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Preferential Repair Incision of Cross-Links *versus* Monoadducts in Psoralen-Damaged Plasmid DNA by Human Cell-Free Extracts†

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ABSTRACT: Upon UVA irradiation psoralens covalently bind to DNA as monoadduct and interstrand cross-link. Psoralen photoadducts are processed *via* an excision repair reaction that has been reproduced *in vitro* with transcriptionnally active cell-free extracts. A derived *in vitro* assay that allows direct quantification of the incised sites has been set up and used to compare the efficiency of the incision reaction on monoadducts and interstrand cross-links. The incision reaction was performed with HeLa cell-free extracts on angelicin or 8-methoxypsoralen (8-MOP)-modified plasmid DNA substrates carrying known amounts of mono- and biadducts, within various relative ratios. In the case of 8-MOP modified plasmids consisting in a mixture of mono- and biadducts on the same DNA molecule, the incision signal was mainly due to the presence of interstrand cross-links. The extent of incision was linear with the number of cross-links up to about 4 cross-links per plasmid and then reached a plateau. The sensitivity of incision defined as the increase of incision by 2-fold over the background level corresponded to about 1 cross-link per plasmid molecule, and about 7% of the total cross-links were repaired under our assay conditions. The incision activity on angelicin monoadducts yielded only 27% when compared to that on 8-MOP cross-links. Furthermore, 8-MOP cross-links lowered the incision extent of angelicin monoadducts when the two photoadducts were present on distinct plasmid DNA molecules. These data are in line with the more rapid excision of psoralen interstrand cross-links *vs* monoadducts observed *in vivo*.

A wide variety of DNA lesions are removed by nucleotide excision repair (NER)[1] which largely contributes to cellular survival. The molecular mechanism is similar from bacteria to humans (Hoeijmakers, 1993a,b; Sancar, 1995). In the current model, the NER process includes two major steps: (i) damage recognition, asymmetric incision of the damaged strand on both sides of the lesion, excision of the damaged oligonucleotide, and (ii) DNA repair synthesis filling in the gap using the complementary strand as template, and ligation. The sequential NER steps have been reproduced *in vitro* with damaged plasmid DNA incubated in the presence of transcriptionally active cell-free extracts (Sibghat-Ullah et al., 1989; Wood et al., 1988). In this *in vitro* assay, the repair activity in protein extracts was measured by the extent of DNA repair synthesis in damaged plasmid as detected by radiolabeled repair patches. It has been estimated that the repair signal is produced by the removal of 1−10% of the lesions contained in plasmid DNA (Sibghat-Ullah et al., 1989; Wood et al., 1988). Despite the use of *in vitro* conditions for the repair reaction with cell extracts, NER on plasmid DNA resembles genomic repair since a defective repair capacity has been observed in extracts from repair-deficient *Xeroderma pigmentosum* (XP) cells belonging to complementation groups from A to G (Hansson et al., 1990, 1991; Reardon et al., 1993; Wood, 1989). Moreover, the complete NER reaction has been reproduced *in vitro* with purified proteins (Aboussekhra et al., 1995; Mu et al., 1995; Shivji et al., 1995).

In order to determine the size of the DNA repair patch, chemically purified DNA damages have been cloned into plasmid DNA at single site. Using cell-free extracts in an NER reaction, the length of the excised oligonucleotide bearing the lesion, and previously labeled, was determined as 27−29 nucleotides (Huang et al., 1992; Svoboda et al., 1993). The use of substrates carrying unique lesion pointed out a difference in the efficiency in excision of various DNA adducts. For instance, among the cisplatin-induced adducts, the major 1,2 d(GpG) adduct was not (Szymkowski et al., 1992) or was poorly repaired (Huang et al., 1994) although the minor 1,3 d(GpG) lesion was efficiently repaired (O'Donovan et al., 1994). Similarly, among the UVC-induced cyclobutane pyrimidine dimers and 6−4 photoproducts, the latter was repaired while the former was not (Szymkowski et al., 1993a). However, the repair efficiency might be different on a unique lesion of a specific class than on a mixture of these lesions on the same DNA molecule.

We addressed the question of a differential repair efficiency *in vitro* of a mix of bulky DNA adducts, i.e., monoadducts (MA) and interstrand cross-links (ICL). In order to obtain plasmid DNA modified with a known number of lesions per molecule but differing in their ratio of MA to ICL, we took advantage of the unique photochemistry of psoralen derivatives. Psoralens intercalate into DNA and, upon UVA (320−400 nm) irradiation, undergo photocy-

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[1] Abbreviations: NER, nucleotide excision repair; MA, monoadduct; ICL, interstrand cross-link; 8-MOP, 8-methoxypsoralen.

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cloaddition with pyrimidines in a sequence-specific manner, to form both pyrone-side or furan-side MA and ICL (Cimino et al., 1985; Sage & Moustacchi, 1987). The ratios between these photoadducts depend on the irradiation protocol. For instance, a single irradiation of DNA in the presence of 8-methoxypsoralen (8-MOP) with 390−405 nm light largely favors the formation of furane-side MA, preferentially at 5′TpA cross-linkable sites (Sage & Moustacchi, 1987; Tessman et al., 1985). A second irradiation (340−365 nm) converts the majority of furan-side cross-linkable MA into biadducts, leading to a high proportion of ICL. In addition, some psoralen derivatives like angelicin can only produce monoadducts. These two molecules were employed here to prepare DNA substrates with a high proportion of either MA or ICL.

