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Repair synthesis by human cell extracts in DNA damaged by *cis*- and *trans*-diamminedichloroplatinum(II)

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ABSTRACT

DNA damage was induced in closed circular plasmid DNA by treatment with *cis*- or *trans*-diamminedichloroplatinum(II). These plasmids were used as substrates in reactions to give quantitative measurements of DNA repair synthesis mediated by cell free extracts from human lymphoid cell lines. Adducts induced by both drugs stimulated repair synthesis in a dose dependent manner by an ATP-requiring process. Measurements by an isopycnic gradient sedimentation method gave an upper limit for the average patch sizes in this *in vitro* system of around 140 nucleotides. It was estimated that up to 3% of the drug adducts induce the synthesis of a repair patch. The repair synthesis is due to repair of a small fraction of frequent drug adducts, rather than extensive repair of a rare subclass of lesions. Nonspecific DNA synthesis in undamaged plasmids, caused by exonucleolytic degradation and resynthesis, was reduced by repeated purification of intact circular forms. An extract made from cells belonging to xeroderma pigmentosum complementation group A was deficient in repair synthesis in response to the presence of *cis*- or *trans*-diamminedichloroplatinum(II) adducts in DNA.

INTRODUCTION

The platinum(II) coordination complex *cis*-diamminedichloroplatinum(II) (*cis*-DDP) has cytotoxic and mutagenic properties and is an important cancer chemotherapeutic agent with clinical activity against several malignancies (1). In contrast, the stereoisomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP) is much less cytotoxic and mutagenic (2, 3). Considerable evidence implicates reactions with DNA as the cause of the cytotoxic effects of *cis*-DDP (2, 3). Both *cis*-DDP and *trans*-DDP form covalent bonds with purine base residues in DNA. The N^7-atoms of guanine (G) and to a lesser extent adenine (A) are the preferred reaction sites (3). The major lesions induced by treatment of isolated mammalian DNA with *cis*-DDP have been characterized as 1,2 intrastrand cross-links between adjacent bases in GG and AG sequences, which correspond to 60−65% and 20−25% of the total amounts of platinum adducts, respectively (4, 5, 6, 7). Rarer lesions are 1,3 intrastrand cross-links between G residues separated by one base residue in GNG sequences (5−6% of platinum adducts), interstrand cross-links between G residues in opposing strands of the DNA helix (1−2%), and platinum-DNA monoadducts (2−3%) (6, 7, 8). The inactive isomer *trans*-DDP is incapable of forming 1,2 intrastrand cross-links between neighboring bases. Instead, a high proportion of DNA monoadducts are induced, which make up 80−85% of the products following a 1−2 h drug incubation (9, 10). Some of the monoadducts slowly rearrange to diadducts, with 50% of *trans*-DDP adducts remaining as monoadducts after a 24 hour incubation (9). The diadducts formed by *trans*-DDP include DNA interstrand cross-links (2% of *trans*-DDP adducts in isolated

mammalian DNA) (11), and probably 1,3 intrastrand cross-links or cross-links between further separated base residues (12).

The significance of repair of DNA lesions for cellular tolerance to *cis*-DDP is demonstrated by studies of repair deficient bacteria (13, 14, 15, 16), yeast cells (17), Chinese hamster cells (18) and human xeroderma pigmentosum cells (19, 20, 21). It has been shown *in vitro* that the *E. coli* UvrABC nuclease incises the 8th phosphodiester bond 5′ and the 4th phosphodiester bond 3′ to GG intrastrand cross-links, thus excising an oligomer containing the adduct (16).

Enhanced DNA repair may be a contributing factor to resistance of tumors to clinical therapy with *cis*-DDP. Thus, it has been shown that some *cis*-DDP-resistant tumor cells remove *cis*-DDP-DNA adducts in genomic DNA more efficiently than sensitive tumor cells (22, 23), and also can have a higher capacity to reactivate *cis*-DDP damaged plasmids (24). In other investigations increased DNA repair synthesis induced by *cis*-DDP has been demonstrated in resistant tumor cells (25, 26, 27). It has been proposed that the lower cytotoxicity of *trans*-DDP is due to a more rapid repair of *trans*- than *cis*-DDP-DNA adducts (28), but other investigators have been unable to find any difference in the rate of removal of *cis*-DDP and *trans*-DDP adducts from the DNA of mammalian cells (29).

Recently an *in vitro* assay for investigations of nucleotide excision repair by extracts from human cells has been developed, and it was shown that extracts from normal cells catalyze repair synthesis in DNA damaged by UV and psoralens (30). These observations have recently been confirmed (31). Preliminary results have also been reported by us indicating repair synthesis in response to *cis*-DDP adducts (32) and by Sibghat-Ullah *et al.* for adducts induced by the *cis*-DDP analog *cis*-diaminocyclohexanedichloroplatinum(II) (31). In the present study we have used this assay to investigate quantitatively the DNA repair synthesis performed by cell extracts in response to both *cis*- and *trans*-DDP induced DNA damage.

MATERIALS AND METHODS

Cells and extracts

EB-virus transformed human lymphoid cell lines GM1953 from a normal individual and GM2345 from an individual with xeroderma pigmentosum (complementation group A) were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, N.J.). Cells were tested and found to be free of *Mycoplasma*. Cultures were grown in RPMI 1640 medium supplemented with 15% fetal calf serum. Whole cell extracts were prepared essentially by the method of Manley *et al.* (33), as previously described (30). After preparation extracts were immediately frozen. When stored at −80°C extracts retained their activity in *in vitro* repair reactions for more than one year.

Preparation of plasmids and treatments with cis-DDP, trans-DDP and UV

Plasmids pAT153 and pBR322 were prepared by the alkaline lysis method (34) from *E. coli* strain DH5 (*recA, hsdR*). Plasmid pCS58 containing a 3.1 kb insertion of the *ada* gene region of *E. coli* in plasmid pAT153 was provided by Dr. B. Sedgwick (35) and was purified from *E. coli* strain CSR603 (*recA uvrA phr*). Following banding in ethidium bromide/cesium chloride, closed circular plasmids were further purified by neutral sucrose gradient centrifugation. Stock solutions of *cis*-DDP and *trans*-DDP (Sigma) were prepared in distilled water at 0.1 mg/ml, and stored at −80°C. Immediately before treatment, stocks were diluted in TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The platinum(II)

coordination complexes initially bind to DNA as monoadducts that slowly rearrange to form diadducts (3). To allow this process to occur, plasmid pAT153 (0.1 mg/ml in TE-buffer) was incubated in the dark at 37°C for 12 h with different concentrations of *cis*-DDP and *trans*-DDP. The reactions were stopped by adding NaCl to 0.5 M. DNA was precipitated with 2 volumes of ethanol at −70°C in the presence of 2.5 M ammonium acetate, washed in 70% ethanol, dried and redissolved in TE-buffer. For experiments with UV irradiated plasmid, pAT153 DNA (50 μg/ml in TE-buffer) was irradiated in 10 μl drops with 350−450 J/m^2 254 nm (peak) germicidal UV at a fluence rate of 0.5 W/m^2 as measured with a Latarjet dosimeter.

Measurements of platinum adducts in plasmid DNA

Plasmid pAT153 was treated with *cis*-DDP and *trans*-DDP, ethanol precipitated, washed in 70% ethanol and dried, as described above. The plasmid DNA was then dissolved in 11.6 M HCl and hydrolyzed in the dark at 37°C overnight. The concentration of hydrolysed DNA in each sample was determined by the absorbance at 260 nm, and the platinum content determined by flameless atomic absorption spectroscopy using a Perkin Elmer Model 306 atomic absorption spectrophotometer equipped with a graphite furnace (29).

