Repair of abasic sites by mammalian cell extracts

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Hamster cell extracts that perform repair synthesis on covalently closed circular DNA containing pyrimidine dimers, were used to study the repair of apurinic/apyrimidinic (AP) sites and methoxyamine (MX)-modified AP sites. Plasmid molecules were heat-treated at pH 5 and incubated with MX when required. The amount of damage introduced ranged from 0.2 to 0.9 AP sites/kb. Extracts were prepared from the Chinese hamster ovary CHO-9 cell line and from its derivative, 43–3B clone which is mutated in the nucleotide excision repair (NER) ERCC1 gene. AP and MX-AP sites stimulated repair synthesis by CHO-9 cell extracts. The level of synthesis correlated with the number of lesions and was of similar magnitude to the repair stimulated by

4.3 u.v. photoproducts/kb. Repair of AP and MX-AP sites was faster than the repair of u.v. damage and was independent of ERCC1 gene product. The high level of repair replication was due to a very efficient and rapid incision of plasmids carrying AP or MX-AP sites, performed by abundant AP endonucleases present in the extract. The calculated average repair patch sizes were: 7 nucleotides per AP site; 10 nucleotides per MX-AP site; 28 nucleotides per (6–4) u.v. photoproduct or cyclobutane pyrimidine dimer. The data indicate that AP and MX-AP sites are very efficiently repaired by base-excision repair in mammalian cells and suggest that MX-AP sites may also be processed via alternative repair mechanisms.

INTRODUCTION

The base–sugar bonds in DNA are highly susceptible to chemical and enzymic hydrolysis. It has been estimated that several thousand bases detach every day from the DNA backbone in mammalian cells under physiological conditions (Lindahl, 1993). The result of this base loss is the formation of apurinic/apyrimidinic (AP) sites. AP sites in the DNA template block DNA replication and, if bypassed by DNA polymerase, generate single base-pair substitutions (Gentil et al., 1992). Depurination/depyrimidination represents therefore a major source of ‘spontaneous’ damage and this is particularly significant in mammalian cells because of the very large size of their genome and the long S-phase period of DNA replication (Lindahl, 1993). Moreover a variety of DNA-damaging agents produce AP sites by chemical or enzymic hydrolysis of modified DNA bases.

Recent studies *in vitro* have investigated the repair mechanism of an AP site generated by the excision of a single uracil residue from double-stranded oligonucleotides (Dianov et al., 1992). These studies have shown that both *Escherichia coli* and human cell extracts carry out repair of the AP site mainly by replacing a single nucleotide. Similarly, Matsumoto and Bogenhagen (1989; 1991) by using *Xenopus laevis* oocyte extracts on depurinated plasmid DNA, have found that the base excision repair of a synthetic analogue of an AP site is localized within a very short patch of 3 or 4 nucleotides surrounding the AP site. Following the resynthesis step [probably catalysed by DNA polymerase β in mammalian cells (Dianov et al., 1992)], excision and ligation proceed in a coordinated manner to complete removal (Dianov et al., 1992; Matsumoto and Bogenhagen, 1989; 1991). In *E. coli*, the long-patch nucleotide excision repair (NER) pathway initiated by the multiprotein complex Uvr ABC excinuclease may also act on AP sites and base-damage analogues, even if they do not result in gross perturbations of

DNA structure (Snowden et al., 1990). Whether sites of base loss may be a substrate for nucleotide excision in eukaryotic cells has not been clarified yet. In yeast it has been shown that the DNA helicase activity of the NER *RAD3* gene is inhibited by small adducts and AP sites and possibly sequestered at these lesion sites (Naegeli et al., 1993).

In this study the ability of mammalian cell extracts to repair both natural and modified AP sites carried by circular DNA duplexes was investigated. For this purpose the repair system, *in vitro*, developed by Wood et al. (1988) was applied to study repair of plasmid DNA molecules carrying heat-induced AP sites or methoxyamine (MX)-modified AP sites in a hamster cell-free system.