Psoralen-modified plasmid DNA undergoes *in vitro* a DNA excision/resynthesis reaction with cell-free extracts that has been attributed to NER (Sibghat-Ullah et al., 1989; Wood et al., 1988; Svoboda et al., 1993). A higher level of repair synthesis was observed for ICL than for MA (Reardon et al., 1991). However, the respective involvement of each type of lesions in the measurement of the global reaction could not be achieved since the length of the repair patch might vary. In order to alleviate this drawback, we specifically monitored the incision reaction *in vitro*. We report here the determination of the incision reaction of MA and ICL by using a label reaction from which the signal is only dependent upon the number of nicks generated by the incision reaction. We show that 8-MOP ICL were efficient substrates for incision by HeLa cell-free extracts. Angelicin MA were repaired much less efficiently than 8-MOP ICL. Furthermore, cross-links lowered the repair efficiency of MA when the two photoadducts were present either on the same or on a distinct DNA molecule.

MATERIALS AND METHODS

Cell-Free Extract. The HeLa S3 cell line was obtained from the stock of European Molecular Biology Laboratories (Heidelberg, Germany). Cells were cultured in suspension in RPMI 1640 medium (Gibco BRL) supplemented with glutamine (2 mM), 7% fetal calf serum (Gibco BRL), penicillin (2×10^5 U/L), and streptomycin (50 mg/L). Cells were regularly tested and found to be free of contamination by Mycoplasma (Mycoplasma detector kit, Boehringer). Cells were collected at a density of about 6×10^5/mL. Whole cell extract was prepared according to the method of Manley (Manley et al., 1983) with minor modifications as previously described (Wood et al., 1988) and were immediately frozen and stored at −80 °C.

Plasmid DNA Substrates. The 2959 bp plasmid pBluescript KS^+ (Stratagene) and the related 3738 bp pHM14 plasmid (gift from Dr. R. D. Wood, ICRF, UK) were prepared by the alkaline lysis method from *Escherichia coli* JM109 (relevant genotype: *recA1, endA1, gyrA96, hsdR17*). Both plasmids were purified on cesium chloride gradient.

8-MOP-adducted DNA substrates containing various amounts of photoadducts and different proportions of ICL were prepared as follows. A reaction mixture containing 4 mg/mL pBS plasmid DNA, plus 8-MOP and [^{3}H]-8-MOP (2.96 TBq/mmol, Amersham), at a molar ratio of 1 psoralen for 10 phosphates, in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, first received 36, 108, or 216 kJ m^{-2} of monochromatic 405 nm UVA at a dose rate of 40 J m^{-2} s^{-1} as previously described (Sage & Moustacchi, 1987). Unbound 8-MOP was eliminated after two ethanol precipitations of DNA, followed by seven washes of the precipitate with 70% ethanol. This treatment yields about 4, 8, and 13 photoadducts per plasmid and favors the formation of MA (about 65% of the lesions, see Table 1). A second irradiation at a dose of 72 kJ m^{-2} of 365 nm UVA light was applied to induce the conversion of cross-linkable MA into ICL (up to 74% of cross-links). The extent of ICL in each sample was determined after enzymatically restricting the plasmid, labeling DNA fragments, and quantitating cross-linked molecules following electrophoresis on agarose gel as previously described (Calsou et al., 1992). Plasmid substrates containing only MA were prepared using angelicin, a monofunctional psoralen derivative. pHM plasmid DNA was irradiated in the presence of angelicin at a ratio of 1 angelicin per 8 nucleotides, in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, with 365 nm UVA light, at doses between 9 and 72 kJ m^{-2}. The extent of photoaddition was estimated using uvrABC as a tool (gift from Dr. Van Sluis, Leiden, The Netherlands). Adducted plasmids were restricted, 5′-labeled, and incubated in the presence of either uvrABC or uvrAB, denatured before electrophoresis on an agarose gel. The disappearance of a 220 bp DNA fragment when DNA has been incubated with uvrABC, in comparison with the sample incubated with uvrAB only, allows to determine the extent of photoadducts according to a Poisson distribution. These plasmids carry respectively 2, 4.4, 8.5, and 17 monoadducts for incident doses of 9, 18, 36, and 72 kJ m^{-2} of UVA radiation. In addition, pBS or pHM plasmid were also UVA-irradiated in the absence of psoralen derivatives, at either 405 or 405 nm plus 365 nm and at the highest doses used, as a control for potential UVA-induced damage such as base modifications which could be recognized in the repair reaction and would contribute to the repair signal. They correspond thereafter to the samples containing 0 photoadduct per plasmid. Following damaging treatment, plasmids were purified on two neutral sucrose gradients to remove the nicked form (30−50% of the starting material) of plasmid DNA.

Nicked Plasmid Substrate. DNase I-nicked plasmid was prepared as follows: pBS plasmid was treated with 1 ng/mL pancreatic DNase I (Gibco BRL) at 25 °C for various incubation times within 30 min. The average number of nicks per circle was calculated according to the Poisson distribution from the remaining fraction of closed-circular plasmid, as determined by ethidium bromide/agarose gel electrophoresis and densitometry of the photographic negative of the gel (scanning laser densitometer, Biocom, France); signals for closed-circular DNA were corrected by a factor of 1.5 to compensate for reduced binding of ethidium bromide.

