Induction of UV-Damage Recognition Protein by Cisplatin Treatment†

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ABSTRACT: The biological functions of DNA damage recognition proteins are not well understood. Using the band shift assay, we detected in nuclear extracts from human carcinoma cell lines damage recognition protein which bound selectively to UV-damaged double-stranded DNA. No consistent correlation was found between steady-state levels of the UV-damage recognition protein and either cisplatin cytotoxicity or DNA repair activity. However, cisplatin treatment caused accumulation of the UV-damage recognition protein. The cisplatin-responsive induction of UV-damage recognition protein in the nucleus was higher in cisplatin-resistant cell lines than in their parental counterparts. These results imply that the level of inducibility in response to treatment, but not the constitutive binding activity, of UV-damage recognition protein correlates with cisplatin resistance. Inhibition of UV-damage recognition protein expression by actinomycin and cycloheximide suggests that induction of UV-damage recognition protein requires *de novo* RNA and protein synthesis, rather than post-translational modification of pre-existing protein. The increased level of UV-damage recognition protein after cisplatin treatment could be a direct response to adduct formation, since it correlated with the number of Pt−DNA adducts. However, it could also be a secondary effect of DNA replication inhibition following DNA damage, since inhibition of DNA synthesis by aphidicolin and hydroxyurea caused the same induction of UV-damage recognition protein. Inducibility of UV-damage recognition protein binding activity by Pt drug treatment suggests involvement of this protein in drug resistance, although a direct link between its function and DNA repair or damage tolerance has not been demonstrated.

One of the major limitations to cisplatin (CP)[1] treatment of several human malignancies is the acquisition of resistance to this drug in initially responsive tumors. Among a number of mechanisms that contribute to resistance are enhanced DNA repair and increased ability of the cell to tolerate unrepaired lesions on the DNA. DNA damage recognition could be an important step both in DNA repair (by providing effective coupling of different enzymes involved in DNA repair to the target lesion) and in damage tolerance (by facilitating replicative bypass to allow cells to survive in S phase and repair DNA during G2 arrest).

Several mammalian proteins that bind to DNA damaged by chemical or physical agents including CP and UV irradiation have been discovered and characterized (Feldberg, 1980; Feldberg et al., 1982; Carew & Feldberg, 1985; Toney et al., 1989; Fujiwara et al., 1990; Abramic et al., 1991; Robins et al., 1991; Andrews & Jones, 1991; Bruhn et al., 1992; Clugston et al., 1992; Hughes et al., 1992; Billings et al., 1992; Hwang & Chu, 1993; McLaughlin et al., 1993; Takao et al., 1993; Keeney et al., 1993; Chao & Huang, 1993b; Chao et al., 1993; Bissett et al., 1993; Lawrence et al., 1993; van-Assendelft et al., 1993). However, the cellular functions of these damage recognition proteins (DRP) are unclear. They have been proposed to either facilitate or interfere with DNA repair at the stage of lesion recognition, or change sensitivity to the damage by several possible mechanisms (Donahue et al., 1990; Pil & Lippard, 1992; Protic & Levine, 1993; Chao & Huang, 1993a; Jones & Wood, 1993; Chu, 1994).

Among these proteins, UV-damage recognition protein (UV-DRP) (also called XPE binding factor), which has a high affinity for (6−4) photoproducts in UV-irradiated DNA (Hirschfeld et al., 1990; Treiber et al., 1992; Keeney et al., 1993; Reardon et al., 1993), has attracted the most attention. UV-DRP activity is associated with a heterodimer formed from 124 and 41 kDa polypeptides (Keeney et al., 1993; Reardon et al., 1993). It has been postulated that this protein might be the damage recognition unit of the nucleotide DNA excision repair complex (Chu & Chang, 1988; Chu et al., 1990; Hwang & Chu, 1993; Chu, 1994). Several lines of indirect evidence support this suggestion. Repair defective cells from some XPE patients are missing UV-DRP binding activity (Chu & Chang, 1988; Chu et al., 1990), and microinjection of less than 10% of the normal cellular amount of the protein can restore the repair capacity of these cells (Keeney et al., 1994). UV-DRP activity is inducible in primate cells by treatment with agents that induce enhanced DNA repair (Protic et al., 1988, 1989; Hirschfeld et al., 1990; McLenigan et al., 1993). Some UV- and CP-resistant cells with increased ability to repair damaged DNA have been reported to exhibit an increased steady-state level of UV-DRP (Chu et al., 1990; Chu & Chang, 1990; Chao et al., 1991a,b; Chao, 1991, 1992, 1993; Chao & Huang, 1993a). UV-DRP recognizes many types of DNA lesions (Feldberg & Grossman, 1976; Feldberg, 1980; Feldberg et al., 1982;

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[1] Abbreviations: CP or cisplatin, *cis*-diamminedichloroplatinum(II); DRP, damage recognition protein; XPE, xeroderma pigmentosum group E complementing protein; XPA, XP group A complementing protein; FCS, fetal calf serum; OP or ormaplatin, tetrachloro-(*d,l-trans*)-1,2-diaminocyclohexaneplatinum(IV); TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PI, propidium iodide; ds, double-stranded; ss, single-stranded.

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Carew & Feldberg, 1985; Reardon et al., 1993). Some investigators have suggested that UV-DRP recognizes Pt−DNA adducts (Chu & Chang, 1988; Chu, 1994) although others have failed to confirm binding of UV-DRP to platinated DNA (Donahue et al., 1990; Chao et al., 1991a; Chao, 1992; van-Assendelft et al., 1993).

However, recent studies have questioned UV-DRP's role in DNA repair (Keeney et al., 1992; Reardon et al., 1993; Chao, 1993; Chao & Sun, 1993). In addition to the fact that complete lack of UV-DRP activity in XPE patients causes only a mild clinical and cellular DNA repair defect, it was found that most (9 out of 12) XPE patients have normal levels of UV-DRP (Kataoka & Fujiwara, 1991; Keeney et al., 1992); and injection of UV-DRP does not stimulate repair in those cells (Keeney et al., 1994). Some lesions, besides CP, known to be recognized by the nucleotide DNA excision repair complex, appear not to be bound by the UV-DRP (Feldberg, 1980; Reardon et al., 1993). UV-DRP binding activity is also induced by agents that do not cause direct DNA damage (Protic et al., 1989; Hirschfeld et al., 1990; Chao et al., 1993). Correlation between UV-DRP expression and either UV or CP cytotoxicity in human cells appears not to be a universal characteristic (Chao, 1993; Chao & Sun, 1993; McLenigan et al., 1993). Finally, recent data suggest that a key component in recognition of DNA lesions during repair could be the XP group A complementing protein (XPA) (Robins et al., 1991; Jones & Wood, 1993). Since there is good evidence that XPA is involved in an early step of nucleotide DNA excision repair, it was postulated that XPA plays a direct role in the recognition of and incision at damaged sites in DNA. In addition to recognition of UV damage on DNA, XPA also can bind to DNA treated with CP (Jones & Wood, 1993). Taken together, these data suggest that if UV-DRP plays any role in DNA repair, it could not be a sole damage recognition unit of the nucleotide DNA excision repair complex.

To better understand the role of UV-DRP in DNA repair and cell sensitivity to CP, we examined whether this protein was CP damage inducible. Using the band shift assay, we demonstrate here that the level of UV-DRP inducibility in response to CP treatment, but not its constitutive binding activity, correlates with CP resistance. Induction of UV-DRP binding by CP appears to be due to either the formation of Pt−DNA adducts or the inhibition of DNA replication caused by DNA damage.

