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Detection of damage-recognition proteins from human lymphocytes★✪

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Proteins recognizing and binding to damaged DNA (DDB-proteins) were analyzed in human lymphocytes obtained from healthy donors. Using an electrophoretic mobility shift assay several complexes between nuclear extract proteins and damaged DNA were detected: a complex specific for DNA damaged by *N*-acetoxy-*N*-acetylaminofluorene, another complex specific for UV-irradiated DNA, and two complexes specific for DNA damaged by *cis*-dichlorodiammine platinum. All the detected complexes differed in electrophoretic mobility and possibly contained different proteins. Complexes specific for free DNA ends were also detected in protein extracts from lymphocytes.

Living organisms have developed several repair systems dealing with a broad range of DNA lesions. Among them are: direct repair, base excision repair (BER), nucleotide excision repair (NER), homologous recombination, non-homologous end-joining, and mismatch repair. Damage recognition is the first step in any repair pathway. In some repair systems (i.e. direct repair and BER) damage is recognized by proteins with enzymatic activ-

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Abbreviations: AAAF, *N*-acetoxy-*N*-acetylaminofluorene; BER, base excision repair; BPDE, benzo(a)pyrene diol epoxide; *cis*-DDP, *cis*-dichlorodiammine platinum(II); DDB, damaged DNA-binding; NER, nucleotide excision repair; PBS, phosphate buffered saline; RPA, replication protein A; XP, xeroderma pigmentosum; XRCC, X-ray repair cross complementation group.

ity. In other systems damage is recognized by specific proteins that recruit other components of the repair machinery. NER is an important universal pathway that removes a broad spectrum of bulky DNA lesions including those induced by UV-irradiation, polycyclic chemicals or the anticancer drug *cis*-dichlorodiammine platinum (*cis*-DDP). It is currently postulated that, depending on the type of damage and the NER sub-pathway, different protein complexes may take part in damage recognition. Among such complexes in eukaryotic cells are XPA/RPA, XPC/HR23B and UV-DDB (XPE) (rev. in [1]). The repair of DNA double-strand breaks engages DNA-dependent protein kinase. The binding of this protein to DNA is mediated through the Ku70/Ku86 heterodimer that shows high affinity to free DNA ends (review in [2]). A large number of DNA-binding proteins show elevated affinity to damaged DNA but they are probably not involved directly in DNA repair. Among them are the abundant chromatin proteins HMG-1/2 [3] and histone H1 [4], which show preferential binding to DNA damaged by *cis*-DDP. Poly(ADP-ribose) polymerase (PARP) binds to single or double strand ends, however its involvement in DNA repair still remains elusive [5]. It has been proposed that such proteins may compete with *bona fide* DNA repair proteins, making the repair less efficient [6].

Defects in DNA repair genes result in several serious diseases that are caused by gradual accumulation of non-repaired DNA lesions. Patients with xeroderma pigmentosum (XP) suffer from severe UV-sensitivity, which is caused by defects in several genes involved in different steps of NER (review in [7, 8]). Several other human disorders: Cockayne syndrome [9], ataxia telangiectasia [10], Nijmegen breakage syndrome [11] are also caused by defects in DNA repair genes. Polymorphisms in some genes involved in repair of damage induced by radicals and ionizing radiation (XRCC1, XRCC3 and XRCC5) have been found in cancer patients and correlate with their increased radiosensitivity [12]. Polymorphism in repair genes can be detected also in healthy individuals. For example, amino-acid substitutions in XPD, XPF and XRCC1 have been found in healthy population [13].

It has been postulated that polymorphisms in repair genes influence repair capacity and affect susceptibility of carriers to cancer. However, to prove this hypothesis genetic and functional data should be completed, which would show how a particular genetic change could affect the cellular repair capacity. Among the tests used for investigating DNA repair the incorporation of radioactive precursors to DNA is frequently applied, either in whole cells [14] or in cellular extracts *in vitro* [15]. Since recognition of a damage is the first step of the repair process, examination of the level and activity of damage-recognition proteins is another convenient test for potential efficiency of DNA repair. Such a test may be a potent clinical screening procedure if peripheral blood lymphocytes are used. The aim of this work was to detect and characterize damaged DNA binding (DDB) proteins in human peripheral blood lymphocytes, the cells widely used in human studies as a "surrogate" tissue.