Measurements of DNA interstrand cross-links

DNA interstrand cross-links were measured by the ethidium bromide binding fluorescence assay described by Lown *et al.* (36) with minor modifications. Samples of plasmid pAT153 (5−10 μg) treated with *cis*-DDP and *trans*-DDP were linearized with EcoRI. The completeness of the digestion was confirmed by electrophoresis. The DNA was then added to 3 ml of 0.4 mM EDTA, 20 mM dipotassium hydrogen phosphate (pH 11.8), with 1 μg/ml ethidium bromide (Sigma). The samples were divided in two 1.5 ml portions, one of which was boiled for 5 min and rapidly cooled in ice-water. Samples were then left to equilibrate for 5 min in a water bath at 24°C, and their fluorescence was measured with a Perkin Elmer LS 3 spectrophotometer using an excitation wavelength of 525 nm and an emission wavelength of 600 nm. Boiling was efficient in denaturing untreated linearized plasmids and reduced the ethidium bromide fluorescence to 2−5% of that of nondenatured plasmid. The ratio of fluorescence in boiled samples to that in unboiled samples, after correction for the background fluorescence, indicated the fraction of plasmids with at least one interstrand cross-link. The average number of interstrand cross-links per plasmid was calculated according to the Poisson distribution from the fraction of non-cross-linked plasmids. Some samples were also analysed for interstrand cross-links by electrophoresis on 1% alkaline agarose gels (34). The two assays gave very similar results.

In vitro repair reactions

Repair reactions were performed as previously described (30). Standard 50 μl reaction mixtures contained 300 ng plasmid pAT153 which was either untreated or damaged with *cis*-DDP, *trans*-DDP or UV, 300 ng untreated plasmid pBR322, 45 mM HEPES-KOH (pH 7.8), 60 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dGTP, dCTP and TTP, 8 μM dATP, 2 μCi [α-^{32}P]dATP (3000 Ci/mmol), 40 mM phosphocreatine, 2.5 μg creatine phosphokinase (Type I, Sigma), 3.4% glycerol, 18 μg bovine serum albumin and (typically) 150 μg extract protein. Reactions were incubated at 30°C for 6 h, unless otherwise indicated, and then stopped by adding EDTA to 20 mM. After a 10 min incubation at 37°C with 80 μg/ml RNAse A, SDS was added to 0.5%, and proteinase K to 190 μg/ml. Tubes were incubated for 30 min at 37°C, and the mixture was extracted with phenol/chloroform. DNA was ethanol precipitated and washed once with 1 ml 70% ethanol. Plasmids were linearized with EcoRI in 30 μl buffer,

and electrophoresed in a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was photographed under near-UV transillumination with Polaroid type 55 positive/negative film. After drying of the gel an autoradiograph was made. In order to quantify the incorporation of [^{32}P]dAMP into DNA, band intensities in the photographic negative and the autoradiograph were measured with an LKB UltroScan XL scanning laser densitometer. To calibrate the densitometry results, bands were excised from the gel in several experiments and analyzed by scintillation counting. The amount of [^{32}P]dAMP incorporated per reaction was corrected for the amount of DNA recovered from the reaction, as measured by densitometry of the photographic negative.

Measurements of repair patch size

Repair patch synthesis was analyzed by equilibrium sedimentation in alkaline cesium chloride gradients, essentially as described by Smith et al. (37). *In vitro* repair reactions using normal GM1953 cell extracts and plasmids treated with *cis*-DDP and *trans*-DDP were performed as described above, except that 20 μM 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) (Pharmacia, Uppsala, Sweden) was substituted for TTP in the reaction mixture and [α-^{32}P]dCTP was used instead of [α-^{32}P]dATP. Following treatment with RNAse A, SDS and proteinase K, the reaction samples were extracted with buffered phenol, purified by centrifugation through a Sepharose G50 (Pharmacia) column, and extracted with chloroform/isoamyl alcohol (24:1). The DNA was ethanol precipitated, washed in 70% ethanol, dried and resuspended in TE-buffer. Before equilibrium sedimentation the DNA samples were digested with EcoRI or XhoII restriction endonucleases. Plasmid pAT153, uniformly labeled with ^{3}H as a density marker for 'light' DNA was prepared from *E. coli* strain AB2487/pAT153 (*recA*, *thyA*) grown in minimal medium in the presence of [^{3}H]thymidine. The DNA was linearized with EcoRI before equilibrium sedimentation. The (−) strand of M13mp18 DNA was synthesised *in vitro* with BrdUTP instead of TTP, in the presence of [α-^{32}P]dCTP, using *E. coli* DNA polymerase I Klenow fragment (Stratagene), with single stranded viral DNA as a template, primed by a universal sequencing oligonucleotide. The product was purified on a NENSORB 20 cartridge (New England Nuclear). This DNA was used as a marker of fully bromouracil-substituted 'heavy' DNA. DNA samples from *in vitro* repair reactions were mixed with 'light' marker DNA and centrifuged in 3 ml alkaline cesium chloride gradients (pH 12.5, η = 1.4060) in a Beckmann SW50.1 rotor at 35,000 rpm for 95 h at 25°C. Fractions were collected from the bottom of the tubes and analysed by scintillation counting.

Average repair patch sizes were estimated by the formula $P = (\Delta D/\Delta D_0) \times S$, where P is the average patch size, S the weight average size of the DNA fragments containing a repair patch, and ΔD and ΔD_0 are the density shifts of DNA fragments containing a repair patch and of fully bromouracil-substituted DNA, respectively. A correction was made for the small difference in density of fully bromouracil-substituted M13mp18 (−) strand (ϱ = 1.7909) from that of plasmid pAT153 (ϱ = 1.7849).

RESULTS

Measurements of platinum adducts, interstrand cross-links and apurinic sites in cis-DDP and trans-DDP treated plasmids

Platinum-adducts in pAT153 plasmids treated with *cis*-DDP and *trans*-DDP were measured by atomic absorption spectroscopy. The relationship between drug concentration during incubation and the average number of platinum-adducts per plasmid was linear for both drugs over the employed dose range (Figure 1A). The efficiency of binding to DNA was

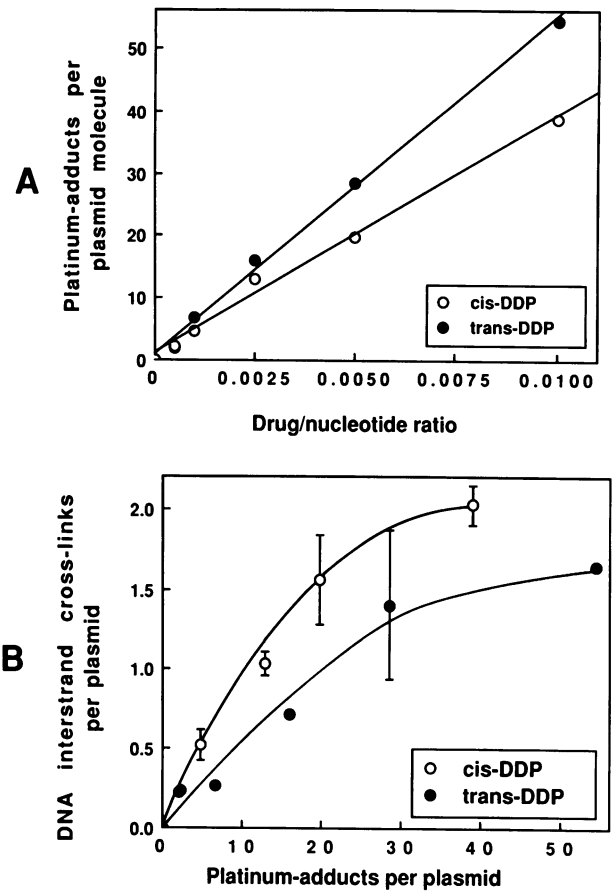


Figure 1. (A) The number of platinum adducts, and (B) DNA interstrand cross-links induced in pAT153 plasmids treated with different concentrations of *cis*-DDP (○) or *trans*-DDP (●) for 12 hours; bars: standard deviation.