MX-AP sites have been largely used to explore the substrate-recognition properties of AP endonucleases of different sources. Interestingly, the major *E. coli* AP endonucleases, like exonuclease III, endonuclease III and IV, are highly tolerant to the MX modification of the C'1 aldehyde (Rosa et al., 1991; Wallace, 1988), while the major 5' mammalian AP endonucleases and AP lyases cleave this substrate very inefficiently (Rosa et al., 1991; Sanderson et al., 1989). Therefore, in mammalian cells, base excision repair of MX-adducted AP sites may be poor and alternative pathways may need to be called into action for repair of such lesions.

In order to test the possible involvement of nucleotide excision in recognition and repair of base-loss sites, the repair, *in vitro*, assay was also performed with NER-deficient mammalian cell extracts.

EXPERIMENTAL

Chemicals

Methoxyamine hydrochloride was purchased from Sigma. Stock

Abbreviations used: AP, apurinic/apyrimidinic; CHO, Chinese hamster ovary; Fpg, formamidopyrimidine–DNA glycosylase; MX, methoxyamine; NER, nucleotide excision repair.

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solutions were prepared in water, neutralized to pH 7, and stored at -20°C .

Cell extracts

Cell extracts were prepared from the Chinese hamster ovary cell line, CHO-9 and its derivative 43-3B, which is defective in the *ERCC1* gene product (Wood and Burki, 1982). The procedure was essentially that published by Manley et al. (1980) as described in Wood et al. (1988). Each extract was prepared from a pellet of about 10^9 exponentially growing cells cultured in 850 cm² roller bottles and harvested by trypsinization. The final protein concentration was typically 15–20 mg/ml. After preparation, cell extracts were immediately stored at -80°C or in liquid nitrogen.

Plasmid substrates

Closed circular pBR 322 (4.3 kb), purified on sucrose gradients, was used as undamaged control DNA. pBR 328 (4.9 kb) was irradiated with u.v. (254 nm) light (500 J/m^2) to yield 4.3 u.v. (cyclobutane + 6–4) photoproducts/kb and treated with an excess of *E. coli* Nth protein (0.04 mg of protein per mg of DNA) to incise DNA containing u.v.-induced hydrated and ring-fragmented pyrimidines. Closed circular irradiated plasmid-DNA was then purified by sucrose gradient centrifugations (Biggerstaff et al., 1991). Plasmid pUC 12 (2.7 kb) was depurinated by incubation in 0.01 M sodium citrate, 0.1 M sodium chloride, pH 5 at 70°C for various times (15, 30, 45 and 60 min). Part of the depurinated plasmid DNA was treated with MX (30 mM final concentration) essentially as described by Talpaert-Borlé and Liuzzi (1983) to generate *O*-alkoxyamine adducted-AP sites. These modified AP sites are very stable under neutral conditions at 30°C (Fortini et al., 1993). Pure supercoiled plasmid forms were then isolated on sucrose or CsCl gradients. The extent of the reaction of MX with AP sites was monitored by alkaline gel-electrophoresis analysis. More than 90 % of the MX-AP supercoiled plasmids were protected by alkaline-rupture, indicating the high efficiency of interaction of MX with AP-DNA. The number of AP or MX-modified AP sites was determined by digestion of the plasmid DNAs with *E. coli* endonuclease III, followed by 1 % agarose gel electrophoresis and densitometric scanning of the relative amounts of supercoiled and nicked forms. The number of AP sites was calculated by Poisson analysis.

Repair replication *in vitro*

Repair replication reactions were carried out by the method of Wood et al. (1988). Briefly, a mixture of 0.4 μg of undamaged pBR 322 and 0.4 μg of AP sites-containing pUC 12 or 0.4 μg of u.v.-irradiated pBR 328 were incubated at 30°C for 6 h (unless otherwise indicated) with 100–200 μg of protein of cell extract in a final volume of 50 μl . The incubation buffer contained 40 mM Hepes/KOH (pH 7.9), 75 mM KCl, 5 mM MgCl_2 , 0.5 mM dithiothreitol, 20 μM each of dGTP, dCTP and TTP, 8 μM dATP, 2 μCi [α -³²P]dATP (3000 Ci/mmol, Amersham), 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase (type I, Sigma), 3.4 % glycerol, 18 μg of BSA. Reactions were stopped by 20 mM EDTA + dry ice and plasmid purified as previously described (Wood et al., 1988). The DNA from each reaction was linearized with *EcoRI* restriction enzyme and electrophoresed overnight on a 1 % agarose gel in buffer containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Data were quantified by densitometric scanning of autoradiography carried out by an LKB Ultrascan XL laser densitometer. The amount of [³²P]dAMP incorporated was corrected for the amount of DNA