MATERIALS AND METHODS

Cells and Cell Treatment. The 2008, C13*, A2780, and A2780/CP human ovarian carcinoma cell lines were kindly provided by Dr. P. Andrews (Georgetown University). The human cervical carcinoma HeLa, GH1 and the human fibrosarcoma HT1080, HT2 cell lines were a gift from Dr. G. Chu (Stanford University). The XP cell lines GM02415 (group E) and GM2252 (group B) and the green monkey kidney CV1 cell line were purchased from the UNC Lineberger Comprehensive Cancer Center tissue culture facility. 2008, C13*, A2780, and A2780/CP cell lines were cultured in RPMI-1640 medium, which contained 10% fetal calf serum (FCS); HeLa, GH1, HT1080, and HT2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS; GM2252 cells were maintained in RPMI-1640 containing 15% FCS; GM02415 and CV1 cell lines were grown in Eagle's minimum essential medium with 20% and 10% FCS, respectively. All cells were maintained in the presence of antibiotics.

The CP-resistant A2780/CP, HT2, and GH1 cell lines have enhanced ability to repair Pt−DNA adducts compared to parental counterparts (Behrens et al., 1987; Lai et al., 1988; Masuda et al., 1990; Chu & Chang, 1990; Parker et al., 1991), while the 2008 and its resistant derivative cell line C13* do not differ in repair capacity (Zhen et al., 1992; Jekunen et al., 1994). The A2780/CP and C13* cell lines are characterized by increased replicative bypass (Mamenta et al., 1994).

Cells were grown to about 85% confluence before the experiments were started. Since some of the drug treatments decreased cell viability significantly, such conditions allowed the retention of a sufficient number of viable cells for the analysis of UV-DRP levels after drug treatment. At the end of most experiments, cells in control plates reached confluence, which caused an increase in the G1 phase and a decrease in S phase compartment sizes, but had no effect on UV-DRP steady-state level. To determine the dose−response and time course for UV-DRP induction, cells were treated with different concentrations of CP or ormaplatin (OP) for 1 h or irradiated with a UV Stratalinker 1800 (Stratagene) and then incubated in fresh medium for 6−48 h (unless otherwise specified). To determine the mechanism of UV-DRP induction, the control or CP-treated A2780 cells were incubated for 40 h in the medium containing actinomycin D (3.5 μg/mL) or cycloheximide (1 μg/mL). To study the effect of cell cycle distortion and DNA synthesis inhibition on UV-DRP level, the A2780 cells were incubated for 40 h in the medium supplemented with 0.5% fetal calf serum or treated with aphidicolin (5 μM), hydroxyurea (2 mM), or cyclopentanone prostaglandin PGA_2 (5 and 10 μg/mL) for 24−40 h.

Cytotoxicity was determined by the 3 day growth inhibition assay (Andrews & Albright, 1992). ID_{10}, ID_{50}, and ID_{90} refer to the doses of DNA damaging agent required to inhibit cell growth by 10%, 50%, and 90%, respectively, 72 h after treatment. The results of the growth inhibition assay strongly correlated to cell viability measured by trypan blue exclusion. Levels of Pt bound to DNA in parental and CP-resistant cell lines were measured in isolated DNA immediately after drug treatment using a Perkin-Elmer 560 atomic adsorption spectrophotometer with an HGA 500 graphite furnace and an AS-1 autosampler.

DNA Probe. Synthetic oligonucleotide T_4C (Treiber et al., 1992) used either as probe or competitor was chosen because the T_4C sequence efficiently forms a TC (6−4) photoproduct and is a superior substrate for the UV-DRP binding activity. The oligonucleotide was synthesized on an Applied Biosystems 380-A DNA synthesizer and purified by HPLC at the Nucleic Acids Core Facility (UNC Lineberger Comprehensive Cancer Center). It was 5′ end labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP, purified by ethanol precipitation, and annealed with complementary strand by heating at 70 °C for 10 min and then cooling slowly (over a 2−3 h) to room temperature. The resulting duplex was purified by 15% native polyacrylamide gel electrophoresis. For quantitation, the gel was stained with 0.5 μg/mL ethidium bromide and the amount of DNA determined in comparison with a known quantity of standard oligonucleotide using a Macintosh image processing system for gel

quantitation (UNC Lineberger Comprehensive Cancer Center). The duplex DNA was then eluted and purified from the gel using a QIAEX Gel Extraction Kit (QIAGEN Inc.). The yield of purified oligonucleotide was determined on the basis of recovered radioactivity. The purified DNA was stored in annealing buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) to prevent denaturation. Probes were irradiated with a UV Stratalinker 1800 (Stratagene) until the desired dose was achieved (usually 20 kJ/m^2).

Band Shift Assay. Nuclear extracts were prepared as described in Hennighausen and Lubon (1987). To achieve maximum enrichment of the UV-DRP, several salt concentrations (100−600 mM NaCl) for extraction of the nuclei were examined. It was found that the salt concentration (400 mM NaCl) of the standard extraction buffer (Hennighausen & Lubon, 1987) was in the optimal range to allow efficient detection of UV-DRP binding activity. Protein concentration was determined by the Bradford assay. DNA−protein complexes were detected as previously described (Hennighausen & Lubon, 1987; Protic & Levine, 1993). Briefly, 0.3 ng of DNA probe was incubated with 2−10 μg of nuclear extract, 2−6 μg of poly(dI-dC), and up to a 5000-fold excess of unlabeled competitor in binding buffer (Hennighausen & Lubon, 1987), in a total volume of 20 μL at 20 °C for 30 min, and then analyzed on a 5% nondenaturating polyacrylamide gel. Gels were imaged and quantified by using a Molecular Dynamics Phosphoimager. Each reaction was normalized to the free probe (i.e., percentage binding = $B/B + F$) before relative values were calculated. Binding in nuclear extracts from untreated cells was generally in the range of 10−20% and was set to 100%. Representative results were obtained by averaging data from at least 8 band shift assays obtained from 2−7 different extract preparations. Differences in induction of UV-DRP binding by DNA damaging agents and DNA synthesis inhibitors varied not more than 7% from extract to extract.

Cell Cycle Analysis. The DNA/cell cycle analysis was performed according to Schaefer et al. (1993). Briefly, after incubation with BrdUrd for 1 h, cells were fixed in 70% ethanol, resuspended in 0.08% pepsin/0.1 N HCl for 20 min at 37 °C, and then in 2 N HCl for 20 min at 37 °C, neutralized by 0.1 M sodium borate, and washed with IFA buffer (Schaefer et al., 1993) containing 0.5% Tween 20. After incubation with 100 μL of anti-BrdUrd fluorescein isothiocyanate (1:5 in IFA buffer, 30 min over ice in the dark), the cells were washed with IFA/0.5% Tween 20, treated with 5 μg/mL RNase A at 37 °C for 15 min, and incubated overnight with 25 μg/mL propidium iodide (PI) in PBS. The cellular DNA content and the amount of incorporated BrdUrd were simultaneously measured using a FACScan flow cytometer (Becton Dickinson). A two parameter histogram of the cell cycle phase distribution and calculations of the percentage of cells in G1, S, and G2 phases were obtained by analyzing 10^4 nuclei for each experimental sample, using Cytomation hardware and software (Cytomation, Inc., Fort Collins, Co).