1.4 times higher for *trans*-DDP compared to *cis*-DDP (72% of *trans*-DDP molecules versus 51% of *cis*-DDP molecules binding to DNA). Approximately 7−8% of *cis*-DDP adducts and 4−5% of *trans*-DDP adducts were DNA interstrand cross-links, up to a level of 20−30 total adducts per plasmid (Figure 1B). When plasmids damaged with *trans*-DDP were electrophoresed on 1% agarose gels we frequently saw small amounts of slowly migrating DNA species, which are likely to represent plasmid molecules connected by intermolecular cross-links (see Figure 2A).

Plasmids treated with *cis*-DDP or *trans*-DDP containing up to 39 adducts per circle showed negligible susceptibility to cleavage by *E. coli* endonuclease IV (38), corresponding to 0.02−0.13 apurinic sites per plasmid. This indicates that the platinum drugs very rarely form apurinic sites or lesions that are converted to apurinic sites (39). No induction of single strand breaks by treatment of plasmid DNA with *cis*-DDP or *trans*-DDP was detected.

Comparison of DNA repair synthesis induced by cis-DDP, trans-DDP and UV adducts

Figure 2A illustrates an experiment measuring DNA repair synthesis by an extract made

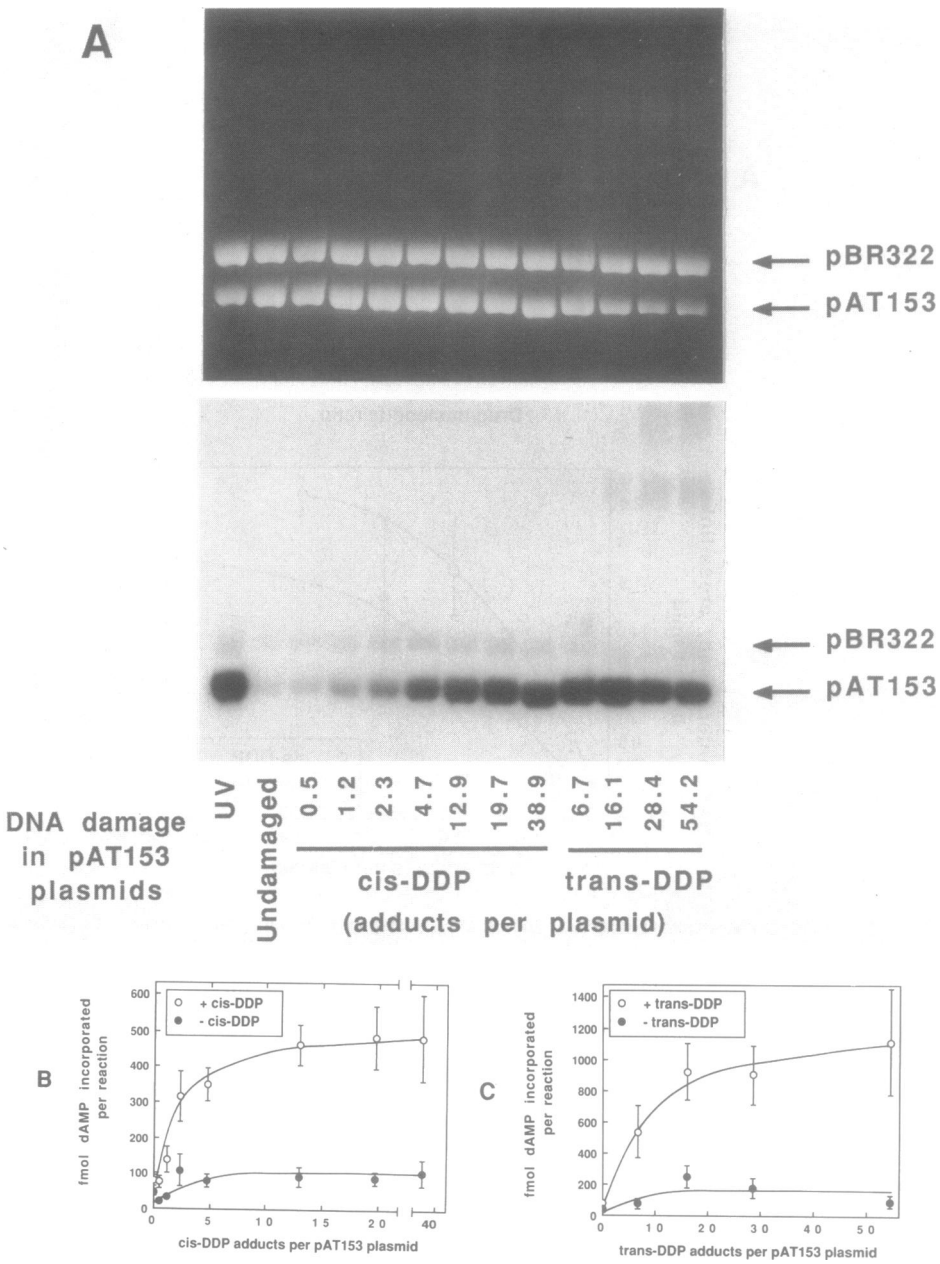

A
pBR322
pAT153
pBR322
pAT153
DNA damage in pAT153 plasmids
UV
Undamaged
0.5
1.2
2.3
4.7
12.9
19.7
38.9
6.7
16.1
28.4
54.2
cis-DDP
trans-DDP
(adducts per plasmid)
B
fmol dAMP incorporated per reaction
+ cis-DDP
- cis-DDP
cis-DDP adducts per pAT153 plasmid
C
fmol dAMP incorporated per reaction
+ trans-DDP
- trans-DDP
trans-DDP adducts per pAT153 plasmid

Table 1. Repair synthesis in damaged plasmid DNA and background incorporation in undamaged DNA.

damage	adducts/molecule	fmol dAMP incorporated per reaction damaged pAT153	undamaged pBR322
cis-DDP	19.7	550 ± 170	63 ± 6
trans-DDP	16.1	990 ± 410	57 ± 26
UV	16	930 ± 400	59 ± 17

Values given are the mean of 3 experiments ± standard error. Plasmids used in these experiments had been purified by ethidium bromide/cesium chloride gradient centrifugation followed by two consecutive neutral sucrose gradients. A UV fluence of 450 J/m^2 yielded approximately 12 cyclobutane dimers and 4 (6–4) photoproducts per plasmid molecule.

from normal GM1953 cells in pAT153 plasmids damaged with *cis*-DDP, *trans*-DDP or UV. A similar amount of undamaged pBR322 is included in the reactions to show the background incorporation into undamaged plasmid. Quantitative results from several experiments are illustrated in Figures 2B–D. A low level of damage-induced DNA repair synthesis was detected in plasmids containing an average of only one *cis*-DDP adduct per molecule. With increasing amounts of *cis*-DDP adducts the repair synthesis increased until a plateau was reached with approximately 13 adducts per plasmid. Increasing repair synthesis was also observed in reactions with increasing amounts of *trans*-DDP adducts. At similar levels of adducts per plasmid *trans*-DDP induced a higher level of repair synthesis than *cis*-DDP, and the plateau level of DNA repair synthesis induced by *trans*-DDP adducts was higher than that for *cis*-DDP adducts. As shown in Table 1, plasmids containing approximately 20 *cis*-DDP or 16 *trans*-DDP adducts gave levels of repair synthesis comparable to irradiation with 450 J/m^2 UV-light, which yields approximately 12 cyclobutane pyrimidine dimers and 3–4 (6–4) photoproducts per plasmid (40). This indicates that the DNA lesions induced by all three agents stimulate DNA repair synthesis with similar efficiencies.