recovered from each reaction, as measured by densitometry of a photographic negative of the gel. When the conformational state of repaired plasmid DNA had to be investigated, the reaction volume and the amount of all components were doubled and half of the recovered DNA was not linearized prior to gel electrophoresis analysis (Frosina and Wood, 1990; Frosina and Rossi, 1992).

Detection of AP sites and MX-modified AP sites in repaired plasmids

Damaged plasmid DNA (400 ng), subjected to repair *in vitro* by CHO-9 or 43-3B extracts, was recovered from the reaction mixture and incubated with *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) protein (0.4 $\mu\text{g}/\text{ml}$) to reveal the presence of potentially unrepaired AP sites or MX-AP sites. Fpg-sensitive sites were detected as an increase in form II (nicked circular) plasmid molecules as compared with undigested samples. Form I supercoiled and form II nicked plasmid molecules were resolved by 1 % agarose gel electrophoresis.

Detection of nicking activity at AP sites and MX-AP sites

400 ng of plasmid DNA were incubated at 30°C for 10 min with 10 and 20 μg protein of CHO-9 or 43-3B whole-cell extract (or with the extract buffer) and reactions were stopped by freezing on dry ice. Nicked forms were then resolved by 1 % agarose gel electrophoresis.

RESULTS

Repair of AP and MX-AP sites by CHO-9 cell extracts

Figure 1(a) shows DNA repair-replication stimulated by similar levels of AP sites or MX-AP sites (+ AP) on pUC 12 plasmids. The repair incorporation increased with increasing number of AP sites (lanes 1 and 2) or MX-AP sites (lanes 3 and 4) as expected from damage-dependent synthesis. MX-adducted AP sites (lanes 3 and 4) stimulated more incorporation than AP sites (lanes 1 and 2). Little or no unspecific incorporation was detected on control undamaged pBR 322 plasmids (– AP, lanes 1–4). When the amount of incorporated radioactive material was plotted as a function of the number of lesions, a linear dose-response relationship was observed (Figure 1b) with both natural (circles) and modified (squares) AP sites. The repair synthesis stimulated by MX-AP sites was 1.5 ± 0.3 -fold higher than the synthesis induced by AP sites (mean $\pm$ S.E.M. of three independent experiments). Hamster cell extracts are therefore able to repair with a great efficiency sites of base-loss in depurinated plasmids.

The repair response was not dependent on the size of the plasmid. Similar repair responses were indeed observed when the 5.2 kb pSV2gpt plasmid was used instead of the 2.7 kb pUC 12 plasmid (results not shown). Two independent preparations of depurinated pUC 12 plasmids were made, with two different methods for purification of supercoiled forms: sucrose and CsCl gradient centrifugations. With both methods, similar dose-effect responses were observed (results not shown).

The repair of AP sites is expected to consist of several steps: incision, excision, resynthesis and ligation. The ligation reaction should proceed rapidly after repair synthesis. In fact when the repair-replication products were resolved on agarose gel electrophoresis without linearization with *EcoRI* (Figure 1c), almost 90 % of radioactivity comigrated with closed circular forms, thus indicating that depurinated plasmids undergo a complete excision repair process.