RESULTS

Detection of UV-DRP in Human Carcinoma Cell Lines. Nuclear extracts from human ovarian and cervical carcinoma and fibrosarcoma cell lines were screened for the presence of the UV-DNA damage recognition protein by band shift assay using UV-irradiated T_4C oligonucleotide. After elimination of the nonspecific protein binding by addition of an excess of poly(dI-dC), one band with mobility different from that of free probe was detected. Figure 1A shows the B_{UV} band formed by UV-irradiated T_4C after incubation with nuclear extract from the A2780 human ovarian carcinoma cell line. Binding to UV-treated double-stranded DNA was damage-specific. The UV-DRP did not bind to labeled undamaged or damaged single-stranded DNA (gels not shown). In addition, no shift to B_{UV} was found using labeled undamaged (Figure 1) or platinated (not shown) double-stranded DNA. Competition assays were performed to ensure the specificity of UV-DRP binding. The presence of up to a 5000-fold excess of damaged or undamaged single-stranded unlabeled competitor did not affect binding (Figure 1A). An excess of undamaged (Figure 1A) or platinated (not shown) double-stranded oligonucleotide also did not compete with labeled probe for UV-DRP binding. In contrast, a 50-fold excess of UV-irradiated unlabeled double-stranded probe totally inhibited the binding (Figure 1A). These specificity data suggest that the UV-DRP detected in

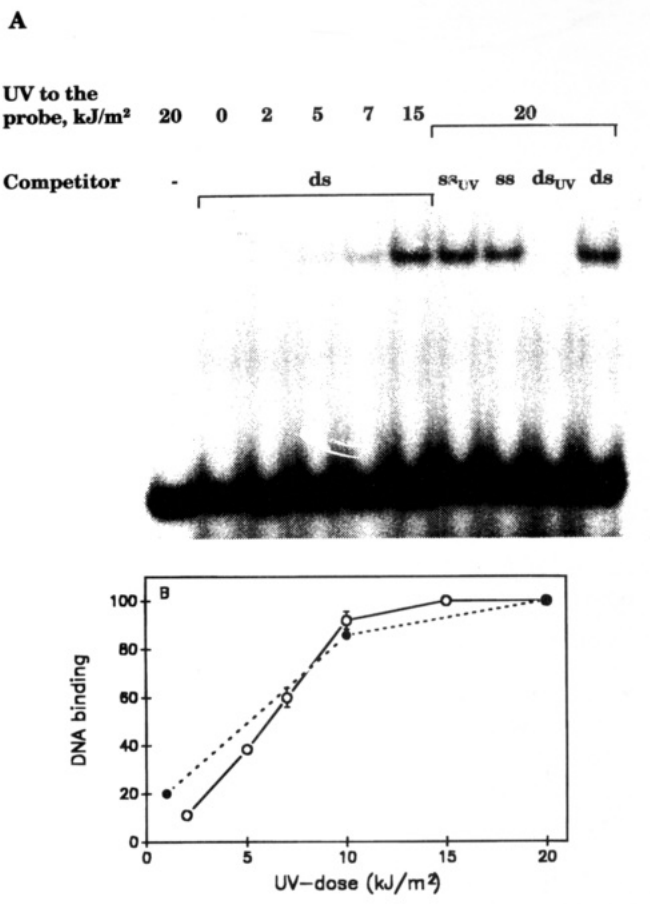

FIGURE 1: Specificity and dose−response analysis of UV-DRP binding. (A) Band shift assay of T_4C oligonucleotide probe either unirradiated or irradiated with increasing doses of UV light and 5 μg of untreated A2780 nuclear extract proteins in the presence of a 5000-fold excess of the unlabeled T_4C competitor (ss/ds: unirradiated single or double stranded; ss_{UV}/ds_{UV}: UV-irradiated single or double stranded oligonucleotide). Lane 1: Control lane without nuclear extract. (B) Summary of UV dose−response data from experiments with T_4C irradiated with increasing doses of UV light. The highest UV-DRP binding is assigned to 100%. (○) Quantitation of the dose−response analysis; (●) from Treiber et al. (1992). Results are the means ± SEM of data obtained from 8 band shift assays with 2 different extract preparations. When the error bars are not shown, they are smaller than the figure symbols.

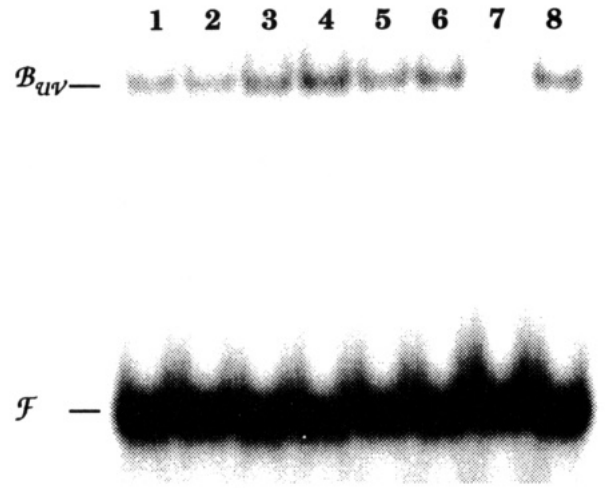

FIGURE 2: Detection of UV-DRP binding activity in nuclear extracts from A2780 (lane 1), A2780/CP (lane 2), 2008 (lane 3), C13* (lane 4), HeLa (lane 5), XPB (lane 6), XPE (lane 7), and CV1 (lane 8) cells. 10 μg of nuclear extract from each cell line was used in the binding reaction. *F*: the position of free UV-T_4C probe. B_{UV}: UV-DRP−DNA complex.

this study was similar to the UV-DRP described previously (Chu & Chang, 1988, 1990; Protic et al., 1989; Hirschfeld et al., 1990; Chao et al., 1991a; Chao, 1992; Treiber et al., 1992; Keeney et al., 1993; van-Assendelft et al., 1993). To further confirm these findings, we performed several control studies. As shown in Figure 2, B_{UV} has the same mobility for all human cell lines tested and for monkey kidney CV1 cell line. UV-DRP binding activity was absent in nuclear extracts from the XPE cell line and was inducible by UV light in CV1 cells (data not shown), which agrees with published data (Protic et al., 1989; Hirschfeld et al., 1990). Using T_4C oligonucleotide irradiated with increasing doses of UV light, we found a dose-dependent increase in the intensity of the shifted band, with a plateau at 10 kJ/m^2 (Figure 1). This is consistent with the UV dose−response analysis of UV-DRP binding to T_4C oligonucleotide described by Treiber et al. (1992) (Figure 1B).