MATERIALS AND METHODS

Cells and protein extracts. Peripheral blood was collected from healthy volunteers 22–28 years old. Lymphocytes were isolated under sterile conditions by centrifugation on a Ficoll gradient (Histopaque 1077, Sigma). In some experiments cells were incubated for 24 h at 37°C and γ-irradiated on ice in a ^{60}Co beam at a dose of 2 Gy. Protein extracts were prepared according to [16], with some modifications. Briefly, freshly isolated lymphocytes were centrifuged, suspended in 3 volumes of buffer consisting of 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitor mixture (Complete^{TM}, Boehringer) and incubated 10 min on ice. Nonidet P-40 was then

added to a final concentration of 0.6%, cells were briefly vortexed and centrifuged for 15 min at 3000 ***g***. Supernatant was collected as a cytoplasmic extract. Pellet was suspended in an equal volume of low-salt buffer consisting of 20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 2 mM KCl, 0.2 mM EDTA and protease inhibitor mixture, and then an equal volume of high-salt buffer (of same composition as above, except that for the presence of 0.8 M NaCl) was added. The mixture was incubated for 30 min on ice, centrifuged for 30 min at 16000 ***g*** and supernatant was collected as a nuclear extract. Total extract was prepared as follows: PBS-washed lymphocytes were suspended in extraction buffer consisting of 10 mM Tris/HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and protease inhibitor mixture, incubated for 20 min on ice, vortexed and centrifuged for 30 min at 16000 ***g***. The supernatant is referred to as total extract.

DNA probes. A synthetic double-stranded 36 bp-long oligonucleotide (5′-AATTCGTAGG CCTAAGAGCA ATCGCACCTG TGCGCG-3′, with blunt ends) was used as a molecular DNA probe. The oligonucleotide (at 10 μM concentration) was incubated for 4 h at 37°C with 40 μM AAAF or BPDE (Midwest Research Institute), or for 20 h at 37°C with 3 μM *cis*-DDP (Ebewe), and then purified by phenol/chloroform extraction and ethanol precipitation. Alternatively, the oligonucleotide was UV-irradiated (5 kJ/m^2) using a 254 nm UV-crosslinker (Stratagene). The treatment with AAAF, BPDE or UV introduced single lesions into about 15% of the DNA molecules (on average), which was verified by the ^{32}P-postlabeling method [17]. The treatment with *cis*-DDP introduces a similar amount of damage, calculated as reported elsewhere [18]. The oligonucleotide was end-labeled by transfer of ^{32}P from [γ-^{32}P]ATP using T4 polynucleotide kinase, and purified by polyacrylamide gel electrophoresis [19].

Electrophoretic mobility shift assay (EMSA). Radioactive oligonucleotide (25 ng) was incubated with proteins of different lymphocyte extracts (5 μg) for 30 min at 4°C. The binding buffer consisted of 20 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol and 150 mM NaCl. Protein-oligonucleotide complexes were formed in a final volume of 20 μl in the presence of non-radioactive DNA competitors added before the addition of proteins. Sonicated *E. coli* DNA was used as non-specific competitor at an 80-fold excess (2 μg). Either undamaged or damaged oligonucleotide was used as a homologous competitor. Protein-oligonucleotide complexes were resolved by electrophoresis on 6% polyacrylamide gel in 0.5 × TBE running buffer (45 mM Tris/borate, 1 mM EDTA). Gels were dried and autoradiographed.

RESULTS AND DISCUSSION

In this study we have tested whether human lymphocytes contain proteins that specifically bind to DNA damaged by various genotoxic agents. We have used an electrophoretic mobility-shift assay to detect DNA-binding proteins in cellular extracts. In this method, proteins having an affinity for a radio-labeled control or damaged DNA are detected by gel electrophoresis in the form of retarded bands containing protein-DNA complexes. A synthetic double-stranded 36 bp-long oligonucleotide, either undamaged or damaged *in vitro* by AAAF, *cis*-DDP or UV radiation was used as a radioactive probe. AAAF forms covalent DNA adducts, mostly at guanine residues [20]. *Cis*-DDP induces mostly 1,2-d(GpG) or d(ApG) intrastrand crosslinks [21]. Major lesions induced by UV-C radiation are cyclobutane pyrimidine dimers and (6–4) photoproducts [22]. We utilized procedures that introduced a single lesion in about 15% of the DNA molecules [17]. All such DNA lesions are known to be repaired in NER system [1].