Increasing amounts of extract gave increasing DNA repair synthesis up to 150 μg protein

Figure 2. Repair of plasmids containing *cis*-DDP or *trans*-DDP adducts or UV induced DNA lesions by extracts from GM1953 cells. Standard repair reactions contained 150 μg of GM1953 extract protein, and 300 ng each of undamaged pBR322 plasmid and pAT153 plasmid either irradiated with 450 J/m^2 UV or with different amounts of *cis*-DDP or *trans*-DDP adducts. After incubation at 30° C for 6 h, the DNA was isolated, linearized with EcoRI, and separated on an agarose gel.

(*A*) Results of a typical experiment. Upper panel: Photograph of a gel, showing DNA fluorescence. Lower panel: Autoradiograph of the same gel, showing incorporation of [^{32}P] dAMP. The plasmids used in this experiment had been purified by centrifugation in ethidium bromide/cesium chloride and two consecutive neutral sucrose gradients.

(*B*) Incorporation of dAMP into *cis*-DDP damaged (○) and undamaged (●) plasmids. For all quantifications reported, incorporation of radioactive material is corrected for the relative DNA content in each band. Symbols indicate mean values of 2–6 separate experiments, bars: S.E.M.

(*C*) Incorporation of dAMP into *trans*-DDP damaged (○) and undamaged (●) plasmids. Symbols indicate mean values of 3–7 separate experiments; bars: S.E.M.

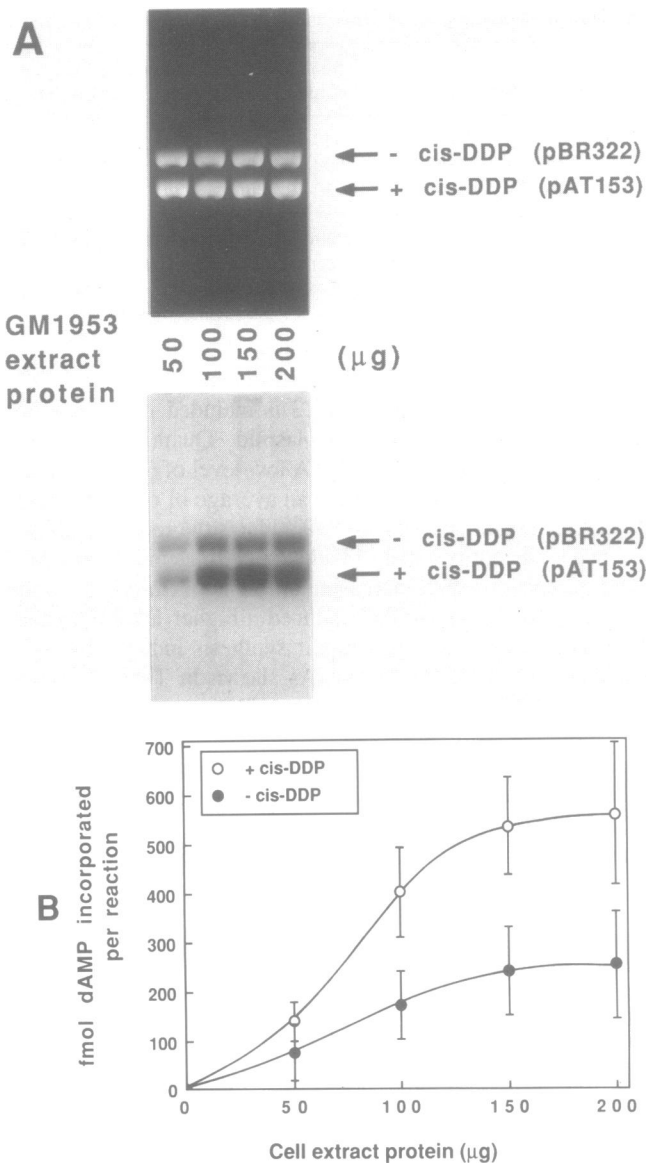


Figure 3. *In vitro* repair of pAT153 plasmids containing *cis*-DDP adducts by different amounts of extract from GM1953 cells. Both plasmids used in these experiments were purified by ethidium bromide/cesium chloride centrifugation followed by a single neutral sucrose gradient. Upper panel: DNA fluorescence. Lower panel: autoradiograph.
(*A*) Result of a representative experiment using pAT153 plasmids containing 12.9 *cis*-DDP adducts per molecule. (*B*) Quantitation of the incorporation of [^{32}P]dAMP into *cis*-DDP damaged (12.9−19.7 adducts per molecule) (○) and undamaged (●) plasmids. Mean values of 3 separate experiments, bars: standard error.

per 50 μl reaction volume (Figure 3). No additional repair synthesis was obtained by further increasing the amount of protein, and an inhibition was seen when the cell extract exceeded 20% of the reaction volume. To obtain maximum DNA repair, 150 μg of extract protein per 50 μl reaction volume was generally used in experiments. Most repair synthesis occurred within the first 3 hours (Figure 4). To allow reactions to go to completion, incubations were for a total of 6 hours. Repair synthesis was undetectable in the absence of ATP, as shown previously for UV-damaged DNA (30). Omission of the ATP regenerating system (phosphocreatine and creatine phosphokinase) from the reaction mixture considerably reduced repair synthesis (data not shown).

Synthesis of repair patches

From the results of equilibrium sedimentation experiments, average repair patch sizes were estimated. GM1953 cell extract was used in reaction mixtures where plasmids had averages of 4.7 or 39 *cis*-DDP adducts (Figure 5), or an average of 6.7 *trans*-DDP adducts per molecule (Figure 6). In gradients containing plasmids linearized with EcoRI 70–85% of the radioactivity was recovered as a distinct peak that was only slightly displaced from the peak containing 'light' marker DNA (Figures 5C,E and 6B). The shift of this peak in the gradient containing DNA with 39 *cis*-DDP adducts per plasmid was about 0.5 fraction (Figure 5E), corresponding to a maximum average repair patch size of approximately 150 nucleotides. In gradients containing plasmids with 4.7 *cis*-DDP and 6.7 *trans*-DDP adducts the displacement was not more than one fraction (Figures 5C and 6B). EcoRI cleavage of pAT153 gives one fragment of 3741 base pairs, so that it is difficult to resolve short repair tracts by monitoring a density shift; values obtained with linearized plasmids are therefore maximum estimates of patch size. Somewhat better resolution was obtained by XhoII cleavage of pAT153, which yields 8 fragments (11, 12, 17, 86, 728, 768, 827 and 1292 base pairs) with a weight average size of 931 base pairs. Gradients containing XhoII

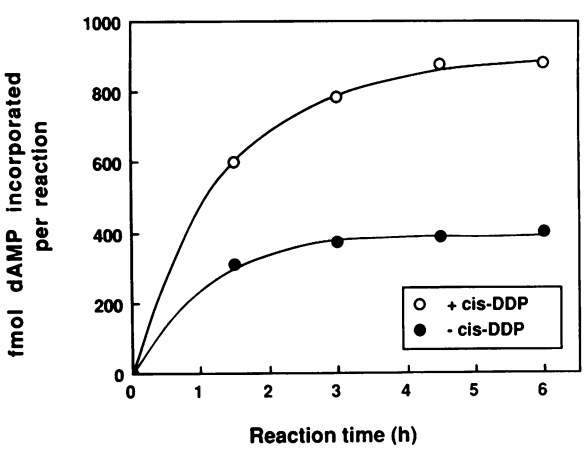


Figure 4. Time course of repair synthesis. Standard repair reactions containing pAT153 plasmids with an average of 12.9 *cis*-DDP adducts and undamaged pBR322 were incubated for 0–6 hours with 150 μg of GM1953 extract protein. The graph shows the incorporation of [^{32}P]dAMP into *cis*-DDP damaged (○) and undamaged (●) plasmids. Plasmids used in this experiment were purified as in Figure 3.