Incision Assay. Standard 50 μL reaction mixtures contained 300 ng each of damaged pBluescript KS^+ and UVA-treated pHM14 closed-circular plasmids, 150 μg of extract protein in the reaction buffer containing 45 mM Hepes-KOH (pH 7.8), 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 60 mM KCl, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase (type I, Sigma), 3.4% glycerol, and 18 μg of bovine serum albumin. 4.5 μM aphidicolin was included as 1 μL of the suitably diluted solution in Me_2SO (Calsou & Salles, 1994a). Reaction was

carried out at 30 °C for 2 h. After termination of the reaction by the addition of EDTA to 25 mM, the mixture was treated with 200 μg/mL proteinase K (37 °C, 30 min) in the presence of 0.5% SDS. Plasmid DNA was purified by phenol−chloroform extraction under gentle mixing conditions and ethanol precipitated. DNA was then incubated at 25 °C for 10 min in 10 μL reaction mixture containing 90 mM Hepes-KOH, (pH 6.6), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 74 kBq of [α-^{32}P]ddATP (110 TBq/mmol, Amersham), 2 mM dithiothreitol, 20 μM each of dGTP, dCTP, and dTTP, 2 μM ddATP, and 1 U of *E. coli* DNA polymerase I large fragment (Gibco BRL). Reaction was terminated by addition of EDTA to 50 mM and unlabeled dATP to 1 mM. The mixture was treated with 50 μg/mL bovine pancreatic ribonuclease A (37 °C, 10 min). DNA was purified by phenol−chloroform extraction, ethanol precipitated, then linearized with *Hin*dIII or *Bam*H1 as indicated, and electrophoresed as described (Wood et al., 1988), on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Data were quantified by autoradiography, scintillation counting of excised DNA bands, and densitometry of the photographic negative of the gel to normalize for plasmid DNA recovery in each reaction sample (scanning laser densitometer, Biocom, France).

RESULTS

Quantitation of the Incision Sites in DNA in Vitro. The extent of label incorporation during the repair synthesis step of an NER reaction *in vitro* (Wood et al., 1988) might be inconclusive for quantitating the number of repaired lesions. Indeed, a potential variation in the size of the repair patch might occur depending on the type of lesion, i.e., MA repaired by a single excision step *vs* ICL that could interfere with the polymerization step after the first incision or lead to two repair patches per lesion in the case of complete removal. To overcome this inherent drawback we have reported a modification of the repair assay that allows to quantify the incision step of the repair reaction (Calsou & Salles, 1994a,b). In this derived assay, the reaction was stopped at the excision step, and incised intermediates were purified and subsequently labeled in a polymerization reaction with the Klenow fragment of *E. coli* polymerase I (Figure 1). However, this procedure might also lead to variations in the extent of polymerization during the repair of an MA or an ICL repair. We needed an assay that would precisely relate the amount of incorporated label to the number of incised sites. Consequently, in the new assay, the 3′-OH nicks generated during the incision−excision step were directly labeled in the polymerization reaction performed in the presence of ddATP instead of dATP (see Materials and Methods and Figure 1). This labeling procedure was set up using DNase I-treated plasmid DNA containing a known number of nicks (Figure 2). Background nicks in DNase I-untreated plasmid DNA probably lacked a 3′-OH end and were not labeled; however, the extent of incorporation was proportional to the number of nicks introduced by the DNase I treatment and did not vary as the incubation time with Klenow polymerase increased from 10 min up to 30 min as shown in Figure 2. Moreover, the extent of incorporation was identical in nicked or gapped plasmid duplex (data not shown).

Preparation of 8-MOP-Photoadducted Plasmid DNA and Detection of Incision Intermediates in an NER Reaction.

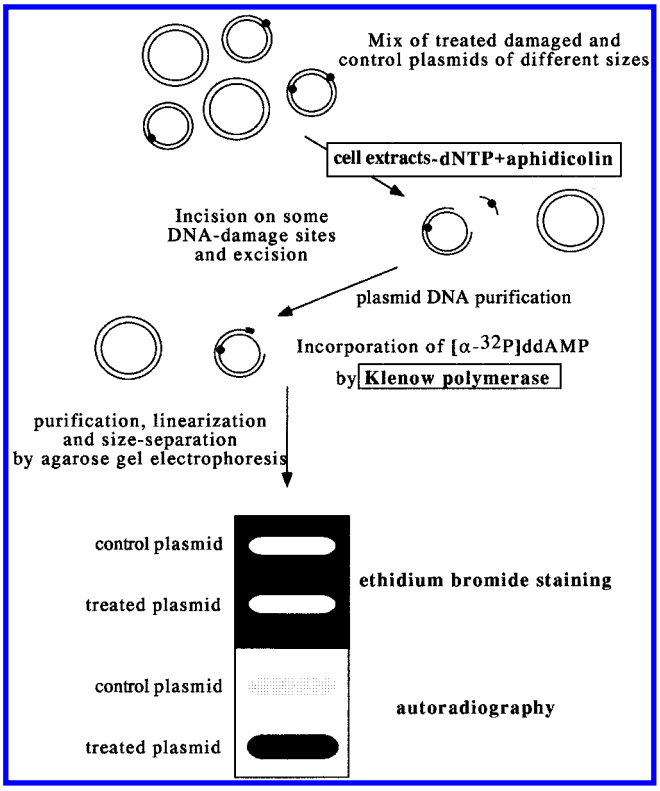

FIGURE 1: Schema of the incision assay. Small dots on plasmids represent DNA lesions.

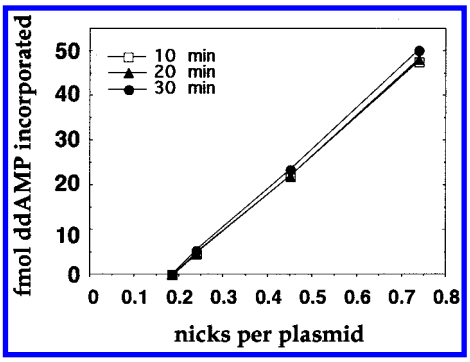

FIGURE 2: Incorporation of [^{32}P]ddAMP in DNase I-nicked plasmids by Klenow polymerase. 300 ng of DNase I-nicked pBS plasmid was labeled with [^{32}P]ddATP in the presence of 1 U of Klenow polymerase as described in the Materials and Methods section (incision assay) for an incubation time as indicated at 25 °C. After purification, plasmids were linearized by *Hin*dIII and electrophoresed. Incorporation in plasmid DNA was quantified by scintillation counting of the bands excised from the gel and corrected for plasmid DNA recovery in each reaction. DNase I-untreated pBS plasmid contained 0.18 background nick.