In initial experiments we tested three types of extracts to compare the presence and speci-

ficity of damage-recognition proteins in peripheral lymphocytes: nuclear extracts (NE), cytoplasmic extracts (CE) and whole cell extracts (WE) (Fig. 1). To reduce the presence of histones 0.4 M NaCl concentration was used to extract nuclear proteins. Complexes specific for AAAF- and *cis*-DDP-damaged DNA were detected in all three types of extracts, however their highest levels were observed in nuclear extracts. Complexes specific for UV-damaged DNA were detected only in nuclear extracts. According to data shown in Fig. 1A one complex specific for AAAF-damaged DNA (AAAF-DDB, lane 2), two complexes specific for *cis*-DDP-damaged DNA (DDP-DDB, lane 3) and one complex specific UV-damaged DNA (UV-DDB, lane 4), were detected in nuclear extracts (arrowheads in Fig. 1A). All these complexes differed in electrophoretic mobility, which suggests that they differed also in protein composition. The two DDP-DDB complexes were very abundant while the amount of AAAF-DDB complex was very low. Complexes that were formed with undamaged DNA were also detected. One such a complex was detected in nuclear extracts and an additional one in cytoplasmic or whole cell extracts (asterisks in Fig. 1A). When BPDE-damaged DNA was used as a radioactive probe no complexes specific for damaged DNA were detected (not shown). Since the levels of complexes specific for damaged DNA were the highest in nuclear extracts, those extracts were chosen for further experimentation.

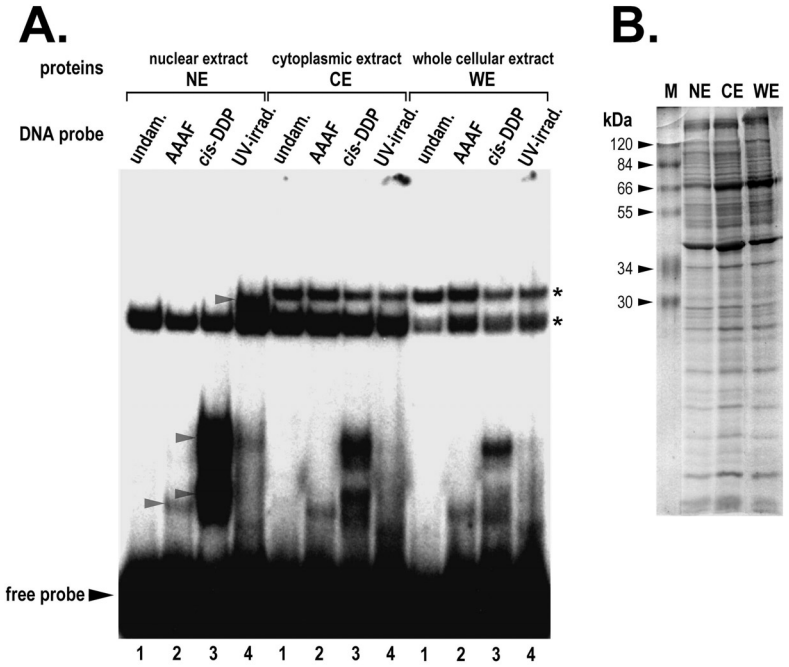

Figure 1. Analysis of damage-recognition proteins in extracts from human lymphocytes.

Panel A. Nuclear (**NE**)], cytoplasmic (**CE**)] and whole cell (**WE**)] extracts were incubated with radioactive oligonucleotide either undamaged (undam.) or damaged by AAAF, *cis*-DDP or UV. *E. coli* DNA was used as a non-radioactive competitor. The complexes were resolved by polyacrylamide gel electrophoresis and dried gels were autoradiographed. Arrowheads show the positions of complexes specific for damaged DNA. **Panel B.** PAGE/SDS analysis of lymphocyte extracts. Lane **M,** molecular mass markers. For designation of the lanes see text.

To test the specificity of detected DDB proteins for different types of DNA damage, labeled oligonucleotide, either damaged by *cis*-DDP or UV-irradiated, was incubated with nuclear extracts in the presence of an 80-fold excess of unlabeled heterologous or homologous competitors (Fig. 2). Both DDP-DDB complexes were fully competed out when *cis*-DDP was used as a competitor (lane 4 in

Fig. 2A). However, when BPDE- or AAAF-damaged oligonucleotides were used as competitors only partial elimination of the radioactive probe from these complexes was observed (lanes 5 and 6 in Fig. 2A), suggesting that the proteins that form the two DDP-DDB complexes also have affinity to DNA damaged by BPDE or AAAF. The complex specific for UV-irradiated DNA (UV-DDB) was fully competed out when UV-damaged oligonucleotide was used as a competitor (lane 3 in Fig. 2B). Oligonucleotides damaged by *cis*-DDP (lane 4) and AAAF (lane 6) were weaker competitors.