Steady-State Level of UV-DRP in Cells with Different Sensitivities to CP. To determine whether resistant cell lines have an increased level of UV-DRP, nuclear extracts from different cell lines were assayed for UV-DRP binding. Table 1 shows the cytotoxicity of UV and CP, and UV-DRP steady-state levels for four pairs of parental and CP-resistant human carcinoma cell lines. The data indicate that constitutive levels of UV-DRP activity were similar in three pairs of CP-resistant and parental cell lines (A2780 and A2780/CP, 2008 and C13*, and HT1080 and HT2). UV-DRP was overexpressed in CP-resistant GH1 cells, which is consistent with published data (Chu & Chang, 1990). To determine whether these data were dependent on assay conditions, nuclear extracts were tested for binding activity as a function of the amount of extract. As demonstrated in Figure 3, UV-DRP binding was enhanced with increasing amount of nuclear extract per binding reaction, but differences in its level between cell lines remained constant. Protic and Levine (1993) earlier showed dependence of UV-DRP activity on buffer salt concentration. Changes in concentration of monovalent (KCl: 50−150 mM) or divalent ($MgCl_2$: 5−20 mM) cations in the binding buffer did not affect the results (not shown). Therefore, the data in Table 1 suggest that development of CP and/or UV resistance and the enhancement in the DNA repair activity cannot be attributed to changes in UV-DRP constitutive levels in 3 out of 4 pairs of cell lines. Thus, the previously described increase in expression of UV-DRP in CP- and UV-resistant cell lines (Chu et al., 1990; Chu & Chang, 1990; Chao et al., 1991a,b; Chao, 1991, 1992; Chao & Huang, 1993a) is not a general phenomenon. This conclusion agrees with reports from other laboratories (Chao, 1993; Chao & Sun, 1993; McLenigan et al., 1993). Lack of correlation between UV-DRP steady-state level and sensitivity to CP or UV does not exclude involvement of this protein in resistance in all cell lines, since acquisition of resistance is a multifactorial process that varies from cell line to cell line. Furthermore, the inducibility of the protein may be more significant for cytotoxicity than its constitutive level. To further determine the role of UV-DRP in the cellular response to CP treatment, and taking into account UV-DRP induction by other DNA damaging agents (Protic et al., 1989; Hirschfeld et al., 1990), we examined changes in the UV-DRP level in different cell lines following CP treatment.

Table 1: Lack of Correlation between Constitutive Levels of UV-DRP and Cell Sensitivity to CP and UV

	UV cytotoxicity		CP cytotoxicity			
cell line	ID_{50}[a] (J/m^2)	RR_{ID50}[b]	ID_{50}[a] (μM)	ID_{90}[a] (μM)	RR_{ID50}[b]	UV-DRP level[c]
A2780	9.0	1	4.9	29.0	1	1 ± 0.03
A2780/CP	20.0	2.22	24.4	115.0	4.98	0.92 ± 0.01
2008	7.3	0.81	3.1	26.0	0.63	1.44 ± 0.03
C13*	6.7	0.74	37.0	212.0	6.94	1.49 ± 0.02
HeLa	15.0	1.67	2.5	12.5	0.51	1.65 ± 0.01
GH1	nd[d]	nd	11.6	58.3	2.37	2.35 ± 0.03
HT1080	10.0	1.11	5.0	30.0	1.02	0.78 ± 0.01
HT2	15.0	1.67	21.2	90.0	4.33	0.74 ± 0.01

[a] ID_{50} and ID_{90} are doses required to give 50% and 90% inhibition of cell growth as described in Materials and Methods. [b] RR_{ID50}, relative resistance at ID_{50} compared to ID_{50} for A2780. [c] UV-DRP level relative to the level in A2780 (20% UV-irradiated T_4C oligonucleotide bound). 10 μg of nuclear extract was used for all cell lines tested. Results are the means ± SEM of data obtained from 8 gel shift assays with 2 different extract preparations. [d] nd, not determined.

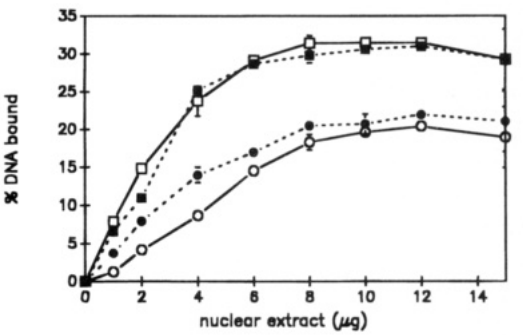

FIGURE 3: UV-DRP binding activity as a function of amount of nuclear extracts added. (●) A2780; (○) A2780/CP; (■) 2008; (□) C13. Results are the means ± SEM of values obtained from 6 band shift assays with 2 different extract preparations. When the error bars are not shown, they are smaller than the figure symbols.

Effect of CP Treatment on UV-DRP Expression. Protic's group previously showed that UV-treated primate cells have induced levels of UV-DRP binding 36−48 h postirradiation (Protic et al., 1989; Hirschfeld et al., 1990). We also observed 1.5- to 2-fold induction of UV-DRP by UV treatment at ID_{90} doses in nuclear extracts from 2008, C13*, A2780, and A2780/CP cell lines (not shown). To test whether UV-DRP from human ovarian carcinoma cell lines

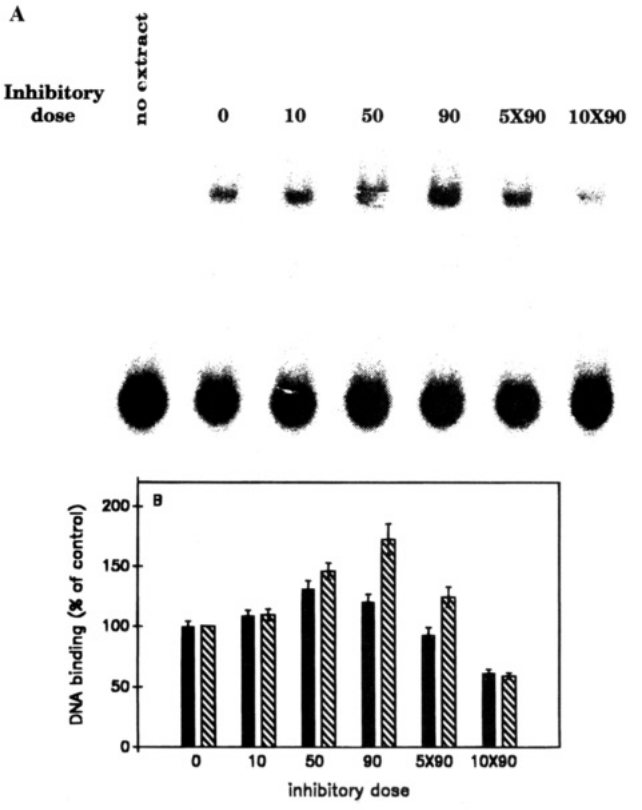

FIGURE 4: Dose-response of UV-DRP induction by CP treatment. Nuclear extracts were prepared from parental A2780 and CP-resistant A2780/CP cells 24 h after treatment with increasing doses of CP. (A) Band shift assay performed with 10 μg of nuclear extract from A2780 cells. (B) Summary of dose−response data from 8 band shift assays with 4 different extract preparations from the A2780 (hatched bar) and A2780/CP (filled bar) cell lines. The UV-DRP binding in nuclear extract from untreated cells is assigned to 100%.

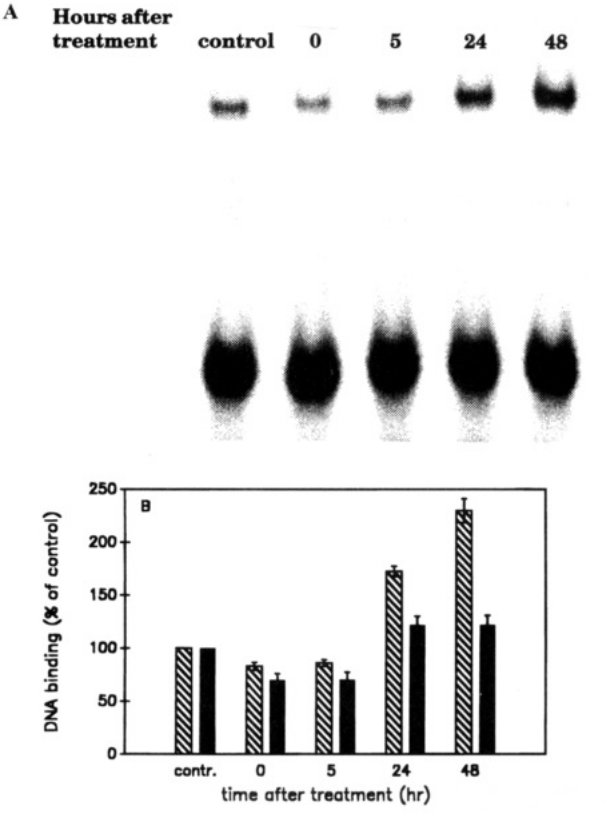

FIGURE 5: Time course of UV-DRP induction by CP treatment. (A) Band shift assay performed with 10 μg of nuclear extract from A2780 cells. Nuclear extracts were prepared from untreated cells or at 0, 5, 24, or 48 h following a 1 h treatment with CP at the ID_{90} dose. (B) Summary of time course data from 8 band shift assays with 3 biological experiments with the A2780 (hatched bar) and A2780/CP (filled bar) cell lines performed as described above. Results are at the drug concentration giving maximal induction for each cell line at 24 h (ID_{90} for A2780 and ID_{50} for A2780/CP). The UV-DRP binding in nuclear extract from untreated cells is assigned to 100%.