Figure 1 Repair of AP or MX-modified AP sites by CHO-9 cell extracts

(a) A mixture of 0.4 μ g undamaged pBR 322 and 0.4 μ g of pUC 12 plasmids containing 0.25 AP sites/kb (lane 1), 0.5 AP sites/kb (lane 2), 0.25 MX-modified AP sites/kb (lane 3) or 0.6 MX-modified AP sites/kb (lane 4), was incubated with 100 μ g of protein of CHO-9 whole-cell extract. Damaged (+ AP) and undamaged (- AP) linearized DNAs were resolved by electrophoresis. The upper panel shows a photograph of the ethidium bromide-stained gel and the lower panel an autoradiograph of the dried gel. (b) Quantification of repair replication as in (a). (○—○) Plasmids carrying AP sites. (□—□) Plasmids carrying MX-AP sites. (c) Autoradiograph of a dried gel as in (a) but with plasmids not linearized at the end of the repair reaction.

Table 1 Repair-replication in supercoiled or linear plasmids carrying abasic sites

Plasmid substrate	Type of damage*	dAMP incorporated (fmol)†	Relative incorporation (%)
pUC12 (supercoiled)	AP sites	111.8	100.0
pUC12 (supercoiled)	MX-AP sites	114.6	100.0
pUC12 (linear)	AP sites	23.9	21.4
pUC12 (linear)	MX-AP sites	38.2	33.3

* pUC12 plasmids carry 0.6 AP sites/kb or 0.4 MX-AP sites/kb.

† Repair-synthesis reactions were performed with 80 μ g of CHO-9 cell extract protein.

ATP is required during repair of AP and MX-AP sites in hamster cell extracts and the optimal ATP concentration was 2 mM (results not shown).

The repair incorporation stimulated by AP sites was decreased when the substrate was linearized (Table 1), similar to what happens with u.v.-irradiated plasmids (Hoeijmakers et al., 1990; Legerski et al., 1987). This might be due to fewer initiated repair events and/or to shorter repair patches.

Repair of AP and MX-AP sites by 43-3B cell extracts

The 43-3B hamster cell line is a derivative of CHO-9 which is highly sensitive to u.v. light and crosslinking agents. The biochemical defect resides in the *ERCC1* gene which is a component of the NER system (Biggerstaff et al., 1993; Van Vuuren et al., 1993). Figure 2 shows the repair synthesis carried out by repair proficient (lanes 1–3) and repair-deficient (lanes 4–6) cell extracts on depurinated plasmids. In this experiment the incubation was carried out for 1 h instead of the standard 6 h repair, because the

repair defect was more clearly detectable with shorter incubation times. Indeed, a clear defect was observed in the repair of u.v. damage by the 43-3B extract, with a residual repair activity of 27% as compared with repair-proficient CHO-9 extract (lanes 1 and 4). On the contrary, no significant difference could be observed between CHO-9 or 43-3B extracts in the repair of AP sites (lanes 2 and 5) or MX-AP sites (lanes 3 and 6). The increased repair incorporation on the MX-AP sites, as compared with unmodified AP sites reported in Figure 1, was not detectable in this experiment, due to the short repair incubation time (1 h versus 6 h) and the lower density of MX-AP sites on the substrate (0.25 MX-AP sites/kb versus 0.35 AP sites/kb). In conclusion, the repair *in vitro* of natural and MX-modified AP sites occurs even in an NER-deficient strain.

Moreover, Figure 2 shows that the repair-incorporation stimulated by 0.35 AP sites/kb (lane 2) is of similar magnitude to the incorporation stimulated by 4.3 photoproducts/kb (lane 1). In order to explain this high level of incorporation on the depurinated plasmids, the efficiency of incision and removal of AP and MX-AP sites was investigated. AP site- and MX-AP site-containing plasmids were incubated with CHO-9 or 43-3B extracts (10–20 μ g of protein) for 10 min at 30 °C and the repair intermediates quick-frozen on dry ice and resolved by agarose gel electrophoresis. Plasmids carrying AP sites were very efficiently incised (Figure 3) with either the CHO-9 (lanes 3 and 4) or the 43-3B extracts (lanes 7 and 8). This enzymic incision is very likely carried out by the high levels of AP endonucleases present in the extracts. Plasmid incision by AP endonucleases is less efficient on MX-AP sites (60% of incision at natural AP sites; lanes 5, 6 and 9, 10), in agreement with previous data (Rosa et al., 1991). Undamaged pBR 322 plasmids were not incised under these conditions. ATP is not required in the incision step although it is essential for repair synthesis and ligation (results not shown).