Plasmid DNA substrates with high proportion of either MA or ICL were prepared as described in the Materials and Methods section. As already observed (Tessman et al., 1985), using 405 nm monochromatic light we were able to produce high proportions of cross-linkable MA (Table 1). ICL were also formed, and their occurrence paralleled the extent of photoaddition. The long irradiation time (15−90 min) and the higher quantum yield to convert 8-MOP furan-side MA into ICL than for initial adduct formation (Tessman et al., 1985) are likely to explain such a presence of ICL. The only species capable of further UVA absorption are furan-side MA, which can be converted into biadducts yielding ICL when properly located (mainly in a 5′TpA context). A second irradiation dose of 72 kJ m^{-2} of 365

Table 1: 8-MOP-Damaged Plasmids

plasmid no.	no. of adducts[a]	UVA irradiation[b]	ICL/plasmid	% ICL/adducts
1	0	405	0	0
2	0	405 + 365	0	0
3	4	405	1.4	34
4	4	405 + 365	2.2	55
5	8	405	3	37
6	8	405 + 365	5.9	74
7	13	405	4.5	35
8	13	405 + 365	7.3	56

[a] The number of psoralen/DNA adduct was calculated from experiments using tritiated 8-MOP, as described in the Materials and Methods. [b] Plasmids 1−2 received 216 kJ m^{-2} of 405 nm UVA radiation with no 8-MOP; plasmids 7−8 received 216 kJ m^{-2} of 405 nm UVA radiation; plasmids 3−4 and 5−6 received respectively 36 and 108 kJ m^{-2}. The second irradiation was performed with 365 nm UVA, at a fluence of 72 kJ m^{-2}.

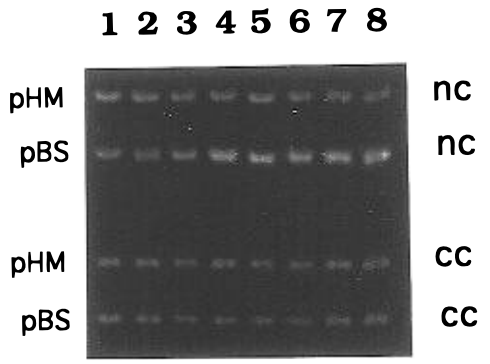

FIGURE 3: Incised repair intermediates of 8-MOP-damaged plasmid DNA. 300 ng each of damaged (pBS) and untreated control (pHM) plasmids was incubated for 2 h at 30 °C with 150 μg of HeLa cell protein extract. Incubation was carried out with aphidicolin and without addition of dNTP. The numbers refer to the damaged pBS plasmids as reported in Table 1. After purification under standard procedure, plasmids were electrophoresed without linearization. The figure shows a photograph of the ethidium bromide-stained agarose gel. nc, nicked-circular form; cc, closed-circular form.

nm UVA radiation allowed us to generate substrates with 74% of photoadducts as ICL (Table 1). Meanwhile the fraction of ICL was reproducibly lower after an initial dose of 405 nm UVA of 216 kJ m^{-2} than after a dose of 108 kJ m^{-2}. This may be due to photoisomerization of a certain proportion of furan-side MA into pyrone-side MA upon UVA irradiation at high doses, as already reported (Tessman et al., 1985). Nevertheless, we were able to generate substrates with an increasing number of ICL within different ratios of MA (Table 1). These substrates were incubated in an incision reaction as previously described (Calsou & Salles, 1994a). The incision step could be broadly estimated from the migration profile of the reaction products on an agarose gel without prior linearization. As shown in Figure 3, there was an accumulation of incised intermediates (nicked form of pBS) depending on the extent of damage, whereas there was no such accumulation in the control pHM plasmid. Moreover, no linear form of the damaged plasmid was seen. However, the direct quantification from these gels could not accurately assess the extent of repair, because of the low efficacy of the repair reaction (Wood et al., 1988).

Determination of the Incised Sites in 8-MOP-Photoadducted Plasmid DNA. The incision reaction was performed with 8-MOP-damaged plasmids where the incised intermediates were purified and radioactively labeled as described above. The extent of incorporation was dependent on the number of photoadducts per molecule (Figure 4A). At constant number of photoadducts, plasmids irradiated only at 405 nm and carrying a high fraction of MA showed a lower extent of incorporation than the same plasmids irradiated at 405 nm plus 365 nm and bearing a high percentage of ICL (Figure 4B). The value of radioactive incorporation obtained from these experiments is proportional to the number of incision events per plasmid circle (Figure 2). When the damage-dependent incision activity (expressed in mol of ddAMP incorporated per mol of pBS plasmid) was plotted *versus* the number of MA (Figure 5A), no simple relationship was observed. In contrast, the extent of incision activity correlated well with the number of ICL (Figure 5B), and an initial linear relationship emerged. In addition, the slope of the curve, which is related to the efficacy of the