responds to CP treatment in a similar way, we performed time course and dose−response analyses of UV-DRP binding in nuclear extracts from A2780 and A2780/CP cells treated with CP. As shown in Figure 4, the relative activity of UV-DRP in nuclear extracts increased with CP dose, reaching a maximum between the ID_{50} and ID_{90} doses. The dose-dependent response suggests that the observed phenomenon is a result of cell treatment with CP. The time course of the induction is shown in Figure 5. The binding activity of UV-DRP increased with time after treatment, reaching the maximum at about 40 h (not shown). The increase in the concentration of UV-DRP in nuclear extracts appeared to be higher for the A2780 cell line than for its CP-resistant counterpart that has enhanced repair activity.

To determine whether other carcinoma cell lines would respond similarly to CP treatment, we tested UV-DRP levels in nuclear extracts from 2008, C13*, HeLa, GH1, HT1080, and HT2 cells exposed to CP at approximately equitoxic doses. The results are shown in Figure 6A. HT1080 and HT2 cell lines appeared to show similar response as A2780 and A2780/CP cells, whereas no increase in UV-DRP concentrations in nuclear extracts from HeLa, GH1, 2008, and C13* cells was observed. A2780/CP, HT2, and GH1 CP-resistant cell lines have acquired an increased level of DNA repair compared to parental A2780, HT1080, and HeLa cells (Behrens et al., 1987; Lai et al., 1988; Masuda et al., 1990; Chu & Chang, 1990; Parker et al., 1991). 2008 and resistant C13* cell lines do not differ significantly in repair activity (Zhen et al., 1992; Jekunen et al., 1994). These results suggested that the acquisition of CP resistance and the alterations in the repair of the DNA damage could not be attributed to the changes in the level of UV-DRP in these cells. However, these data were obtained by standard band shift assay, where equal portions of nuclear extract were used for each data point. Therefore, these experiments, as well as those previously reported (Protic et al., 1989; Hirschfeld et al., 1990; Chao et al., 1991b, 1993; Chao & Huang, 1993b), did not take into consideration changes in amount of other nuclear proteins following DNA damage. It is well established that CP treatment preferentially inhibits cell cycle progression and DNA synthesis compared to RNA and protein synthesis (Sorenson et al., 1990). Thus one would anticipate an increase in total nuclear proteins following CP treatment. Furthermore, not all nuclear proteins would be expected to increase in parallel. For example, Evans and Gralla (1992) have shown that CP treatment induces expression of some genes while inhibiting the expression of others. Therefore, the relative concentration of an individual protein in nuclear extracts may not reflect changes in its absolute amount. By measuring of UV-DRP levels per μg of nuclear proteins (Figure 6A), one is really asking whether the increase in UV-DRP levels is greater or less than the total increase of all other nuclear proteins (which most likely represents the difference between increased accumulation of

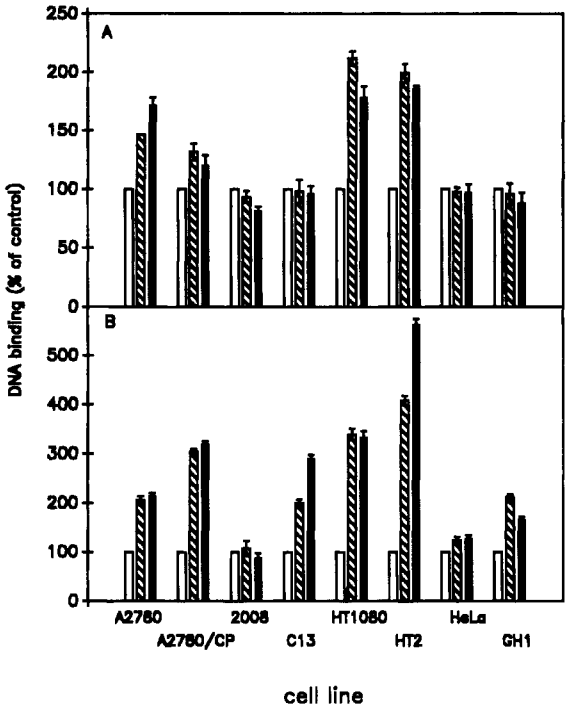

FIGURE 6: Effect of CP treatment on the UV-DRP binding activity in the nuclear extracts from human carcinoma cell lines. (A) Relative concentration of UV-DRP in nuclear extract. (B) Amount of UV-DRP per nucleus. Blank bars: control cells; hatched and filled bars; cells treated with CP at ID_{50} and ID_{90}, respectively. Extracts were prepared 24 h after cell treatment with CP. Data presented as a percentage of untreated control. Results are the means ± SEM of data obtained from 8 band shift assays with 2 different extract preparations. Differences in UV-DRP induction per nucleus between sensitive and resistant cell lines (panel B) were statistically significant at the $p < 0.01$ level for A2780/A2780/CP and 2008/C13* pairs treated with CP at ID_{50} and ID_{90}, for HT1080/HT2 cell lines treated with CP at ID_{90}, and for HeLa/GH1 cell lines treated with CP at ID_{50}.

Table 2: Nuclear Protein Content as a Percent of Untreated Control 24 h after CP Treatment[a]

cell line	ID_{50}	ID_{90}
A2780	141 ± 8	125 ± 6
A2780/CP	216 ± 17	226 ± 15
2008	115 ± 2	108 ± 3
C13	204 ± 14	301 ± 1
HeLa	127 ± 14	132 ± 18
GH1	221 ± 12	189 ± 16
HT1080	167 ± 2	172 ± 15
HT2	173 ± 2	248 ± 20

[a] Isolation of nuclei and determination of protein concentration was as described in Materials and Methods. Results are the means ± SEM of data obtained from 3−5 extract preparations.

some proteins and decreased levels of others). We felt it would be more biologically relevant to ask whether CP treatment would alter UV-DRP levels per nucleus. To make this determination, we treated cells under identical conditions used for measurements of UV-DRP levels, isolated nuclei as described in Materials and Methods, and determined changes in nuclear protein content following CP treatment. As shown in Table 2, we found greater accumulation of nuclear proteins in resistant cells than in parental cells at equitoxic concentrations of CP. Under these conditions the DNA content of the nucleus did not change significantly up to 10 × ID_{90} (flow cytometry measurements, not shown). We used the data on nuclear protein concentration (Table 2) and the previously determined percentage of UV-DRP binding per μg of nuclear extract (Figure 6A) to calculate percentage of UV-DRP binding per nuclei (Figure 6B). When UV-DRP activity was calculated in this manner, it became apparent that the increase in the amount of UV-DRP activity per nuclei was higher in all resistant cell lines than in their parental counterparts. The results were statistically significant at the 1% level of significance. All subsequent data on UV-DRP binding were also normalized to protein content in the nucleus.

