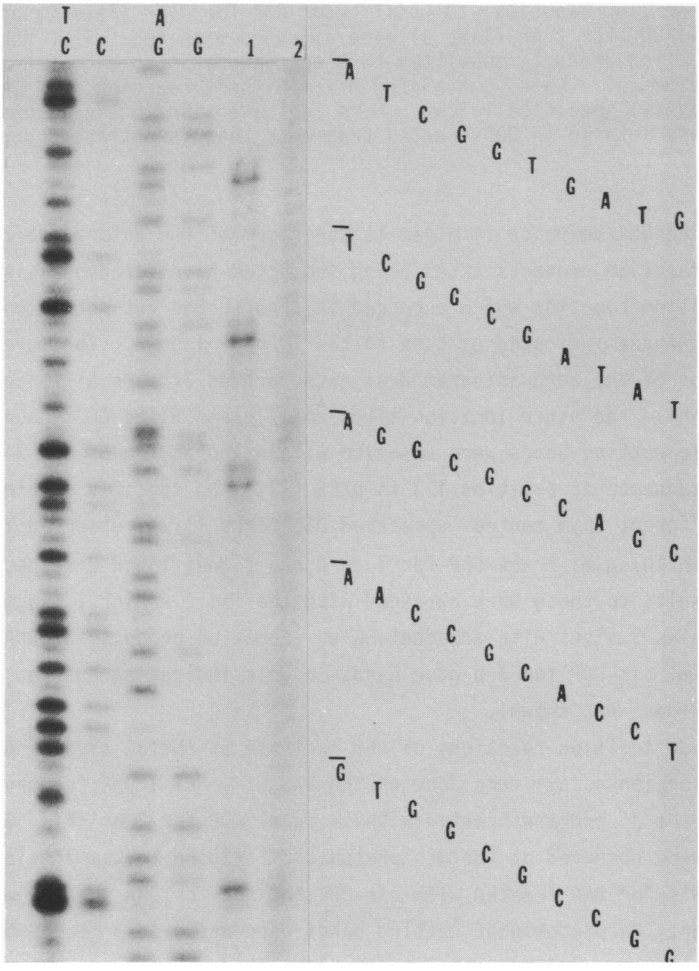

Fig. 8. Reaction products obtained by digestion of DDP-damaged (Bam HI - Sal I)275 DNA fragments with the UVRABC nuclease. The DNA fragments were labeled at the 3' end. DNA treated with 0 DDP (lane 2) and 10 μg/ml cis-DDP for 2 hr (lane 1) were digested with the UVRABC nuclease and the reaction products submitted to electrophoresis on a buffer gradient polyacrylamide gel. Lanes containing Maxam Gilbert reactions are labeled with the base(s) specifically cut.

only the cis isomer. This was evident by the fact that bacteria blocked in excision repair (uvrA, uvrB, or uvrC mutants) were all exceptionally sensitive to cis-DDP. These mutants did, however, exhibit sensitivity to trans-DDP when compared to wild-type cells with respect to rates of increases in colony forming units. Possibly trans-DDP has other biological

targets in addition to those of the cis-compound or causes damage in DNA which is repaired by other pathways in addition to those involving the UVRABC nuclease.

The uvr mutants are blocked at the first step of the excision repair pathway that functions in the recognition and repair of a variety of lesions such as psoralen crosslinked DNA, alkylated DNA and UV-induced pyrimidine dimers (16). A complex reaction for the first step of the excision repair pathways is indicated by the fact that in E. coli at least three proteins, the gene products of uvrA, uvrB and uvrC loci, are requisite for function in damage recognition and incision. A similar complexity is seen in eucaryotes. Human patients with the disease xeroderma pigmentosum are believed to be deficient in excision repair. Similar to uvr mutants of E. coli, cell lines of xeroderma patients have been shown to be comprised of different complementation groups and to be sensitive to UV and various alkylating agents. Accordingly, Fraval et al. (28) reported that xeroderma pigmentosum XP 12 BE cells were 4 times less efficient than normal cells in surviving a given amount of cis-DDP bound to DNA.

Purified uvr gene products were used to reconstitute the UVRABC nuclease for study of its incision activity on DNA treated with DDP. Only the cis compound produced adducts in linear end-labeled DNA which were detected by incision reactions of the UVR nuclease at specific base sequences. However, supercoiled DNA treated with either compound was sensitive to the UVRABC nuclease. Sites of enzyme incision on end-labeled DNA fragments were determined by analysis of the reaction products on polyacrylamide gels in comparison with fragments cut at specific bases by the methods of Maxam and Gilbert (22). When fragments were treated with trans-DDP we did not detect cuts at specific sites by the repair enzyme as the reaction products resembled those of control untreated fragments after digestion. For DNA treated with cis-DDP, the predominant sites of incision were at the 8th phosphodiester bond 5' and at the 4th phosphodiester bond 3' to adjacent GG's. These results indicate that only cis-DDP causes adducts in DNA at runs of GG's which are readily detected by incision reactions of the UVR nuclease. These platinum crosslinked guanines appear to be excised by the UVR nuclease in a manner similar to that observed for pyrimidine dimers in UV-irradiated DNA.