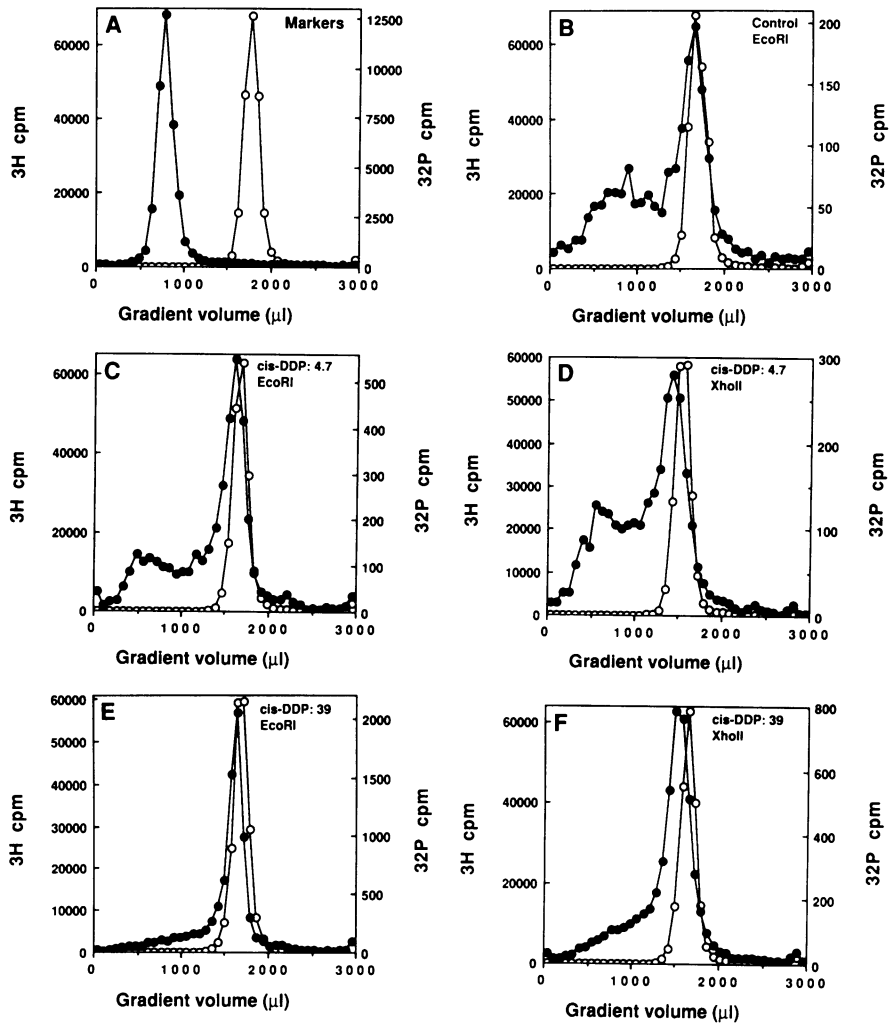


Figure 5. Analysis of DNA repair patch size by equilibrium sedimentation of bromouracil-substituted DNA, for plasmids damaged with *cis*-DDP. Alkaline cesium chloride gradients were centrifuged at 35,000 rpm in a Beckmann SW50.1 rotor; displacement of 'heavy' marker DNA (ΔD_0): 888 μl. Fractions (75 μl) were collected after 95 h centrifugation at 25° C.

(*A*) Markers: ^{3}H-labeled 'light' DNA (pAT153 DNA uniformly labeled with [^{3}H]thymidine and linearized with EcoRI) (○), and ^{32}P-labeled 'heavy' DNA (M13mp18 (−) strand substituted with BrdUTP and labeled with [α-^{32}P]dCTP) (●).

(*B-F*) Repair reactions using GM1953 cell extract were performed in the presence of [α-^{32}P]dCTP and with BrdUTP instead of TTP in the reaction buffer. The plasmids were purified, digested with either EcoRI or XhoII (●), and mixed with ^{3}H-labeled 'light' pAT153 DNA linearized with EcoRI (○) before centrifugation.

(*B*) Undamaged pAT153, linearized with EcoRI.

(*C*) pAT153 containing 4.7 *cis*-DDP adducts per molecule, linearized with EcoRI.

(*D*) pAT153 containing 4.7 *cis*-DDP adducts per molecule, digested with XhoII.

(*E*) pAT153 containing 39 *cis*-DDP adducts per molecule, linearized with EcoRI.

(*F*) pAT153 containing 39 *cis*-DDP adducts per molecule, digested with XhoII.

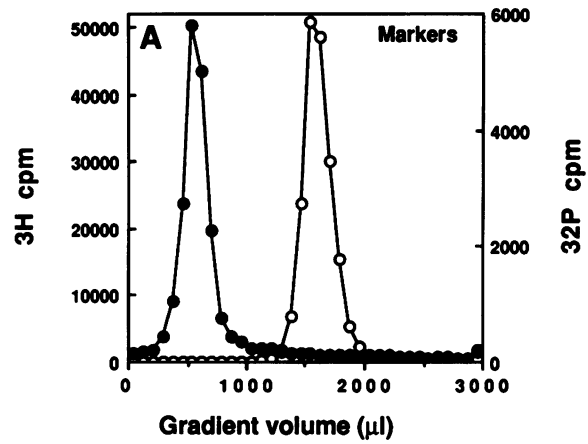
digested plasmids all showed a displacement of the main ^{32}P-containing peak from the ^{3}H peak of 1.5 fractions, corresponding to average patch sizes of about 115 and 160 nucleotides in the experiments with *cis*-DDP and *trans*-DDP adducts, respectively (Figures 5D, F and 6C). These estimates are in the same range as obtained with EcoRI-linearized molecules, suggesting that most labeled plasmids contain only one repair patch.

In addition to the main peak, approximately 25% of the radioactive material in gradients containing EcoRI cut plasmids with 4.7 *cis*-DDP adducts and *trans*-DDP adducts sedimented at a higher density (Figure 5C). This material may represent the result of nick translation or exonucleolytic digestion and resynthesis to yield DNA tracts much longer than average repair patches. This phenomenon was also observed in a gradient containing DNA from a repair reaction with undamaged plasmid (Figure 5B). Approximately ⅔ of the label sedimented as a peak without any detectable density shift from 'light' DNA, thus representing very short stretches of newly synthesized DNA. In addition, approximately ⅓ of the radioactive material sedimented near the expected density of fully substituted DNA. This peak probably contains products of nick translation initiated in the small population of nicked plasmids present even after two consecutive sucrose gradient purification steps.