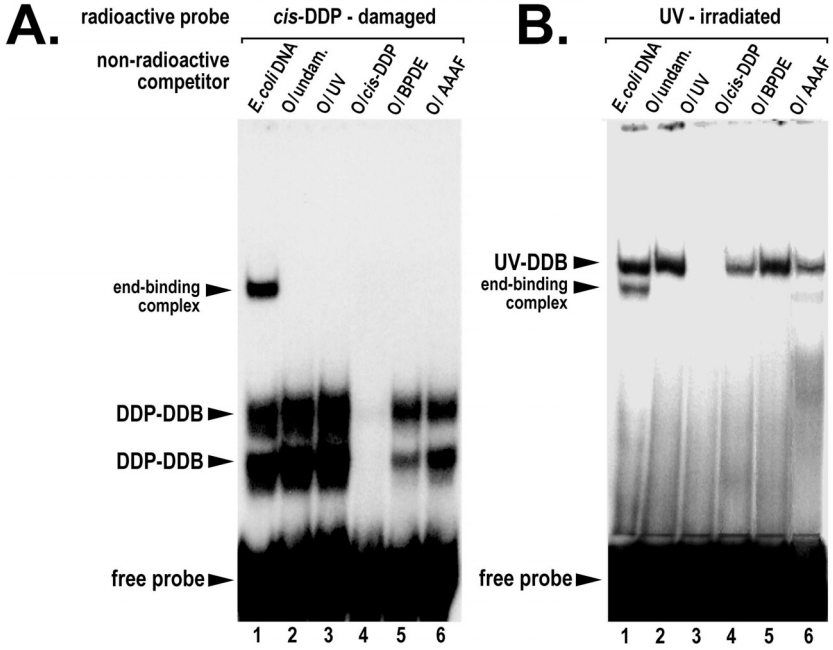

Figure 2. Analysis of the substrate specificity of proteins recognising DNA damaged by *cis*-DDP or UV radiation.

Panel A. Proteins from nuclear extracts were incubated with radioactive oligonucleotide damaged by *cis*-DDP. *E. coli* DNA or homologous oligonucleotide was used as a non-radioactive competitor. Oligonucleotide was either undamaged (**O/undam**.) or damaged by **UV**, ***cis*-DDP**, **BPDE** and **AAAF,** as indicated. **Panel B**. Proteins from nuclear extracts were incubated with UV-irradiated oligonucleotide. Competitor DNA was the same as in panel A. Complexes containing damaged DNA, complex specific for free DNA ends (see in text) and free DNA probe are marked by arrowheads. For other details see text.

This shows that proteins from UV-DDB complex have much lower affinity to these types of DNA lesion than to UV-irradiated DNA.

Major proteins recognizing DNA crosslinks induced by *cis*-DDP belong to the so-called "HMG-box" proteins, however other DDP-DDB proteins were also detected in different cell types [6]. One of the DDP-DDB complexes detected in this study co-migrates with a complex containing purified HMG-1 protein (not shown). However, the identity of the two complexes remains to be established. The major human protein recognizing UV-irradiated DNA (UV-DDB) was purified as a 127-kDa polypeptide (p127) that can form a complex with a 48-kDa polypeptide (p48), which is mutated in some xeroderma pigmentosum group E cells [23]. As the p127/p48 complex is so far the only human protein with such high affinity to and specificity for UV-induced lesions (500 000-fold preference for (6–4) photo-products-containing DNA over undamaged DNA), we assumed that the UV-DDB complex detected in our experiment probably contains this protein. AAAF-DDB complexes were also detected in nuclear extracts from lymphocytes. Analogous complexes, yet much more abundant, were found in human lymphoblastoid HL60 cells and rat hepatocytes.

These complexes contain a chromatin protein of about 23 kDa, which is probably not involved directly in DNA repair ([17], unpublished results).