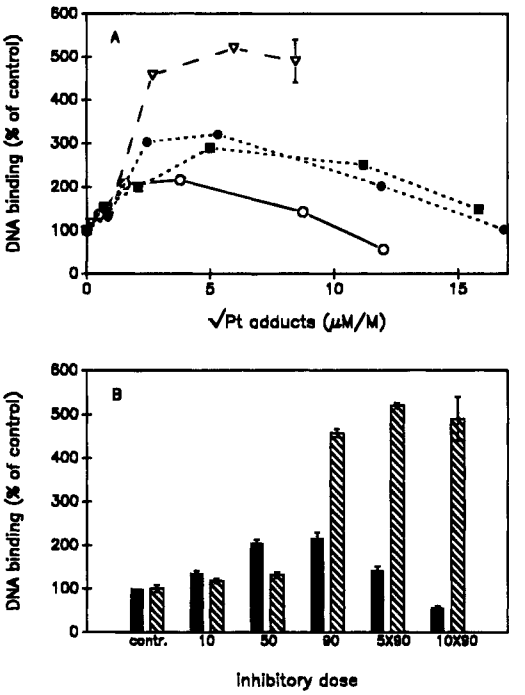

FIGURE 7: Induction of UV-DRP as a function of either DNA damage or cytotoxicity level. (A) Correlation between UV-DRP inducibility and number of Pt adducts on cellular DNA. Extracts were prepared 24 h after cell treatment with CP [(○) A2780; (●) A2780/CP; (■) C13] or OP [(▽) A2780]. (B) Binding of UV-DRP as a function of inhibitory dose. Filled and hatched bars: nuclear extracts from A2780 cells treated with CP and OP, respectively. IDs for CP treatment can be found in Table 1; for OP treatment they are as follows: ID_{10}, 0.2 μM; ID_{50}, 5.19 μM; ID_{90}, 50.6 μM. The UV-DRP binding in nuclear extract from untreated cells is assigned to 100%. When the error bars are not shown, they are smaller than the figure symbols.

In Figure 6B UV-DRP levels were presented for different cell lines at approximately equitoxic concentrations of CP. To better analyze the results with respect to DNA damage, we reploted these data so that induction of UV-DRP, as function of the number of Pt adducts on the cellular DNA, could be compared directly. Because only five doses were taken in this analysis, the precise peak of binding activity cannot be defined, but the data clearly suggest a correlation between the increase in UV-DRP and the level of DNA damage (Figure 7A). The maximum binding activity was observed at about 2.5 × 10^{-5} Pt adducts/nucleotide phosphate. This dependence becomes even more clear from comparison of the effect of platinum carrier ligands on the UV-DRP level. In human ovarian cell lines diaminocyclohexane−Pt adducts are more cytotoxic than *cis*-diammine−Pt adducts (Schmidt & Chaney, 1993). For example, the A2780 cell line requires 2- to 3.5-fold more *cis*-diammine−Pt adducts than diaminocyclohexane−Pt adducts to give comparable inhibition of cell growth (see cytotoxicity data

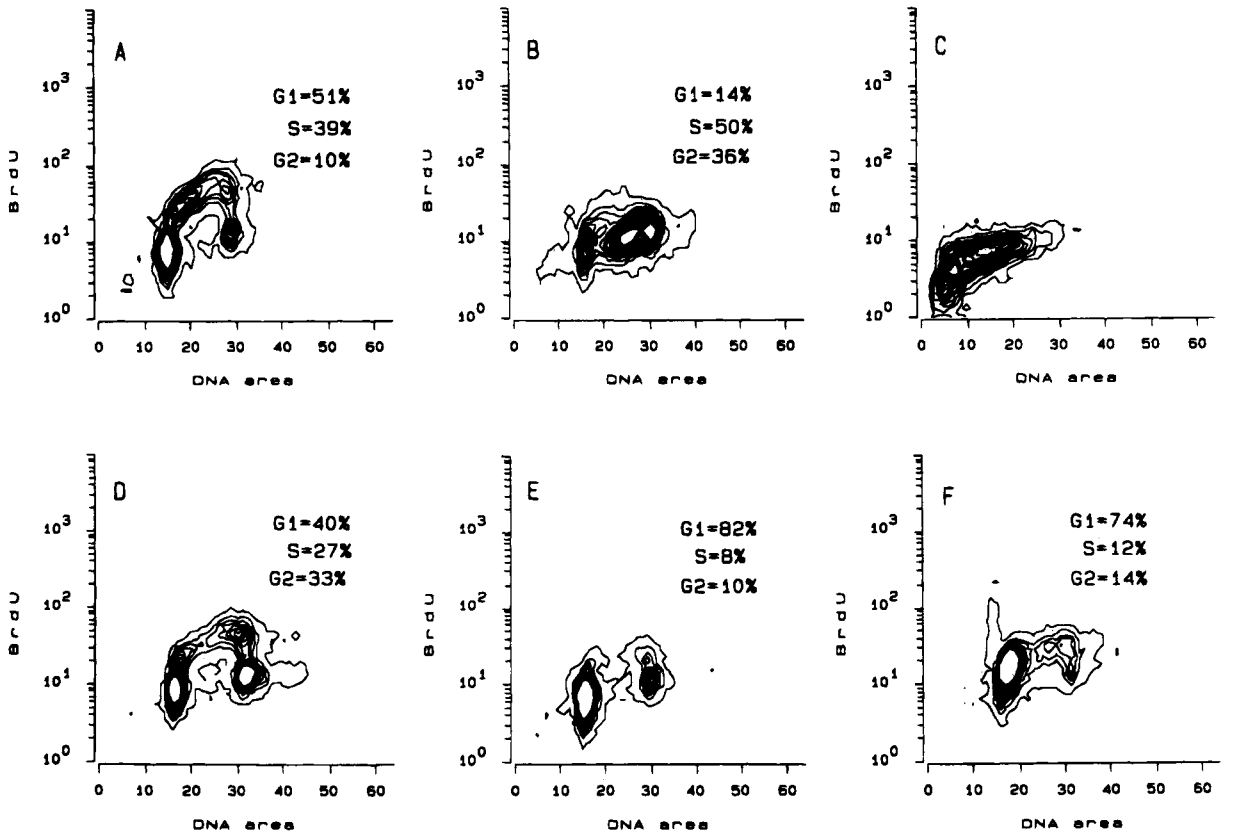

FIGURE 8: Cell cycle analysis of A2780 cells treated with inhibitors of cell cycle progression: x axis, DNA content measured by PI staining; the major population on the left has a G1 DNA content, and the population on the right has a G2 DNA content. Cells to the left of the G1 phase represent apoptotic cells with a fractional DNA content. y axis, new DNA synthesis as measured by BrdUrd incorporation. (A) Control cells. (B and C) Cells that were attached to the plate (B) or floating (C) after incubation in fresh medium for 40 h following 1 h treatment with CP at ID_{90}. (D and E) Exponentially growing (50% confluent) (D) or 85% confluent (E) cells incubated for 24 h in medium containing PGA_2. (F) Cells incubated for 40 h in medium containing hydroxyurea (cells treated with aphidicolin showed the same pattern). Cells actively synthesizing DNA and progressing through S phase have more BrdUrd incorporated into the newly synthesized DNA and could be discriminated from cells arrested in S phase (which have the same DNA content) based on the intensity of staining with anti-BrdUrd fluorescein isothiocyanate. Thus, the increase in the cell population in S phase in panel B represents S phase arrest.

for CP in Table 1 and for OP in Figure 7). When we compared the dose–response for UV-DRP induction in the A2780 cell line for CP and OP (a diaminocyclohexane–Pt compound), maximum increase of UV-DRP binding occurred at approximately the same number of Pt–DNA adducts formed by CP and OP (Figure 7A), but that corresponded to a dose of OP which resulted in much higher cytotoxicity compared to CP (Figure 7B). These results indicate that induction of UV-DRP by Pt drugs correlates with level of DNA damage rather than with survival level of the cells.