Efficiency of repair of cis-DDP and trans-DDP adducts

Estimates of the efficiencies of repair of *cis*-DDP and *trans*-DDP adducts were made from the measurements of repair patch sizes and the levels of damage-specific DNA repair synthesis induced by different numbers of adducts (Figure 2). Using average repair patch sizes of 115 to 160 nucleotides, we estimate that 12% to 22% of plasmids contained a repair patch at the highest levels of drug modification. Because the dose-response curves saturate (Figure 2B−C), the proportion of drug adducts that induce the synthesis of a repair patch decreases with increasing level of adduction. At low levels of modification approximately 3% of *cis*-DDP adducts induced a repair patch. With increasing levels of drug modification the efficiency decreased to about 0.5%. The efficiency of *trans*-DDP adducts in inducing repair patch synthesis was similar to that of *cis*-DDP adducts.

The low efficiency of repair obtained could be due either to efficient repair of a rare subclass of drug-induced lesions, or repair of a fraction of frequent DNA lesions. In order to distinguish between these possibilities platinum-adducted plasmids were incubated in a repair reaction mixture, repurified, and then incubated again in a second reaction. If the repair signal were principally due to efficient repair of rare lesions, these would be depleted during the first incubation, leading to a much reduced level of repair synthesis in the second incubation. Repair occurring exclusively in the second reaction was monitored by omitting [^{32}P]dATP from the reaction mixture during the first cycle of repair, including label only in the second cycle reaction. Incorporation of radioactive material in this second reaction could then be directly compared with incorporation in single reactions that used portions of the same DNA samples. Figure 7 shows the result of such an experiment, using plasmids with 4.7 *cis*-DDP adducts or 6.7 *trans*-DDP adducts. Incorporation of radioactive material was nearly identical for DNA that had been incubated in either one or two reactions. Thus, repair synthesis stimulated by platinum adducts is principally due to repair of a fraction of frequently occurring lesions, rather than efficient repair of rare adducts. When the plasmid DNA was electrophoresed without previous digestion with EcoRI, most label in plasmids damaged with *cis*-DDP was found in closed circular plasmid molecules, indicating that most repair patches are ligated after DNA synthesis (Figure 7). In this experiment a larger fraction of the label was seen in open circular species in plasmids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Markers
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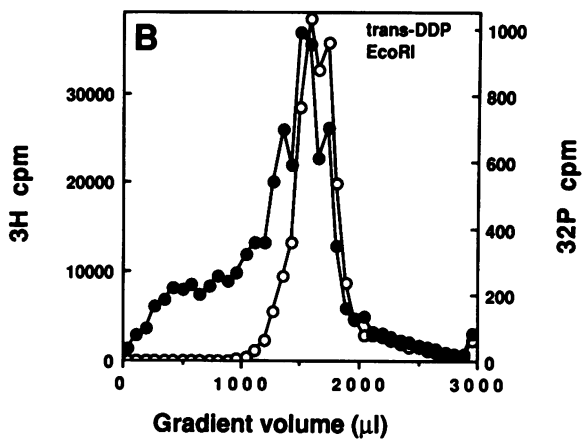
B
trans-DDP
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800
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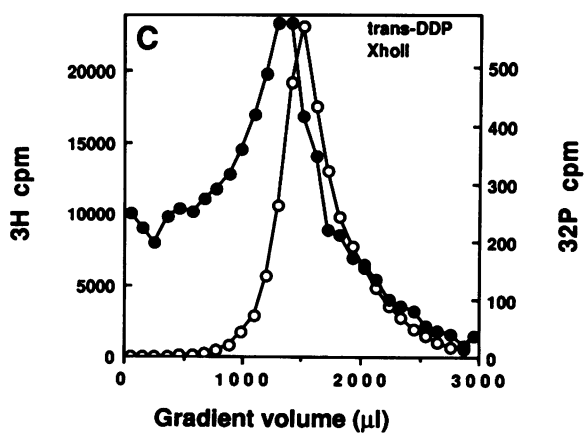
C
trans-DDP
XhoII
3H cpm
32P cpm
Gradient volume (µl)
20000
15000
10000
5000
0
500
400
300
200
100
0
1000
2000
3000

damaged with *trans*-DDP. The radioactive label observed in unligated plasmids is likely to include products of exonucleolytic degradation and resynthesis.

Background incorporation of radioactivity into undamaged plasmids

A key component in the cell-free DNA repair system employed here is the use of plasmid DNA circles purified through consecutive gradient centrifugations (30). However, even with such substrates, the background DNA synthesis in undamaged pBR322 plasmids in repair reactions with *cis*-DDP or *trans*-DDP damaged pAT153 plasmids was high, reaching up to 50% of the synthesis on the damaged plasmids (Figures 3–4). The background incorporation in untreated pBR322 plasmids was reduced 3–4-fold by repurifying the plasmids on a second neutral sucrose gradient before use in repair assays (Table 2). The effect can also be seen by comparing the autoradiographs in Figures 2A and 3A; more highly purified plasmid was used in the experiment in Fig. 2 than in Fig. 3. It is likely that this difference is ascribable to the decrease in nicked circular plasmid species achieved by repurification. Similar high background levels of DNA synthesis, caused by the presence of nicked circular plasmid species, have also been observed in repair reactions with UV-damaged plasmids (P. Robins and R. Wood, in preparation).

An unusual feature of background DNA synthesis can be seen in Figures 2B–C. The level of background DNA synthesis was low when only undamaged plasmids were present in the reaction mixture, but when *cis*-DDP or *trans*-DDP damaged pAT153 plasmids were present, there was increased incorporation of radioactivity into the undamaged pBR322 plasmid. We eliminated the possibility that the background synthesis was due to residual free drug, since addition of *cis*-DDP to repair reactions did not alter the level of background incorporation. It was considered possible that reaction of monoadducts in damaged plasmids with undamaged plasmids might stimulate background repair synthesis, but pre-incubating *cis*-DDP damaged or *trans*-DDP damaged pAT153 plasmids with untreated pBR322 plasmids for 6 hours at 37°C before repair reactions did not increase background DNA synthesis. Another possibility was that repair-related recombination events might occur between the highly homologous pAT153 and pBR322 plasmids, resulting in transfer of DNA containing radioactive material to undamaged plasmids. When reactions were performed with undamaged plasmid pCS58 (derived from pAT153 by insertion of a nonhomologous 3.1 kb fragment of *E. coli* DNA into the HindIII site) in place of pBR322, the same relative level of background incorporation was seen in nonhomologous and homologous portions of the plasmid. Thus it is unlikely that the increased background is due to homologous recombination.

Deficiency of extract from xeroderma pigmentosum cells in performing repair synthesis in response to cis-DDP, trans-DDP or UV damage and inhibition of nonspecific background DNA synthesis by cis-DDP damage

Extracts prepared from the cell line GM2345, derived from an individual with the disease xeroderma pigmentosum (complementation group A) have consistently shown reduced repair

Figure 6. Analysis of DNA repair patch size by equilibrium sedimentation of bromouracil-substituted DNA, for plasmids damaged with *trans*-DDP. Gradients were centrifuged in a Beckmann SW55.0 rotor at 34,800 rpm for 95 h at 25° C; displacement of 'heavy' marker DNA (DD_0): 906 μl.
(*A*) Markers: ^{3}H-labeled 'light' pAT153 DNA (○) and ^{32}P-labeled 'heavy' DNA (●).
(*B*) Plasmid pAT153 containing 6.7 *trans*-DDP adducts per molecule, linearized with EcoRI (●), 'light' pAT153 DNA (○).
(*C*) Plasmid pAT153 containing 6.7 *trans*-DDP adducts per molecule, digested with XhoII (●), 'light' pAT153 DNA (○).