Base loss sites are therefore very efficiently incised by both wild-type and *ERCC1* mutant hamster cell extracts. MX-modified AP sites are a less suitable substrate but, under the

Figure 2 Repair of AP- or MX-modified AP sites by NER-defective 43-3B cell extracts

Plasmid mixtures containing undamaged (—) and damaged (+) DNAs (0.4 µg each) were incubated for 1 h with 200 µg of cell extract. Plasmid DNAs were then purified, linearized and resolved by electrophoresis. (a) Photograph of the ethidium bromide stained gel. Lane 1: u.v.-irradiated pBR 328 containing 4.3 u.v. photoproducts/kb after incubation with CHO-9 cell extracts. Lane 2: pUC 12 plasmid containing 0.35 AP sites/kb, after incubation with CHO-9 cell extracts. Lane 3: pUC 12 plasmid containing 0.25 MX-modified AP sites/kb, after incubation with CHO-9 cell extracts. Lanes 4, 5 and 6: plasmids as in lanes 1, 2 and 3 respectively, except that the incubation was performed with 43-3B *ERCC1* mutant extract. (b) Autoradiograph of the dried gel. (c) Quantification of repair incorporation as in (b).

Figure 3 Incision of plasmids carrying AP or MX-AP sites by CHO-9 or 43-3B extracts

Damaged plasmids were incubated for 10 min with 10 µg (lanes 3 and 5) or 20 µg (lanes 4 and 6) CHO-9 extracts; or with 10 µg (lanes 7 and 9) or 20 µg (lanes 8 and 10) 43-3B extracts. As control, damaged plasmids were also incubated in the reaction mixture without extracts (lanes 1 and 2). Lanes 1, 3, 4, 7 and 8: plasmids containing 0.35 AP sites/kb. Lanes 2, 5, 6, 9 and 10: plasmids containing 0.25 MX-AP sites/kb.

extreme excess of AP endonucleases as in the repair assay *in vitro* (100–200 µg extract), they are likely to be completely incised as natural AP sites.

The occurrence of repair synthesis in damaged substrates is not informative about the extent of lesion removal which has taken place. If AP sites or MX-AP sites are left unrepaired after the repair reaction *in vitro*, treatment with specific AP-cleavage activities should reveal their presence. The Fpg protein was selected for these experiments, it being able to recognize efficiently both natural and MX-modified AP sites (Figure 4). pUC 12 plasmids carrying an average of 0.4 MX-AP/kb (lanes 5, 6, 9 and 10) or 0.9 MX-AP/kb (lanes 7, 8, 11 and 12) were repaired in standard repair reactions with 150 µg of protein of CHO-9 (lanes 5–8) or 43-3B (lanes 9–12) extract. They were then purified and exposed to the AP lyase activity of *E. coli* Fpg protein. Plasmid DNAs were completely insensitive to the nicking activity of Fpg, after being repaired by either the CHO-9 (lanes 5–8) or the 43-3B (lanes 9–12) extracts, thus showing that no detectable MX-AP sites were left unrepaired on the DNA circles. This phenomenon was observed in both the ethidium bromide stained gel (Figure 4a) and the autoradiograph (Figure 4b), confirming that nearly 100% of MX-AP sites-containing plasmids are repaired by the whole-cell extracts. Plasmid circles migrated as relaxed closed circular forms [with higher electrophoretic mobility as compared with supercoiled DNA (lane 13)]. This is the typical conformational state of repaired plasmids, where most of repair synthesis is confined (Figures 4b and 1c). In control lanes, undamaged pBR 322 plasmids were completely insensitive to Fpg (lanes 1 and 2), whilst unrepaired pUC 12 plasmids carrying an average of 0.4 MX-AP lesions/kb were incised (lanes 3 and 4). Similarly, no Fpg-sensitive sites were detected in AP sites-containing