Induction of the UV-DRP Binding Requires de Novo RNA and Protein Synthesis. Cell treatment with 5 × ID_{90} and 10 × ID_{90} doses of CP caused decrease in UV-DRP levels (Figures 4 and 7). It seems likely that such reduction of UV-DRP binding is caused by substantial inhibition of RNA and protein synthesis observed at high doses of the drug. We also found that enhanced accumulation of UV-DRP after cell treatment with ID_{50}–ID_{90} doses of CP could be prevented by RNA and protein synthesis inhibitors. The induction of UV-DRP by CP was diminished after incubation of CP-treated cells in the presence of either actinomycin or cycloheximide (gels not shown). Both these inhibitors also decreased the UV-DRP level in untreated cells. For example, the steady-state level of UV-DRP in control A2780 cells was decreased 38% by cycloheximide at a concentration which caused 65% inhibition of protein synthesis. Induction of UV-DRP activity by CP in the presence of cycloheximide was decreased 73% while protein synthesis was inhibited by 60% under the same conditions. These results suggest that the increase in UV-DRP binding activity comes from newly synthesized proteins, rather than from post-translational modification of pre-existing proteins.

UV-DRP Is Induced by Inhibition of DNA Replication, but Not by Cell Cycle Arrest. CP and OP treatment caused significant inhibition of DNA synthesis and an accumulation of cells in late S and G2 phases (Figure 8B) (Sorenson et al., 1990; Balconi et al., 1988; Fujikane et al., 1989; Demarcq et al., 1992; Perras et al., 1993; Ormerod et al., 1994). To find out the nature of the induction signal for increased UV-DRP expression, we tested the effect on UV-DRP expression in the A2780 cell line of several treatments and conditions which inhibit DNA replication and/or cell cycle progression without directly damaging DNA. We found that DNA synthesis inhibitors strongly affected UV-DRP level. Aphidicolin and hydroxyurea both induced UV-DRP binding activity to about the same level as did CP treatment (Figure 9).

As described in Materials and Methods, experiments on UV-DRP level determination were normally begun at 85% confluence so that sufficient cells would survive the highest doses of CP to allow analysis of UV-DRP levels. In addition, confluent cells more closely resemble the growth rate in *in vivo* tumors. Induction of UV-DRP by CP was not an artifact of the high cell densities used in these

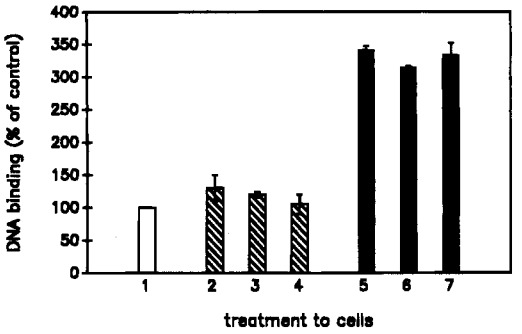

FIGURE 9: Effect of cell cycle arrest (2−4) or DNA synthesis inhibition (5−7) on UV-DRP binding in A2780 cells. Nuclear extracts were prepared from control cells (1), cells treated for 1 h with CP and incubated for 40 h in fresh medium (5), or cells incubated for 40 h in the medium supplemented with 0.5% fetal calf serum (2), or containing PGA_2 (3, 4), hydroxyurea (6), or aphidicolin (7). PGA_2 was added to exponentially growing (50% confluent) (3) or 85% confluent (4) cells. Results are the means ± SEM of data obtained from 8 band shift assays with 2 different extract preparations. Data presented as a percentage of untreated control.

experiments since increase in UV-DRP binding following CP treatment was also observed when the experiments were initiated with exponentially growing cells at 50% confluence. Control cells had normally reached confluence at the end of the 40 h incubation period. These cells continued to grow, but showed an increase in G1 phase and a decrease in S phase cells without affecting UV-DRP level (data not shown). Serum starvation, which also inhibited the cell cycle in G0/G1 phase (the G1 phase population increased to 40% of the control, whereas S phase population decreased to 29% of the control), failed to substantially increase the UV-DRP activity (Figure 9). Since proliferation in the cancer cell lines tested did not display stringent G0/G1 arrest upon serum deprivation, we also chose alternate approaches of obtaining cell cycle arrest. Prostaglandin PGA_2 arrested exponentially growing A2780 cells in the G2 phase of cell cycle (Figure 8D) and inhibited G1 phase progression of 85% confluent cells (Figure 8E) as described previously (Ohno et al., 1988). Similar to serum starvation, PGA_2 had no significant effect on UV-DRP expression under conditions leading to either G1 or G2 arrest (Figure 9). CP treatment caused S and G2 arrest (Figure 8B), whereas the DNA synthesis inhibitors aphidicolin and hydroxyurea initially slowed DNA synthesis in S phase and delayed G2 phase, but by 40 h caused pronounced loss of S phase cells and accumulation of cells preferentially in the G1/S boundary (Figure 8F). Therefore, we conclude that induction of UV-DRP does not appear to be cell cycle related.

DISCUSSION

Using the band shift assay, we detected protein in nuclear extracts from human carcinoma cell lines that have high affinity for UV-damaged DNA (Figure 1). Comparison of our data with published results revealed that the shifted band B_{UV} (Figures 1 and 2) represents the complex formed by UV-irradiated oligonucleotides and a protein (UV-DRP) which was first identified in human placenta by Feldberg and co-workers (Feldberg & Grossman, 1976; Feldberg, 1980; Feldberg et al., 1982; Carew & Feldberg, 1985) and then described by others (Chu & Chang, 1988; Protic et al., 1989; Hirschfeld et al., 1990; Chu & Chang, 1990; Chao et al., 1991a,b; Chao, 1992; Treiber et al., 1992; Keeney et al., 1993; van-Assendelft et al., 1993). We also detected one band with mobility different from that of free probe after incubation of the nuclear extracts with platinated double-stranded DNA (not shown). This binding was damage-specific. Constitutive levels of the protein which recognized CP−DNA adducts were similar in all pairs of parental and resistant cell lines, and no substantial induction of this protein by CP treatment was found (not shown). These results are consistent with data of Chu and Chang (1990), Donahue et al. (1990), Andrews and Jones (1991), and Bruhn et al. (1992). The magnitude of the mobility shift and the specificity for double-stranded platinated probe suggest that the band we observed represented binding of one of the high mobility group (HMG) domain-containing proteins described earlier (Hughes et al., 1992; Pil & Lippard, 1992).