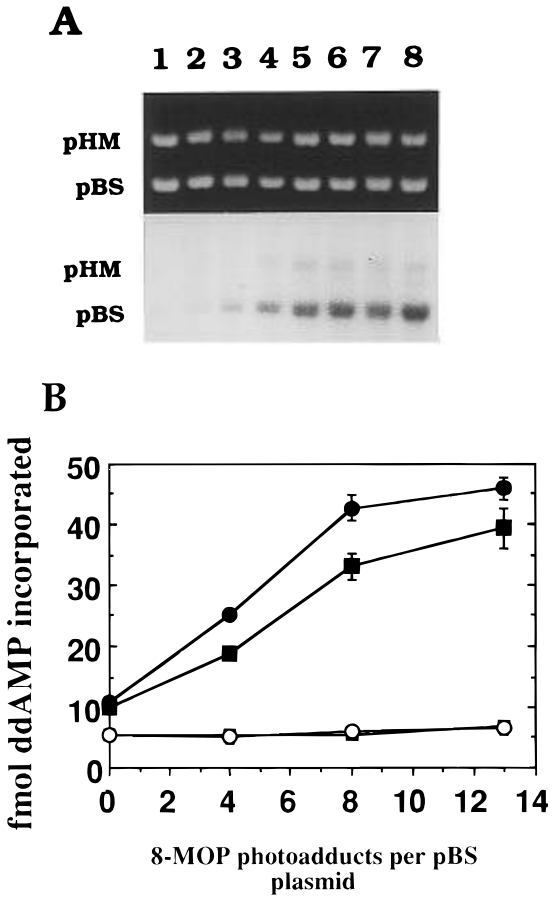

FIGURE 4: Dose−response for damage-dependent incision activity on 8-MOP-photoadducted plasmid DNA. 150 μg of HeLa cell protein extracts were incubated, in the absence of dNTP and with aphidicolin in the presence of 300 ng of pBS plasmid photoadducted to various extents and 300 ng of untreated pHM control plasmid. After 2 h at 30 °C, plasmids were purified and labeled by Klenow polymerase (1 U, 10 min, 25 °C) in the presence of [α-^{32}P]ddATP. Plasmids were then purified, linearized with *Hin*dIII, and electrophoresed on agarose gel. The numbers refer to the damaged pBS plasmids as reported in Table 1. (A) Upper panel: photograph of the ethidium bromide-stained agarose gel. Lower panel: autoradiograph of the dried gel. (B) Quantitative analysis. Incorporation was expressed as femtomoles of ddAMP incorporated into plasmid DNA. For each sample, incorporation was normalized for the amount of DNA recovered. Filled symbols correspond to the treated pBS plasmid and open symbols to the untreated pHM plasmid. Squares correspond to pBS irradiated at 405 nm and circles to pBS irradiated at 405 + 365 nm. Each value is the mean of three independent experiments with SD.

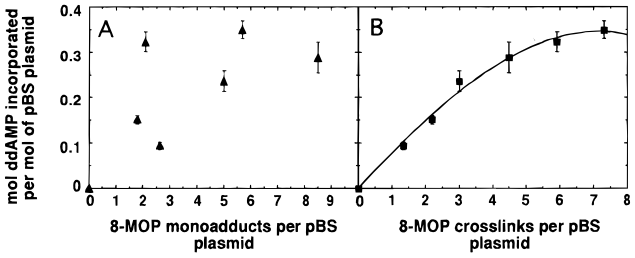

FIGURE 5: Dose−response for damage-dependent incision activity on 8-MOP-photoadducted DNA: role of ICL *vs* MA. For each sample in Figure 4, the amount of background incorporation in untreated pHM plasmid was subtracted from incorporation in treated pBS plasmid. Each value was also corrected for the background incorporation due to UVA irradiation (0 photoadduct per pBS). Damage-dependent incision activity was calculated as mol of ddAMP incorporated per mol of damaged pBS and then plotted *versus* the number of MA (A) or ICL (B) per pBS plasmid calculated from Table 1. Each value is the mean of three independent experiments with SD.

reaction, was equal to 0.074, indicating that 7.4% of ICL were incised under these repair conditions.

Determination of the Incision Activity on MA-Photoadducted Plasmids DNA in the Absence or Presence of 8-MOP-Damaged DNA. In order to determine the incision efficiency on psoralen MA in the absence of ICL, the incision reaction was performed with angelicin-damaged plasmids. We observed a dose-dependent radiolabel incorporation (Figure 6A) which was linear up to 17 monoadducts per plasmid molecule (Figure 6C). However, when the efficacy of the reaction was calculated in the same way as for 8-MOP ICL (see Figure 5B), a value of 2% of MA incised under these repair conditions was obtained. In order to assess a potential *trans* acting effect of ICL on the repair efficiency of MA, an incision experiment was performed on a mixture of two different plasmid DNA substrates, a pBS plasmid containing a mild number of 8-MOP ICL (pBS no. 4, Table 1), and a pHM plasmid bearing an increasing number of angelicin MA (Figure 6B). Although MA were still incised, the efficiency of the reaction on angelicin MA in the presence of 8-MOP ICL was decreased by 48% when compared with the reaction in the absence of ICL (Figure 6C). In addition, the extent of ICL repair remained broadly unchanged.

DISCUSSION

We have attempted to determine the relative repair efficiency of monoadducts (MA) and interstrand cross-links (ICL) produced by psoralen as a single DNA damaging treatment, in an *in vitro* assay using cell-free extracts. As initially set up (Sibghat-Ullah et al., 1989; Wood et al., 1988), the NER assay could not assess conclusive quantitation of repaired lesions due to possible variations in the repair patch size. Psoralen-damaged plasmids with various ratios of MA *vs* ICL were used as substrates in a new biochemical assay designed to quantify the extent of incision by cell-free extracts in an NER reaction. This assay can be briefly described as follows: (1) incision and excision of the damage on plasmid DNA with cell-free extracts, the polymerization step being blocked by both the presence of aphidicolin and the absence of added dNTPs; (2) purification of the incised intermediates; (3) DNA polymerization reaction with the Klenow fragment of pol I in the presence of [α-^{32}P]ddNTP. This labeling reaction produces a signal which depends only on the number of 3′-OH nicks generated by the NER