When either damaged or undamaged oligonucleotide was incubated with nuclear extracts, an additional complex was detected, electrophoretic mobility being slightly higher than that of UV-DDB complexes. Such a complex was visible when *E. coli* DNA, but not oligonucleotide (either damaged or undamaged), was used as a competitor (lanes 1 in Fig. 2). We presumed that the complex was specific for either the sequence or free DNA ends. To verify this we used pUC19 plasmid in either circular or linearized form as a competitor. Data presented in Fig. 3A show that a complex not specific for damaged DNA was competed out when the linear but not the circular form of the plasmid was used as a competitor (lanes 1 and 2). This result is consistent with the interpretation that the complex is specific for free DNA ends, hence we named it the "end-binding complex". When a UV-irradiated probe was used as a control, neither form of the plasmid was effective as competitor of the UV-DDB complex but both behaved as predicted for competitors of the "end-binding complex". The most abundant protein that shows specificity for free DNA ends is the Ku70/Ku86 heterodimer [24]. However, the identity of the "end-binding complex" detected in this work remains to be established. When pUC19 DNA was used as a competitor, an additional complex was detected (lanes 1 and 2 in Fig. 3A, marked with asterisk). Since such a complex was competed out when *E. coli* DNA (containing a more heterogenic se-

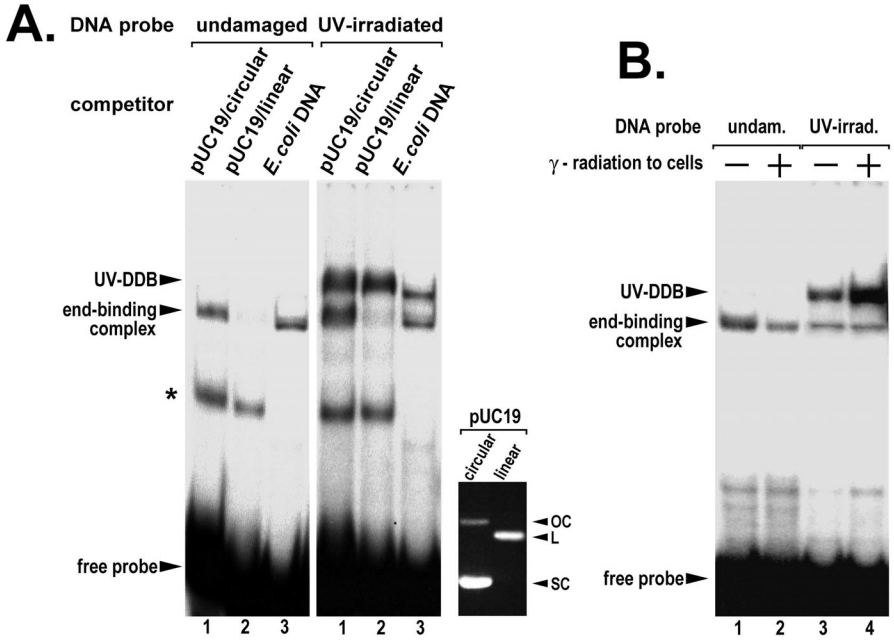

Figure 3. Analysis of the complexes specific for free DNA ends.

Panel A. Undamaged or UV-irradiated oligonucleotide was incubated with nuclear extracts in the presence of 2 μg of non-radioactive competitor: pUC19 plasmid, either circular form (no free ends) or linear form (processed with *Eco*RI restriction enzyme), and *E. coli* DNA. The positions of complexes specific for UV-irradiated DNA, free end-containing DNA and position of free probe are marked by arrowheads. Sequence-specific complex is marked by an asterisk. The inserted picture shows an electrophoretic analysis of circular and linear forms of plasmid used as competitors. Arrowheads show open circular (**OC**), linear (**L**) and supercoiled (**SC**) forms. **Panel B**. Comparison of the amount of complexes specific for UV-irradiated or free end-containing DNA in nuclear extracts from lymphocytes irradiated with γ-radiation (+) or controls (–). *E. coli* DNA was used as a non-radioactive competitor. For other details see text.

quence) was used (lane 3 in Fig. 3A), we assume that it was probably specific for the oligonucleotide sequence.