Figure 4 Loss of Fpg-protein sensitive sites in repaired plasmids

pUC 12 plasmids (0.4 μ g) were incubated in standard repair reactions with 150 μ g of extract proteins, then purified and incubated with 10 ng Fpg protein (even lanes) or Fpg buffer (odd lanes). Lanes 1 and 2: unreplicated, undamaged control pBR 332; lanes 3 and 4: unreplicated pUC 12 plasmids with 0.4 MX-AP sites/kb; lanes 5 and 6: after incubation with CHO-9 extract; lanes 7 and 8: pUC 12 plasmids with 0.9 MX-AP sites/kb after incubation with CHO-9 extract; lanes 9 and 10: pUC 12 plasmids with 0.4 MX-AP sites/kb after incubation with 43-3B extract; lanes 11 and 12: pUC 12 plasmids with 0.9 MX-AP sites/kb after incubation with 43-3B extract; lane 13: nicked and supercoiled pUC 12 plasmids. (a) Photograph of the gel. (b) Autoradiograph of the gel.

Figure 5 Kinetics of repair replication on damaged plasmids

Repair reactions *in vitro*, composed as those described in Figure 2, were performed for different times (from 20 min to 3 h). Plasmid DNA was then purified and [32 P]dAMP incorporation quantified. ($\triangle$ — $\triangle$) u.v.-irradiated pBR 328 after incubation with CHO-9 extract. ($\blacktriangle$ — $\blacktriangle$) u.v.-irradiated pBR 328 after incubation with 43-3B extract. ($\circ$ — $\circ$) pUC 12 plasmids containing AP sites, after incubation with CHO-9 extract. ($\bullet$ — $\bullet$) pUC 12 plasmids containing AP sites, after incubation with 43-3B extract. ($\square$ — $\square$) pUC 12 plasmids containing MX-AP sites, after incubation with CHO-9 extract. ($\blacksquare$ — $\blacksquare$) pUC 12 plasmids containing MX-AP sites, after incubation with 43-3B extract.

plasmids after repair synthesis (results not shown). In conclusion, in the assay *in vitro*, either MX-AP or natural AP sites are removed by CHO-9 or 43-3B extracts, with a system efficiency very close to 100%.

Comparison of repair kinetics of AP sites versus u.v. damage

In an attempt to characterize the nature of the vigorous repair of base-loss sites by mammalian cell extracts, repair-kinetics experiments were performed on plasmids containing AP sites or u.v. lesions. As shown in Figure 5, after only 20 min incubation, the repair of AP sites and MX-AP sites is approximately 70% of the level found after 3 h, with both a wild-type CHO-9 extract and the NER-mutant 43-3B extract. In contrast, the repair of u.v. damage is only about 10% of that found after 3 h, thus indicating that the NER pathway is much slower than the repair of AP sites. As expected, the repair of u.v. damage was deficient in a 43-3B extract, at both 1 and 3 h incubation time (Figure 5). Moreover, it should be noted that in this experiment the lesion densities of u.v. photoproducts was 12-fold higher than the density of AP sites (4.3 versus 0.35 lesions/kb). Therefore, the difference in the repair replication rate of the two kinds of damage is likely to be underestimated.

DISCUSSION

Mammalian cell-free extracts have been successfully used to explore the repair mechanism of bulky DNA adducts built into circular DNA duplexes (Wood et al., 1988; Hansson and Wood, 1989). We have extended this type of studies to less severe perturbations of the DNA structure like natural and modified base-loss sites.

Repair of natural and modified AP sites

DNA AP sites are efficiently repaired by Chinese hamster extracts in the repair system *in vitro*. The level of repair-incorporation stimulated by 0.5 AP sites/kb is in fact comparable to the incorporation observed with a substrate containing 4.3 u.v. photoproducts/kb. What accounts for this different ability to elicit repair synthesis? It is known that a small percentage (about 5%) of u.v.-photoproducts are a substrate for the human NER in the repair system *in vitro* (Szymkowski et al., 1993a; Wood et al., 1988; Wood and Robins, 1989). In contrast, in the case of depurinated DNA, we have shown that nearly 100% of the depurinated plasmid molecules are incised by the hamster cell extracts (Figure 3) and no damage is left on DNA at the end of the repair reaction (Figure 4). Therefore, the efficiency of the repair system in charge of AP sites-removal is a major determinant of the higher level of repair-incorporation at AP sites as compared with u.v. photoproducts.