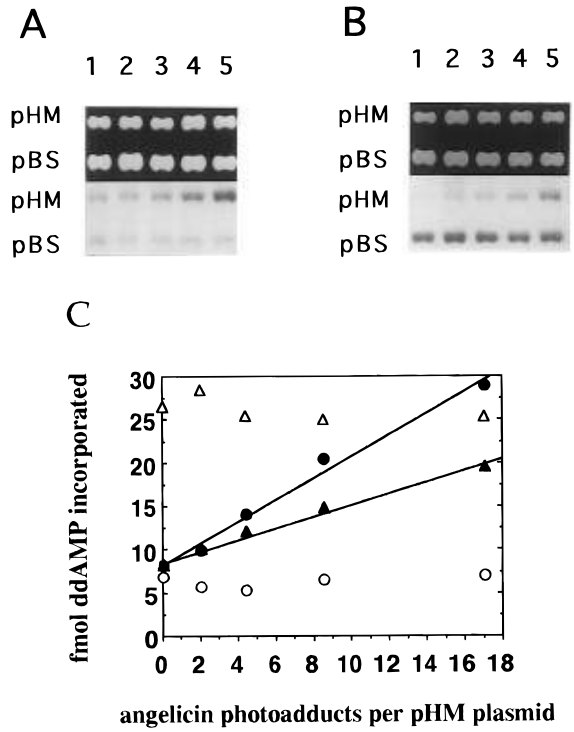

FIGURE 6: Dose−response for damage-dependent incision activity on angelicin-photoadducted plasmid DNA. (A) 150 μg of HeLa cell protein extracts were incubated, in the absence of dNTP and with aphidicolin in the presence of 300 ng of untreated pBS plasmid and 300 ng of pHM plasmid photoadducted with angelicin to various extents. After 2 h at 30 °C, plasmids were purified and labeled by Klenow polymerase (1 U, 10 min, 25 °C) in the presence of [α-^{32}P]ddATP. Plasmids were then purified, linearized with *Bam*H1, and electrophoresed on agarose gel. Lanes 1, 2, 3, 4, and 5: pHM plasmid containing 0, 2, 4.4, 8.5, and 17 angelicin MA, respectively. Upper panel: photograph of the ethidium bromide-stained agarose gel. Lower panel: autoradiograph of the dried gel. (B) Incision reaction as in (A) but in the presence of 300 ng of 8-MOP-treated pBS plasmid (no. 4, Table 1) and 300 ng of pHM plasmid photoadducted with angelicin to various extents. Lanes 1, 2, 3, 4, and 5: pHM plasmid containing 0, 2, 4.4, 8.5, and 17 angelicin MA, respectively. Upper panel: photograph of the ethidium bromide-stained agarose gel. Lower panel: autoradiograph of the dried gel. (C) Quantitative analysis. Incorporation was expressed as femtomoles of ddAMP incorporated into plasmid DNA. For each sample, incorporation was normalized for the amount of DNA recovered. Each value is the mean of two independent experiments. Circles correspond to the quantification of experiments A: (○) untreated pBS control plasmid; (●), MA-modified pHM plasmid. Triangles correspond to the quantification of experiments B: (△) 8-MOP-treated pBS plasmid; (▲) MA-modified pHM plasmid.

reaction. Therefore, it allows the quantification of incised sites. Under these conditions of quantitative label incorporation, the incision activity of HeLa extracts toward psoralen MA and ICL was measured, as well as the efficacy of the NER reaction.

A group reported recently the processing of psoralen MA and ICL by human chromatin-associated proteins which diverged from the conventional NER pattern (Kumaresan et al., 1995). Whether this unique excision pathway obtained with partially purified non-nucleoplasmic fractions is relevant for the repair of psoralen photoadducts in cells remains to be established. Nevertheless, the damage-dependent incision activity that we observed with HeLa whole cell extracts in the presence of psoralen DNA photoadducts can reasonably be attributed to NER. Indeed, under similar conditions,

previous reports showed the inability of extracts from NER-deficient cells to perform the reaction on psoralen plasmid DNA (Sibghat-Ullah et al., 1989; Wood et al., 1988) and established that the excision pattern obtained with psoralen photoadducts was similar to that of other NER substrate lesions (Svoboda et al., 1993).

The extent of radiolabel incorporation follows a linear relationship with the number of photoadducts up to a maximal repair activity for plasmids carrying 5 ICL (Figure 5B) or 17 MA (Figure 6C). A threshold of one ICL per plasmid was necessary to create a signal at least equal to 2-fold the background value. A similar sensitivity was observed with UVC-irradiated plasmid (Calsou & Salles, 1994b). As already reported for UVC photoproducts (Calsou & Salles, 1994b), the extent of incision could be slightly increased with higher protein concentration (data not shown). We can assume an incorporation of one label per incised site from the following points: (1) No double-strand breaks after repair of psoralen-damaged plasmids were observed on ethidium bromide-stained agarose gel (Figure 3) or after radiolabeling and overexposure of the autoradiograph (data not shown); similarly, uvrABC excinuclease induced a dual incision on only one strand (the furan-side adducted strand) in duplex DNA containing a psoralen cross-link at a defined position (Van Houten et al., 1986). (2) It has been reported that the 3′-OH end of the incised damaged oligonucleotide cannot be extended *in vitro* by the Klenow polymerase next to the furan side of a psoralen cross-link (Sladek et al., 1989).

The efficacy of the incision reaction was determined in the case of 8-MOP ICL and angelicin MA as the slope of the radiolabel incorporation *versus* the number of adducts per plasmid. The values obtained were 7.4% of ICL incised compared with 2% of MA, indicating that the incision efficiency on angelicin MA yielded only 27% of that of 8-MOP ICL.