Ionizing radiation is known to introduce double-stranded breaks into DNA. We wanted to test whether the level of putative "end-binding complexes" was affected in γ-irradiated lymphocytes. If protein(s) from such "end-binding complexes" take part in detection of DNA double-strand breaks one might expect that their level in extracts from γ-irradiated cells would be changed, and, in fact the level of putative "end-binding complexes" was slightly decreased in extracts from γ-irradiated cells (Fig. 2B, lanes 1 and 2). More tight binding of proteins from this complex to damage-containing chromatin could explain the lower yield of proteins extracted from this fraction. We found that the amount of UV-DDB complexes was increased in extracts from γ-irradiated cells (lanes 3 and 4 in Fig. 3B). Hwang *et al.* [23] have shown that the UV-DDB activity was enhanced and the amount of p48 protein was increased after γ-irradiation of cells, due to increased expression caused by the p53 protein. This would confirm the previous presumption that the UV-DDB complex detected in our experiments contains the p127/p48 protein complex. The effects of ionizing radiation upon the level of either UV-DDB or "end-binding complexes" were studied in cells from different donors. In all cases radiation resulted in a decreased level of putative „end-binding complexes" and an increase of UV-DDB complexes, yet the extent of these changes varied from one individual to another (not shown).

Several nuclear proteins that form complexes with damaged DNA were detected in human lymphocytes. Some of them (e.g. UV-DDB) are damage-recognition proteins involved in nucleotide excision repair, while others may probably compete with repair proteins making repair slower and less efficient. However, both types of proteins that bind to damaged DNA affect repair processes. Inter-individual variations in level of the detected DDB-proteins, as well as their identity and relations to repair capacity, as studied by the "repair DNA synthesis" tests, will be subject of further investigation.

REFERENCES

1. Wood, R.D. (1996) *Annu. Rev. Biochem.* **65,** 135–167.
2. Haber, J.E. (1999) *Cell* **97**, 829–832.
3. Pil, P.M. & Lippard, S.J. (1992) *Science* **256**, 234–237.
4. Yaneva, J., Leuba, S.H., van Holde, K. & Zlatanova, J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13448–13451.
5. Lindahl, T., Satoh, M.S., Poirier, G.G. & Klungland, A. (1995) *Trends Biochem. Sci.* **20**, 405–411.
6. Chu, G. (1994) *J. Biol. Chem.* **269**, 787–790.
7. Laat, W.L., Jaspers, N.G.J. & Hoeijmakers, J.H.J. (1999) *Genes Dev.* **13**, 768–785.
8. Yasui, A. & McCready, S.J. (1998) *BioEssays* **20**, 291–297.
9. Dianov, G., Bischoff, C., Sunesen, M. & Bohr, V.A. (1999) *Nucleic Acids Res.* **27**, 1365–1368.
10. Rotman, G. & Shiloh, Y. (1997) *BioEssays* **19**, 911–917.
11. Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates III, J.R., Hays, L., Morgan, W.F. & Petrini, J.H.J. (1998) *Cell* **93**, 477–486.
12. Price, E.A., Bourne, S.L., Radbourne, R., Lawton, P.A., Lamerdin, J., Thompson, L.H. & Arrand, J.E. (1997) *Somat. Cell Mol. Genet.* **23**, 237–247.
13. Shen, M.R., Jones, I.M. & Mohrenweiser, H. (1998) *Cancer Res.* **58**, 604–608.
14. Eker, A.P.M., Vermenten, W., Miura, N., Tanaka, K., Jaspers, N.G.J., Hoeijmakers,

J.H.J. & Bootsma, D. (1992) *Mutat. Res.* **274**, 211–224.

15. Jones, C.J., Cleaver, J.E. & Wood, R.D. (1992) *Nucleic Acids Res.* **20**, 991–995.

16. Ferry, A.E. & Pace, B.S. (1998) *Cell. Mol. Biol. Lett.* **3**, 375–378.

17. Rzeszowska-Wolny, J. & Widłak, P. (1999) *Acta Biochim. Polon.* **46**, 173–180.

18. Ushay, H.M., Tullius, T.D. & Lippard, S.J. (1981) *Biochemistry* **20**, 3744–3748.

19. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

20. Kriek, E. (1972) *Cancer Res.* **32**, 2042–2048.

21. Eastman, A. (1986) *Biochemistry* **25**, 3912–3915.

22. Mitchell, D.L. & Nair, R.S. (1989) *Photochem. Photobiol.* **49**, 805–819.

23. Hwang, B.J., Ford, J.M., Hanawalt, P.C. & Chu, G. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 424–428.

24. Featherstone, C. & Jackson, S.P. (1999) *Mutat. Res.* **434**, 3–15.