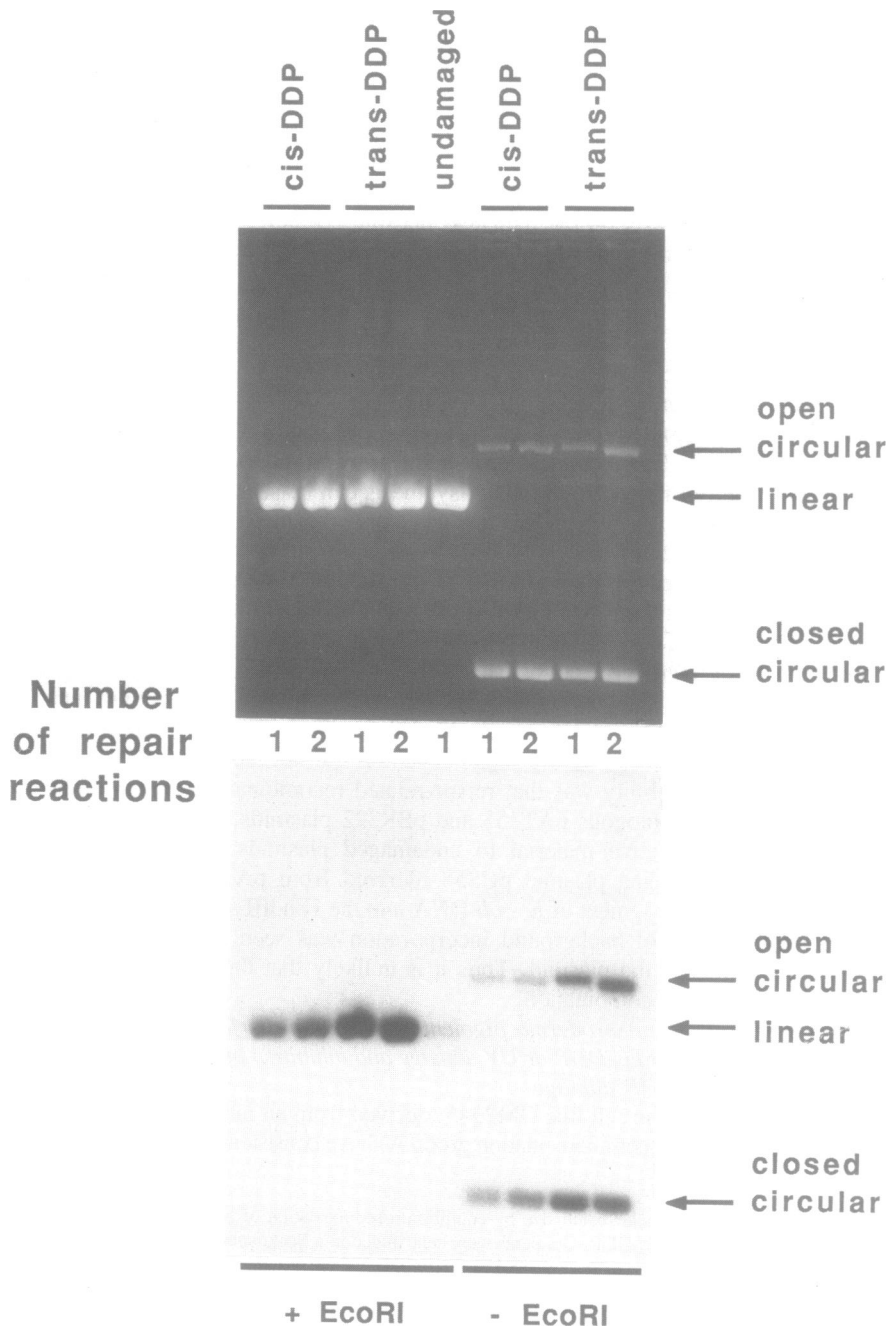
cis-DDP
trans-DDP
undamaged
cis-DDP
trans-DDP
open circular
linear
closed circular
Number of repair reactions
1 2 1 2 1 1 2 1 2
open circular
linear
closed circular
+ EcoRI
- EcoRI

Table 2. DNA synthesis in nondamaged pBR322 plasmids incubated in repair reactions with damaged pAT153 plasmids: reduction of nonspecific synthesis by repurification of plasmid DNA.

No. of neutral sucrose gradients used to purify plasmids	Nonspecific synthesis in pBR322 plasmid DNA (fmol dAMP per reaction) in presence of pAT153 plasmid damaged by:	
	cis-DDP	UV
1 (n = 9)	238 ± 37	195 ± 36
2 (n = 3)	59 ± 6	59 ± 17
Ratio	4.0	3.3

Reactions with *cis*-DDP damaged pAT153 DNA had 12.9−19.7 adducts per molecule, which gave an average incorporation of 739 and 507 fmol dAMP after purification on 1 and 2 sucrose gradients, respectively. Reactions with UV damaged DNA (400−450 J/m^2) had 11−12 cyclobutane pyrimidine dimers and 3−4 (6−4) photoproducts per plasmid molecule, which gave an average incorporation of 1069 and 932 fmol dAMP after purification on 1 and 2 sucrose gradients, respectively. The values are the means ± standard error for the indicated number (n) of experiments.

synthesis in response to UV-induced lesions (30). However, extracts from this cell line can still yield nonspecific DNA synthesis in undamaged plasmids, and this is occasionally higher than the nonspecific activity found with GM1953 normal cell extracts. An experiment with such a GM2345 cell extract demonstrates a further characteristic of nonspecific repair synthesis in damaged plasmids. Figure 8 shows the action of the extract on plasmids damaged by UV, *cis*-DDP or *trans*-DDP. In each case, the extract was defective in performing damage-dependent repair synthesis. Interestingly, the incorporation into pAT153 was significantly inhibited by the presence of *cis*-DDP adducts but not by *trans*-DDP or UV-induced lesions. The autoradiograph in Figure 8 is overexposed to more clearly demonstrate this feature. The result indicates that *cis*-DDP adducts can suppress background DNA synthesis. This conclusion is strengthened by examination of the profiles of the gradients in Figure 5. The higher density peak corresponding to synthesis of long DNA tracts by extract from the normal cell line (Figure 5B,C, and D) is absent or suppressed in plasmids with 39 *cis*-DDP adducts per molecule (Figure 5E and F). Together, these results indicate that *cis*-DDP adducts, when present in sufficient numbers, can inhibit the extension of repair patches associated with nonspecific background DNA synthesis.

DISCUSSION

In the present study we found that 5−8% of the *cis*-DDP adducts and 3−5% of the *trans*-DDP adducts in plasmid DNA were interstrand cross-links, a significantly higher proportion

Figure 7. Comparison of the DNA repair synthesis in *cis*-DDP (4.7 adducts per molecule) and *trans*-DDP (6.7 adducts per molecule) damaged pAT153 plasmids obtained in two sequential repair reactions. Before incubation with GM1953 extract, reaction mixtures were divided into two aliquots and [α-^{32}P]dATP was added to one of them, while the other was incubated without radioactive label. After purification, plasmid DNA incubated without label was reused in second repair reactions with [^{32}P]dATP. DNA was repurified and portions linearized with EcoRI before electrophoresis. Upper panel: DNA fluorescence. Lower panel: autoradiograph.