The value obtained for the incision efficiency likely reflects the efficacy of the whole repair reaction *in vitro* since the incision reaction is the limiting step (Shivji et al., 1992). In the case of 8-MOP, the repair efficiency increased linearly with the number of ICL per plasmid while no correlation was found with the number of MA per plasmid (Figure 5A,B). In addition, the dose-response curve extrapolated to zero in the absence of ICL. In the case of angelicin, the poor incision extent was further decreased if 8-MOP ICL carried on another plasmid molecule were present in the repair reaction mixture (Figure 6). These results indicate that psoralen MA *per se* contribute poorly to the repair signal as measured *in vitro*; in addition, psoralen ICL can compete for the incision of MA by both *cis* and *trans* acting effects. The lack of processivity of the repair complex on damaged plasmid DNA *in vitro* (Szymkowski et al., 1993b) may amplify the repair preference for ICL *versus* MA when both adducts are present on the same plasmid molecule, which is the case for 8-MOP photoadducted DNA.

In vitro experiments using cell-free extracts have already established the following: (1) pure MA could be excised by both the UVRABC complex *in vitro* (Van Houten et al., 1986) and cell-free extracts in an incision reaction designed to determine the repair patch size (Svoboda et al., 1993), although the repair efficiency was not assessed; (2) psoralen cross-links induced a higher level of repair synthesis compared to MA (Reardon et al., 1991); and (3) cisplatin/DNA ICL represent the major contribution to DNA repair synthesis (Calsou et al., 1992) assuming the same patch size for MA and ICL.

Taken together, these data and ours indicate that, although pure MA are recognized and repaired, ICL are preferentially repaired when present with MA. The preferential incision of ICL might overcome the recognition of MA, that could be especially detectable in the case of the low repair extent obtained with cell extracts *in vitro*. This establishes a lower affinity of the repair complex for MA than for ICL, as demonstrated for UVC-induced cyclobutane pyrimidine dimers *versus* 6−4 photoproducts (Szymkowski et al., 1993a). It could be hypothesized that the repair enzymes in human cell extracts recognize or incise ICL better than MA, because of a greater distortion of the double helix induced by the former lesion. Even though the conversion of a MA into ICL requires DNA structural reorganization, a rather similar distortion results from MA and ICL (Spielmann et al., 1995). Interestingly, these authors suggest that psoralen damage confers a greater flexibility to the backbone which would constitute the recognition signal for the repair complex. Futhermore, we have demonstrated elsewhere (Sage & Moustacchi, 1987) that 8-MOP-ICL are essentially formed in alternating $(AT)_n$ sequences which are naturally prone to conformational freedom, whereas MA formation is not as sequence specific. As we suggested previously (Boyer et al., 1988; Sage & Moustacchi, 1987), alternating $(AT)_n$ tracks, where are formed most ICL but only parts of MA, may be more subject to excision repair than other sequences. In addition, it has been reported that the human damage-recognition protein XPE, which enhances the affinity of the recognition complex for certain lesions in the NER reaction, recognizes UVC- and cisplatin-induced lesions but does not discriminate between psoralen-monoadducted DNA and undamaged DNAs (Reardon et al., 1993). With this in view, it might be interesting to use ICL adducts in a recognition assay by XPE binding factors.

What is the relevance of our *in vitro* data with the *in vivo* repair situation? The question of the possible interference between lesions in their repair efficiency is pertinent. It has been shown that, in human cells, the presence of large amounts of psoralen MA blocks ICL (Papadopoulo et al., 1988) and conversely the presence of ICL reduces the removal of MA (Cleaver & Gruenert, 1987; Gruenert & Cleaver, 1985; Vuksanovic & Cleaver, 1987). The ratio between the two photoadducts seems to play a key role in this phenomenon. More generally, it has been observed that, in mammalian cells, MA are more slowly excised than ICL from bulk DNA as well as from actively transcribed genes (80% of ICL, but only 45% MA are removed from the human DHFR gene within 24 h) (Islas et al., 1991, 1994; Vos & Hanawalt, 1987). ICL constitute absolute block for replication and transcription, while MA are not. Indeed, transcription-coupled repair has not been observed for MA in rodent cells (Islas et al., 1994). Also, MA can be bypassed both *in vitro* and *in vivo* by DNA polymerase (Chanet et al., 1983; Piette et al., 1985; Piette & Hearst, 1983) partly in an error-free manner (Vos & Hanawalt, 1987). It appears that MA and ICL are processed in rather different ways. Despite the limitations of the *in vitro* NER assay, i.e., plasmid DNA, and lack of chromosomal structure and of transcription, our *in vitro* data support the conclusion based on *in vivo* experiments.

Genetic and biochemical experiments with purified bacterial proteins (Cheng et al., 1991; Cole, 1973; Sladek et al., 1989; Van Houten et al., 1986a,b, 1988) indicated that, at least in *E. coli*, ICL were repaired in a more complex reaction than MA. A recombination step seems necessary after the dual incision of ICL lesion on one strand for complete removal of ICL. XP variant cells which are impaired in bypass replication exhibit a marked deficiency in the ability to bypass ICL, but partly read through MA, in comparison to normal cells (Misra & Vos, 1993). A difference in the processing of MA *vs* ICL is also illustrated in using the human XPA revertant cell line (XP129) that has regained wild-type resistance to killing by ICL but was as sensitive to MA as the parental XPA (Vuksanovic & Cleaver, 1987).

Our results from repair incision experiments performed with cell extracts on plasmid DNA seem to reflect an *in vivo* situation in mammalian cells. It is therefore possible that in the NER process recognition and/or incision represent the limiting steps of the repair of ICL psoralen DNA adducts *in vivo*.

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REFERENCES

Aboussekhra, A., Biggerstaff, M., Shivji, M. K. K., Vilpo, J. A., Moncollin, V., Podust, V. N., Protic, M., Hubscher, U., Egly, J. M., & Wood, R. D. (1995) *Cell 80*, 859−868.

Boyer, V., Moustacchi, E., & Sage, E. (1988) *Biochemistry 27*, 3011−3018.

Calsou, P., & Salles, B. (1994a) *Biochem. Biophys. Res. Commun. 202*, 788−795.