The estimation of the repair-patch associated with removal of DNA damage may help in the identification of the repair pathway. The u.v.-irradiated (500 J/m²) plasmids contain approximately 3.2 cyclobutane pyrimidine dimers/kb and 1.1 (6–4) photoproducts/kb and about 5% are a substrate for DNA repair, as mentioned above. The (6–4) lesions are repaired 10-fold more efficiently than cyclobutane dimers (Szymkowski et al., 1993b; Wood, 1989). Therefore, the (6–4) photoproduct-dependent incisions introduced during the incubation were: $1.1 \text{ (6–4 dimer)} \times 4.9 \text{ (pBR328 kb)} \times 123 \text{ (fmol of plasmid)} \times 0.05 \text{ (repair efficiency of the system)} = 33.1 \text{ fmoles}$. Likewise, in the case of cyclobutane dimers, the damage-dependent incisions were: $3.2 \text{ (cyclobutane dimers)} \times 4.9 \text{ (pBR328 kb)} \times 123 \text{ (fmol of plasmid)} \times 0.05 \text{ (repair efficiency of the system)} \times 0.1 \text{ (relative repair efficiency of cyclobutane dimers as compared with (6–4) photoproducts)} = 9.6 \text{ fmoles}$. About 300 fmoles of dAMP (1200 fmoles of total dNMPs) were incorporated during the repair events (Figure 2). Therefore, the average patch size for a single (6–4) dimer or cyclobutane dimer is $1200 / (33.1 + 9.6) = 28$

nucleotides. This value is in agreement with the processing of u.v. photoproducts by the NER pathway with a repair patch of 28–30 nucleotides (Huang et al., 1992; Szymkowski et al., 1993a,b).

Similarly, from the number of AP sites incised and the level of repair synthesis, the repair-patch for a single AP site can be estimated. An average size of 6.6 nucleotides was calculated.

This short repair-patch suggests that the base-excision repair is the main mechanism responsible for the repair of AP sites located on circular molecules by mammalian cell extracts. The repair-patch observed with hamster cell extracts is slightly longer than that found by Matsumoto and Bogenhagen (1989) using *Xenopus laevis* oocyte extracts (3–4 nucleotides) and by Dianov et al. (1992) using human cell extracts (1 nucleotide). This might be due to: (1) some inaccuracy in the evaluation of the patch size due to the presence of multiple lesions and/or the determination of the specific activity of the radiolabelled nucleotide; (2) the excision of a longer oligonucleotide stretch by the hamster base-excision system as compared with the amphibian or human repair system; (3) the higher level of incorporation observed in experiments with circular DNA (this study) compared with experiments with linear DNA (Dianov et al., 1992) (Table 1); (4) the use of authentic AP sites as compared with modified AP sites (Matsumoto and Bogenhagen, 1989; 1991); (5) a partial contribution of a long-patch repair mechanism to the overall incorporation stimulated by AP sites. Concerning point (3), it should be noted that the calculated AP site repair patch-size on linear DNA (Table 1) is about 1 nucleotide, in agreement with the value reported by Dianov et al. (1992), who also used linear substrates. This might indicate that repair patches at AP sites are longer with supercoiled circular substrates.