No correlation was found between the constitutive levels of UV-DRP and survival after UV irradiation or CP treatment in most of the parental and CP-resistant cell lines tested (Table 1). These data agree with other reports, which show that constitutive level of DRPs may not necessarily correlate with the sensitivity or resistance of cells to DNA damaging agents, and emphasize the potential importance of the inducible levels of DRPs to cellular cytotoxicity (Chao et al., 1991b, 1993; Chao, 1992; McLenigan et al., 1993).

Previous studies of damage recognition proteins have compared percentage binding of damaged DNA per μg of nuclear extract (Protic et al., 1989; Hirschfeld et al., 1990; Chao et al., 1991b, 1993; Chao & Huang, 1993b). We feel that such quantitative analysis of damage recognition activity is not appropriate for determining inducibility of that activity by DNA damaging agents, because it does not take into account differences in the accumulation of nuclear proteins between different cell lines after treatment. Therefore, we decided to determine the amount of UV-DRP binding activity per nuclei. Such an approach allowed us to compare changes in the absolute amount of UV-DRP in nuclei after drug treatment, rather than the differences in concentration of this protein relative to other proteins in nuclear extracts. Omitting differences in the accumulation of total nuclear proteins may explain the variable results for UV-DRP induction by UV irradiation with different cell lines reported earlier (McLenigan et al., 1993; Protic et al., 1989).

Despite the specificity of UV-DRP for UV-damaged DNA shown by cross competition experiments, we observed UV-DRP induction by CP treatment (Figures 4−7). Inhibition of UV-DRP expression by actinomycin and cycloheximide suggests that the major control for UV-DRP activity lies at the transcriptional level. The increase in UV-DRP binding activity per nuclei was higher in resistant cell lines following CP treatment (Figure 6B). These results suggest that the magnitude of induction, rather than the steady-state level of UV-DRP (Table 1), is characteristic of the CP resistance. For three out of four cell lines tested, the mechanisms of acquired resistance included increased repair activity (Behrens et al., 1987; Lai et al., 1988; Masuda et al., 1990; Chu & Chang, 1990; Parker et al., 1991). However, the maximum difference in UV-DRP induction level was found with the parental 2008 and resistant C13* cell lines, which do not differ in their repair capacity for intrastrand CP lesions in cellular DNA (Zhen et al., 1992; Jekunen et al., 1994). These findings question the involvement of UV-DRP induction in the enhancement of DNA repair activity. Low affinity

of UV-DRP for Pt−DNA adducts (discussed below) also makes this mechanism unlikely and suggests that UV-DRP could play another role. Keeney et al. (1993, 1994) recently came to a similar conclusion based on the abundance of UV-DRP. They showed that microinjection of less than 10% of the normal cellular amount of UV-DRP fully corrected DNA repair in XPE cells lacking UV-DRP. Increasing the amount of this protein beyond the minimum necessary level did not further stimulate repair activity. Thus, if only a small portion of the UV-DRP present in cells is necessary for effective DNA repair, it seems unlikely that an increase in repair activity seen in some CP-resistant cell lines would require additional expression of this protein. The time course for UV-DRP induction by CP (Figure 5) also contradicts the idea that increased amount of UV-DRP is required for enhanced DNA repair. Repair of Pt−DNA adducts appears to be a biphasic process, with most of the repair occurring in the first 6−8 h (Eastman & Schulte, 1988; Masuda et al., 1990), while UV-DRP is induced over a much slower time course. We cannot exclude, however, the possibility that transcriptional activation of UV-DRP is an early event. It is possible that we could not detect an immediate increase in UV-DRP because the newly synthesized protein at early time points was tightly bound to the damaged sites, and its amount in nuclear extracts increased with time due to release from its substrate after the damage is repaired. A similar phenomenon was described by Protic's group (Protic et al., 1989; Hirschfeld et al., 1990; McLenigan et al., 1993) for UV-DRP expression after UV treatment. Further studies (e.g., analysis of expression of UV-DRP mRNA) are needed to resolve this question.

The significance of UV-DRP induction is unclear. An involvement of UV-DRP in the signal transduction pathway for apoptosis has been hypothesized (Chao & Sun, 1993) in analogy with the suggested role of CP-damage recognition protein in CP-induced cell death (Donahue et al., 1990; Pil & Lippard, 1992). Our results do not support this hypothesis. We found similar levels of UV-DRP in attached and detached cells at 48 h after CP treatment. Flow cytometry (Figure 8B,C) showed that most of the detached cells were apoptotic, whereas the attached cells were primarily arrested in the S and G2 phases of the cell cycle. Even if some overlap between subpopulations of early apoptotic cells (which still have high molecular weight DNA) and viable cells occurs in such analysis of DNA content, it is most likely that the majority of the S and G2 arrested cells, which remained attached to the plate 48 h after CP treatment, would eventually survive (Sorenson & Eastman, 1988; Sorenson et al., 1990). Another fact which contradicts the likelihood of UV-DRP involvement in apoptosis is the higher level of its induction in resistant cell lines (Figure 6B).

Our laboratory has previously shown that replicative bypass plays an important role in CP resistance (Gibbons et al., 1991; Mamenta et al., 1994). Model studies in *Escherichia coli* suggest that DRPs may play an essential role in replicative bypass (Rosenberg & Echols, 1990; Slater & Maurer, 1991; Rajagopalan et al., 1992). The fact that the greatest differences in UV-DRP level were found in the 2008/C13* pair (which shows a 4.8-fold increase in the replicative bypass ability for the resistant line compared to sensitive) (Figure 6B) causes us to speculate that UV-DRP might play a role in increased tolerance of CP damage in resistant cells by enhancing replicative bypass of Pt−DNA adducts. While this report as well as previous works (Donahue et al., 1990; Chao et al., 1991a; Chao, 1992; van-Assendelft et al., 1993) showed low, if any, affinity of UV-DRP to CP damage, it is possible that UV-DRP in association with other proteins could recognize Pt−DNA adducts and facilitate replicative bypass. However, a direct link between UV-DRP and postreplicative repair has not been demonstrated, and biological functions of UV-DRP remain to be identified.

What signal causes induction of UV-DRP? We found a strong correlation between induction of UV-DRP and the level of Pt−DNA adducts (Figure 7). However, this induction could have also been a result of DNA synthesis inhibition following DNA damage. Protic et al. (1989) previously reported an increase in UV-DRP binding by aphidicolin. In our study UV-DRP was induced in response to inhibition of DNA replication by both aphidicolin and hydroxyurea, which do not directly produce DNA damage (Figure 9). Nevertheless, we saw considerably greater induction of UV-DRP at 40 h treatment with aphidicolin and hydroxyurea than at 24 h, and a 40 h incubation in the presence of these inhibitors is sufficient time for considerable accumulation of DNA strand breaks (Coyle & Strauss, 1970; Skog et al., 1992). Therefore, it is also possible that the increase in UV-DRP binding is a secondary effect of the DNA damage caused by inhibition of DNA synthesis. On the other hand, significant change in UV-DRP level is most likely not a consequence of cell growth arrest *per se*. Growth cessation by serum depletion or by growing cells to saturation density as well as cell treatment with PGA_2, which arrested cells at different phases depending on growth conditions, had no significant effect on UV-DRP activity (Figure 9). Equal induction of UV-DRP by CP (which arrested cells in late S and G2 phases) and aphidicolin or hydroxyurea (which block cells at the G1/S boundary) (Figures 8 and 9) also supports a hypothesis that induction is not simply due to a redistribution of cells into a part of the cell cycle which happens to express UV-DRP at high levels. Thus, it is very likely that either the formation of Pt−DNA adducts or the inhibition of DNA replication caused by the DNA damage acts as the signal for the UV-DRP induction following CP treatment.

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