Other platinum adducts may be acted upon by the UVR nuclease but occur less frequently than intrastrand crosslinks at adjacent GG's. Eastman (29), for example, has detected a number of different adducts in DNA treated with

cis-DDP. Such adducts may have been responsible for bands on audiogradiograms which were visualized only by overexposure of the film (Fig. 6, lane 4). Alternatively, the repair enzyme may have different reactivities with respect to the particular damage occurring in DNA. For example, one of the bands in the overexposed lane was produced by cuts 7 bases to the 5' side of a run of 3 C's and thus could be due to incision reactions in the complementary strand opposite runs of DDP crosslinked GG's. Another site of incision was 7 bases to the 5' side of a run of pyrimidines and may have been due to UV-induced damage although we attempted to minimize UV exposure in the purification of DNA. Cutting was observed for a few AG base sequences and may indicate that cis-DDP reacted with some of these sites dependent upon their adjacent base sequence.

Recently, DNA treated with cis-DDP has been enzymatically digested and the platinum adducts quantitated and in some cases characterized (29,30). The major adduct observed by Fichtinger-Schepman et al. (30) was cis-$Pt(NH_3)_2d(pGpG)$ which accounted for about 60% of the total bound platinum. Thus, it is not surprising that we were able to detect cutting by the UVR nuclease at these adducts. A second major adduct occurring at about 1/3 the frequency of the intrastrand crosslink between guanines was cis-$Pt(NH_3)_2d(pApG)$. Possibly under our reaction conditions the frequency of this adduct was too low to be detectable by the UVR nuclease reaction or this adduct occurred at only a few of the AG sequences in the DNA. The occurrence of an adduct at particular bases in DNA has been found to be affected by the adjacent base sequence (14,31,32). This has been observed with the inhibition of restriction enzyme reactions (31) and exonuclease III digestion (14,32) at sites of platinum adducts. This may explain why we failed to see a cutting band for one pair of adjacent GG's in the Bam HI - Sal I fragment. Alternatively, the failure to find an incision band may be due to the presence of a second platinum adduct in this G rich fragment which could interfere with the incision reaction. Minor adducts such as cross-linked guanines separated by a third base, $Pt(NH_3)_3dGMP$ due to monofunctional reaction of platinum compounds or cis-$Pt(NH_3)_2d(GMP)_2$ due to interstrand crosslinks of two guanines occur at low frequencies (30). Incision reactions by the repair nuclease for such adducts would most likely not be detected.

Additionally, these results indicate that adducts in supercoiled DNA may be more sensitive to the incision reactions of the UVR nuclease than adducts in linear DNA. Plasmid DNA with as few as 5 platinum adducts caused

by treatment with either cis-DDP or trans-DDP was sensitive to the UVRABC nuclease and the supercoiled form I molecules were converted to the relaxed form by incision reactions of the repair nuclease. Since trans-DDP does not cause intrastrand crosslinks between adjacent GG's, the UVRABC nuclease may be acting on regions of local denaturation in DNA caused by different platinum adducts. Evidence for such denatured regions are inherent in the observation that both forms of the isomer caused plasmid DNA to become hypersensitive to S1 nuclease (33, unpublished observation of Popoff and Beck). Production of topological distortions by platinum adducts would be expected to have greater consequences in supercoiled DNA than in linear DNA. Topological perturbation of DNA by DDP is indicated by the fact that unwinding of supercoiled DNA results after its reaction with either drug (34).

It is significant that even though both drugs form adducts in supercoiled DNA which are sensitive to the UVRABC enzyme, only the cis-compound is exceptionally lethal to uvr mutants. It may be the case that only cis-DDP reacts with adjacent guanines in DNA to form intrastrand crosslinks that must be removed from the DNA for cell survival by the repair enzyme and that are detected in assays using end-labeled linear DNA. This confirms the hypothesis of Stone et al. (12-13) that only cis-DDP causes crosslinking of adjacent guanines. However, both drugs cause adducts which are sensitive to the repair nuclease since supercoiled DNA treated with either drug is cut by the nuclease. Further studies are necessary to reveal the nature and significance of these adducts. These results suggest that the topology of DNA may be an important factor in the UVRABC excision nuclease reactivity.

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