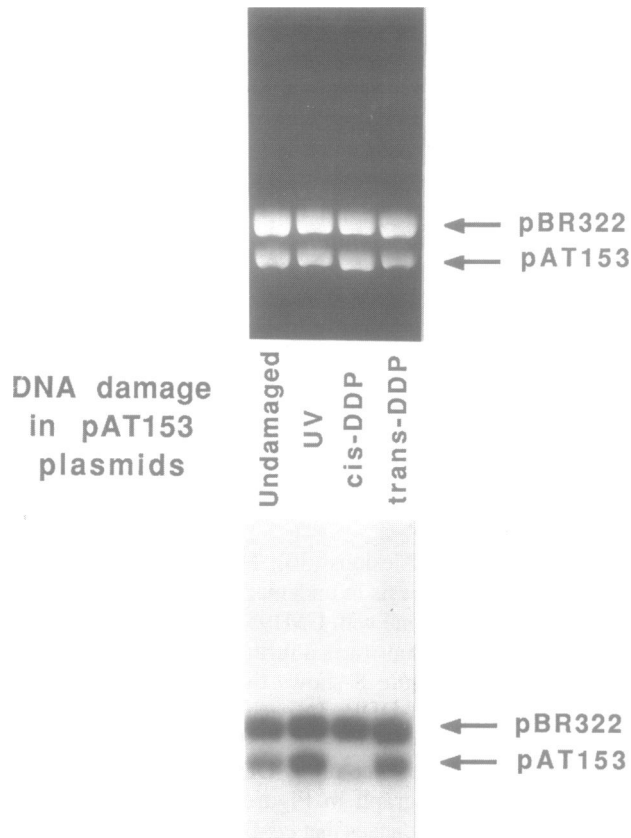

Figure 8. Repair reactions using an extract from GM2345 xeroderma pigmentosum complementation group A cells. Standard repair reactions were performed with 150 μg GM2345 extract protein in 50 μl reaction volumes containing undamaged pBR322 plasmids and pAT153 plasmids that were either undamaged, UV-irradiated (450 J/m^2), treated with *cis*-DDP (19.7 adducts per molecule) or *trans*-DDP (28.4 adducts per molecule). Upper panel: DNA fluorescence. Lower panel: autoradiograph.

than the 1−2% reported by other investigators (5, 6, 8, 11). A possible explanation for this difference is that most data in the literature have been obtained by incubating genomic DNA of mammalian origin with drug, while we used bacterial plasmid DNA. It has been suggested that for stereochemical reasons CG sequences may be the main site of formation of interstrand cross-links (41). It is well established that CG sequences are underrepresented in the DNA of vertebrates. In calf thymus DNA, for example, CG sequences occur with a frequency of 1.4% versus an expected frequency of 4.6% (42). In contrast, the base sequence of plasmid pAT153 showed that it contains slightly more CG sequences than expected from the content of cytosine and guanine base residues (7.6% versus 7.2%). Thus, there are 5- to 6-fold more CG sites available for formation of interstrand cross-links in plasmid pAT153 than in DNA from mammalian cells, which could account for the 3- to 4-fold higher yield of these lesions.

Repair synthesis could be detected in plasmids containing only a single *cis*-DDP adduct

on the average (Figures 2A, B). With both *cis*- and *trans*-DDP, increasing DNA repair synthesis was obtained with increasing levels of drug adducts per molecule. The plateau in repair synthesis observed at high levels of adduction is unlikely to result from blockage of repair synthesis by platinum adducts (43), since for *cis*-DDP, the plateau was reached at a level of approximately 13 adducts per plasmid. This corresponds to an average inter-adduct distance of 580 nucleotides, while the average repair patch size was much shorter. Furthermore, most incorporation of radioactivity was seen in closed circular, ligated plasmids (Figure 7), which would not be the case if patch synthesis had been blocked prior to completion by unrepaired DNA adducts. When plasmids treated with *cis*-DDP (13−20 adducts per molecule) or *trans*-DDP (7−25 adducts per molecule) were UV irradiated (350 J/m^2) and used in repair reactions, the additional repair synthesis obtained was comparable to that seen in plasmids damaged with UV only, indicating that the presence of *cis*-DDP or *trans*-DDP adducts does not interfere with the synthesis of repair patches at UV-induced lesions (data not shown).

The total DNA synthesis in damaged plasmids measured in the *in vitro* repair assay is the sum of unspecific background DNA synthesis and damage-induced repair synthesis, which in turn depends both on the number of repair events initiated and the size of the repair patches. The lower level of DNA synthesis in *cis*-DDP compared to *trans*-DDP damaged plasmids in the same experiments (Table 1) could be caused by less frequent incision at *cis*-DDP adducts than *trans*-DDP adducts, by the reduced background DNA synthesis in plasmids containing *cis*-DDP adducts (Figures 5E and 8), by a slightly smaller average patch size for *cis*-DDP adducts, or by a combination of these factors.

Interpretation of the estimated repair patch sizes of 115 to 160 nucleotides for *cis*-DDP and *trans*-DDP adducts, derived from the isopycnic sedimentation analysis, is complicated by the occurrence of some radioactive material at densities near that of fully substituted DNA. Thus, the repair patch sizes obtained for *cis*-DDP and *trans*-DDP treated plasmids may not be significantly different from one another, and should be regarded as upper estimates. During excision repair of UV-damaged chromosomal DNA in cultures of human cells, repair patch lengths of 20−40 nucleotides have been observed (44). The patch size increases to 50−90 nucleotides in the presence of aphidicolin, apparently because gaps formed after excision of adducts are extended by exonucleases when DNA polymerases α and δ are inhibited. To our knowledge no estimate has been reported of patch sizes for the repair of *cis*-DDP or *trans*-DDP damage *in vivo*. In the cell-free system, initial gaps created by incision near DNA damage are also susceptible to enlargement by exonucleases present in the extracts.

From the level of damage-induced DNA repair synthesis and the estimated repair patch sizes the efficiency of repair of drug adducts was calculated and found to be similar for both drugs (0.5 to 3%, depending on the level of adduction). Thus, the dramatic difference in cytotoxicity of the isomers does not seem to be due to a large difference in removal of *cis*-DDP and *trans*-DDP adducts. The relatively low efficiency of repair is comparable to that seen with other systems using crude cell extracts for SV40 replication (45) or transcription *in vitro* (33). Since both *cis*-DDP and *trans*-DDP induce a mixture of different drug lesions, it is not certain which types of adduct induce repair synthesis. The experiment in which drug damaged plasmids were used in two sequential assays (Figure 7) suggests that only a fraction of potentially repairable drug lesions are removed during the course of a single reaction. We therefore favour the hypothesis that a minority of the more frequent DNA lesions are repaired, *e.g. cis*-DDP induced DNA 1,2 intrastrand cross-links and *trans*-

DDP induced DNA monoadducts. Studies of the repair of DNA substrates containing a single defined *cis*-DDP or *trans*-DDP lesion are required in order to provide more definitive information.

The plateau in DNA repair synthesis in response to platinum adducts (Figure 2B and C) is consistent with the possibility that the limiting factor in the repair reactions is not the number of adducts, but a factor in the cell extract which is depleted at these levels of DNA damage. Recently, a protein that binds specifically to *cis*-DDP damaged DNA has been reported (46). The binding of such a factor to an adduct may be the initial step in the assembly of a multi-component complex that incises a DNA strand at the site of damage. We observed that an extract made from a cell line representative of the excision repair deficiency syndrome xeroderma pigmentosum (complementation group A) was defective in damage-dependent repair synthesis in response to the presence of *cis*-DDP or *trans*-DDP adducts in plasmid DNA (Figure 8). This observation indicates that the cell free system employed here may be a valuable tool in the search for proteins involved in the repair of *cis*-DDP and *trans*-DDP adducts in DNA. This is of practical interest, since differences in DNA repair capacity are likely to be of importance for the varied response of tumors to clinical therapy with *cis*-DDP.

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