With reference to point (5), it is interesting to consider the data obtained with alkoxyamine-modified AP sites. MX-modified AP sites are a better substrate for repair-incorporation by mammalian cell extracts than AP sites. In fact, MX-modified AP sites stimulate a repair-replication response that is about 1.5-fold higher than that stimulated by AP sites (Figure 1). On the other hand, plasmids carrying MX-AP sites are incised by CHO-9 extracts with a lower efficiency as compared with unmodified AP sites (Figure 3). Nonetheless, no MX-modified AP sites are left unrepaired on the circular genomes at the end of the repair reaction (Figure 4), indicating that under the repair conditions *in vitro* all the modified plasmids are a substrate for the hamster repair enzymes. The calculation of the average repair-patch for a single MX-AP site gives a size of 10 nucleotides. This result suggests that, even if base-excision repair is still the main mechanism active on AP site-analogues, the modification by MX might also determine some events of long patch synthesis.

The kinetics of repair is also different for AP sites as compared with u.v. photoproducts. The repair of AP sites is accomplished in 20 min, while u.v. damage is repaired in 3 h. Moreover, in these experiments the lesion density of AP sites is lower than that of u.v. photoproducts; therefore the higher incorporation at AP sites compared with u.v. damage is more striking than that appearing in Figure 5.

There should be a significant difference in the ability of the NER complex to locate, incise and repair lesions as compared with the AP site repair process.

Base-excision repair is a fast and efficient system especially devoted to cope with one of the major sources of endogenous damage in cells i.e. AP sites. NER is a slower mechanism, acting on a much broader spectrum of lesions (u.v. dimers and bulky chemical adducts) that quantitatively affect the genome integrity to a lower extent, compared with the several thousands of AP sites arising each day in the DNA of a mammalian cell (Lindahl, 1993).

The specific requirement for ATP in repair of depurinated plasmids is associated with the excision, resynthesis and ligation steps, not with the incision. Instead, with u.v.-irradiated DNA the presence of ATP seems to be associated with early steps of repair, like incision and/or oligonucleotide displacement (Wood et al., 1988).

Is the nucleotide excision involved in repair of AP sites or AP site-analogues?

To investigate the potential role of nucleotide excision in AP site repair we have used an *ERCC1* mutant hamster cell line, 43-3B. The product of the *ERCC1* gene is required in the incision step of the nucleotide excision (Biggerstaff and Wood, 1992), and it has recently been shown that the protein assembles with one or more other peptides (coded by genes *ERCC4*, *XPFC* and *ERCC11*) to form a multi-protein enzymic repair complex (Biggerstaff et al., 1993; Van Vuuren et al., 1993). In agreement with data by others (Van Vuuren et al., 1993), we found that the repair defect of the 43-3B mutant was detectable *in vitro* only when u.v.-irradiated plasmids were incised with *E. coli* Nth protein and rebanded, to get rid of plasmids carrying hydrated and ring-fragmented pyrimidines. The latter are indeed the substrate of an Nth-like activity present in mammalian cells that seems very efficient in Chinese hamster cells. Repair-replication initiated by this enzymic activity is sufficient to obscure the defect in NER of pyrimidine dimers. When u.v.-irradiated plasmids treated with *E. coli* Nth protein were used in the repair assays, the residual repair activity of 43-3B extract was 27% of that of parental CHO-9 cell extracts, in agreement with previously published data (Van Vuuren et al., 1993; Biggerstaff and Wood, 1992). An influence of the incubation time was also observed as the above value of residual activity was observed after 1 h incubation, while at 20 min it was 72% and at 3 h it was 32%.

When NER-deficient 43-3B cell extracts were tested on plasmids carrying AP and MX-AP sites, no defect in repair replication was detected, in comparison with the wild-type CHO-9 extract (Figure 2). Although any effect of *ERCC1* would likely be masked by the vast excess of AP endonucleases, these data indicate that the involvement of nucleotide excision in the repair of AP or MX-AP sites, if any, is very limited. The 1.5-fold increase in repair synthesis observed on MX-AP sites as compared with AP sites, might be due to an interference of MX-modification with the post-incision repair steps leading to more extensive nick-translation events.

In conclusion, we have shown that circular molecules containing AP or MX-AP sites are efficiently repaired by hamster cell extracts. Base-excision repair is the main mechanism of removal of these non-coding lesions. The construction of a substrate containing a single AP site or MX-AP site at a defined position will allow the repair patch at these lesions to be established precisely.

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