Calsou, P., & Salles, B. (1994b) *Nucleic Acids Res. 22*, 4937−4942.

Calsou, P., Frit, P., & Salles, B. (1992) *Nucleic Acids Res. 20*, 6363−6368.

Chanet, R., Cassier, C., Magana-Schwencke, N., & Moustacchi, E. (1983) *Mutat. Res. 112*, 201−214.

Cheng, S., Sancar, A., & Hearst, J. E. (1991) *Nucleic Acids Res. 19*, 657−663.

Cimino, G. D., Gamper, H. B., Isaacs, S. T., & Hearst, J. E. (1985) *Annu. Rev. Biochem. 54*, 1151−1193.

Cleaver, J. E., & Gruenert, D. C. (1987) *J. Invest. Dermatol. 82*, 311−315.

Cole, R. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1064−1068.

Gruenert, D. C., & Cleaver, J. E. (1985) *Cancer Res. 45*, 5399−5404.

Hansson, J., Grossman, L., Lindahl, T., & Wood, R. D. (1990) *Nucleic Acids Res. 18*, 35−40.

Hansson, J., Keyse, S. M., Lindahl, T., & Wood, R. D. (1991) *Cancer Res. 51*, 3384−3390.

Hoeijmakers, J. H. J. (1993a) *Trends Genet. 9*, 173−177.

Hoeijmakers, J. H. J. (1993b) *Trends Genet. 9*, 211−217.

Huang, J.-C., Svoboda, D. L., Reardon, J. T., & Sancar, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3664−3668.

Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J., & Sancar, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10394−10398.

Islas, A. L., Vos, J. M. H., & Hanawalt, P. C. (1991) *Cancer Res. 51*, 2867−2873.

Islas, A. L., Baker, F. J., & Hanawalt, P. C. (1994) *Biochemistry 33*, 10794−10799.

Kumaresan, K. R., Hang, B., & Lambert, M. W. (1995) *J. Biol. Chem. 270*, 30709−30716.

Manley, J. L., Fire, A., Samuels, M., & Sharp, P. A. (1983) *Methods Enzymol. 101*, 568−582.

Misra, R. R., & Vos, J.-M. H. (1993) *Mol. Cell. Biol. 13*, 1002−1012.

Mu, D., Park, C. H., Matsunaga, T., Hsu, D. S., Reardon, J. T., & Sancar, A. (1995) *J. Biol. Chem. 270*, 2415−2418.

O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C., & Wood, R. D. (1994) *Nature 371*, 432−435.

Papadopoulo, D., Averbeck, D., & Moustacchi, E. (1988) *Photochem. Photobiol. 47*, 321−326.

Piette, J. G., & Hearst, J. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5540−5544.

Piette, J., Decuyper-Debergh, D., & Gamper, H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7355−7359.

Reardon, J. T., Spielmann, P., Huang, J.-C., Sastry, S., Sancar, A., & Hearst, J. E. (1991) *Nucleic Acids Res. 19*, 4623−4629.

Reardon, J. T., Nichols, A. F., Keeney, S., Smith, C. A., Taylor, J. S., Linn, S., & Sancar, A. (1993a) *J. Biol. Chem. 268*, 21301−21308.

Reardon, J. T., Thompson, L. H., & Sancar, A. (1993b) *Cold Spring Harbor Symp. Quant. Biol. 58*, 605−617.

Sage, E., & Moustacchi, E. (1987) *Biochemistry 26*, 3307−3314.

Sage, E., Drobetsky, E. A., & Moustacchi, E. (1993) *EMBO J. 12*, 397−402.

Sancar, A. (1995) *J. Biol. Chem. 270*, 15915−15918.

Shivji, M. K. K., Kenny, M. K., & Wood, R. D. (1992) *Cell 69*, 367−374.

Shivji, M. K. K., Podust, V. N., Hubscher, U., & Wood, R. D. (1995) *Biochemistry 34*, 5011−5017.

Sibghat-Ullah, Husain, I., Carlton, W., & Sancar, A. (1989) *Nucleic Acids Res. 17*, 4471−4484.

Sladek, F. M., Munn, M., Rupp, W. D., & Howard-Flanders, P. (1989) *J. Biol. Chem. 264*, 6755−6765.

Spielmann, H. P., Dwyer, T. J., Sastry, S. S., Hearst, J. E., & Wemmer, D. E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2345−2349.

Svoboda, D. L., Taylor, J.-S., Hearst, J. E., & Sancar, A. (1993) *J. Biol. Chem. 268*, 1931−1936.

Szymkowski, D. E., Yarema, K., Essigmann, J. M., Lippard, S. J., & Wood, R. D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 10772−10776.

Szymkowski, D. E., Lawrence, C. W., & Wood, R. D. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9823−9827.

Szymkowski, D. E., Hajibagheri, N. A. M., & Wood, R. D. (1993b) *J. Mol. Biol. 231*, 251−260.

Tessman, J. W., Isaacs, S. T., & Hearst, J. E. (1985) *Biochemistry 24*, 1669−1676.

Van Houten, B., Gamper, H., Hearst, J. E., & Sancar, A. (1986a) *J. Biol. Chem. 261*, 14135−14141.

Van Houten, B., Gamper, H., Holbrook, S. R., Hearst, J. E., & Sancar, A. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8077−8081.

Van Houten, B., Gamper, H., Hearst, J. E., & Sancar, A. (1988) *J. Biol. Chem. 263*, 16553−16560.

Vos, J. M. H., & Hanawalt, P. C. (1987) *Cell 50*, 789−799.

Vuksanovic, L., & Cleaver, J. E. (1987) *Mutat. Res. 184*, 255−263.

Wood, R. D., Robins, P., & Lindahl, T. (1988) *Cell 53*, 97−106.

Wood, R. D. (1989) *Biochemistry 28*, 8287−8292.

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