962–966 Nucleic Acids Research, 1995, Vol. 23, No. 6

Late induction of human DNA ligase I after UV-C irradiation

Alessandra Montecucco, Elena Savini, Giuseppe Biamonti, Miria Stefanini, Federico Focher and Giovanni Ciarrocchi*

Istituto di Genetica Biochimica ed Evoluzionistica, CNR, via Abbiategrasso 207, 27100 Pavia, Italy

Received December 5, 1994; Revised and Accepted February 3, 1995

ABSTRACT

We have studied the regulation of DNA ligase I gene expression in UV-C irradiated human primary fibroblasts. An increase of ~6-fold both in DNA ligase I messenger and activity levels was observed 24 h after UV treatment, when nucleotide excision repair (NER) is no longer operating. DNA ligase I induction is serum-independent and is controlled mainly by the steady-state level of its mRNA. The activation is a function of the UV dose and occurs at lower doses in cells showing UV hypersensitivity. No increase in replicative DNA polymerase α activity was found, indicating that UV induction of DNA ligase I occurs through a pathway that differs from the one causing activation of the replication machinery. These data suggest that DNA ligase I induction could be linked to the repair of DNA damage not removed by NER.

INTRODUCTION

Lesions to DNA arise constantly from the interaction of the genetic material with many different kinds of damaging agents, either present in the environment or deriving from endogenous processes (1,2). As a consequence, different kinds of lesions are produced that share the property of altering the DNA structure (3,4) and consequently interfere with aspects of DNA metabolism, such as transcription, replication and recombination. In mammalian cells, DNA damage elicits complex responses that include changes in growth rate (5,6) and induction of a variety of genes associated with growth control (checkpoint genes) (7), enhanced protection (7,8) and altered mutagenesis (9,10). Surprisingly, in contrast to bacteria and yeast, only a limited number of mammalian genes involved in DNA repair have been thus far shown to be induced by damaging agents (11,12).

Because of its relevance in human skin cancer (13), UV light is a model genotoxic agent widely used for studying both DNA repair mechanisms and stress responses in mammalian cells. UV-induced DNA damage removal in mammalian cells is a process that occurs over a period of several hours and shows several levels of complexity. Different kinds of lesion have been shown to be removed with different kinetics during DNA repair metabolism: for example, 6-4 photoproducts are removed more rapidly than cyclobutane pyrimidine dimers (CPDs) (14). In addition, repair of DNA lesions is generally heterogeneous with respect to different genomic domains. This is due in large part to the preferential repair of transcribed DNA strands (15). Further differences in the DNA repair rate depend on the location of the damage within the gene itself. An impressive correlation has been found between 'slow spots', where DNA repair lags, and hot spots for mutations in the *p53* gene (16).

The most general repair mechanism which responds to a variety of types of DNA lesion is nucleotide excision repair (NER) (4). All known excision repair processes require the rejoining by a DNA ligase activity of the patched gap left by repair DNA polymerases. Three distinct forms of DNA ligase have been reported so far in mammalian cells (17–20). While little is known about DNA ligase II and III, there are clear indications in favour of the involvement of DNA ligase I in both DNA replication and NER (21). However, in a recent report DNA ligase III has been found to co-purify with DNA repair protein Xrcc1 (22). We previously observed that the steady-state level of DNA ligase I mRNA increases 3-fold 24 h after UV treatment of human primary fibroblasts (23) and a similar increase in DNA ligase I activity has been reported by Mezzina and Sarasin (24). These data suggested that DNA ligase I could belong to a DNA repair system induced late after UV damage and probably devoted to the removal of DNA damage not removed by NER (25). In this paper we analyse in more detail the induction of DNA ligase I in response to UV treatment of human primary fibroblasts from both healthy and xeroderma pigmentosum (XP) donors.

MATERIALS AND METHODS

Probes

The 1257 bp partial cDNA of human DNA ligase I was prepared according to the procedure previously reported (23). The c-*fos* probe was extracted from plasmid pc-fos-1 (26). The human β-actin cDNA was extracted from plasmid pHFβA-1 (27).

Cells and culture conditions

Fibroblast strains from one healthy individual (C3PV), one patient belonging to group C of the NER-defective form of XP (XP9PV) (28) and one XP variant patient (XP14PV; unpublished observations) were used in this study. The cells were routinely

* To whom correspondence should be addressed

grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA) containing 10% foetal calf serum (FCS), 50 μg/ml gentamicin and 2 mM L-glutamine (complete medium). All cell strains were examined according to established procedures and found to be mycoplasma-free.

To obtain starved cells, confluent fibroblasts were grown for 5 days in DMEM supplemented with 0.25% FCS.

In irradiation experiments the cells were exposed to UV-C radiation (254 nm) using a Philips TUV 15 W lamp as previously described (23). The c-*fos* specific induction at early times in response to UV irradiation was taken as a measure of the efficiency of the treatment (23).

Unscheduled DNA synthesis and S-phase cells percentage in fibroblasts

Fibroblasts were plated in complete medium in 30 mm dishes containing a coverslip. Five days after reaching confluence, cells were UV irradiated with a dose of 20 J/m^2 and re-incubated in complete medium. At different times during post-UV cell incubation (0–40 h), cultures were labelled with [^{3}H]thymidine ([^{3}H]TdR, specific activity 2 Ci/mmol; Amersham, UK) at a final concentration of 1 μCi/ml in the medium and fixed 8 h later. Control cells were treated in the same way except for irradiation. Autoradiography was performed with Ilford emulsion; after 14 days at 4°C, the slides were developed and stained (28). S-phase nuclei were heavily labelled and easily distinguished from non-S-phase cells. The percentage of S-phase cells was evaluated by scoring at least 1000 cells/culture, while the UDS was measured by evaluating the mean number of grains on 25 non-S-phase cells.

Cell extracts

Fibroblast pellets (25–50 mg) collected at different times after UV treatment were resuspended in 5 vol of ice-cold 10 mM potassium phosphate buffer (pH 6.8) containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride. Cells were kept on ice for 10 min, then sonicated at 100 W three times for 5 s. Disrupted cells were centrifuged at 10 000 r.p.m. in an Eppendorf centrifuge for 15 min and supernatants were frozen in aliquots at –70°C.

Aliquots of fibroblast extracts were assayed for DNA ligase and DNA polymerase α (29) activities. In particular DNA ligase activity was measured in a poly(dA)–oligo(dT) assay (18,30).

Protein concentrations in fibroblast supernatants were determined by the fluorimetric method (31).

Analysis of DNA ligase I mRNA steady-state levels

Total RNA preparations (32), RNA gels and Northern blot hybridisations were performed as previously described (23). To compare relative transcript levels, samples were normalised to equal amounts of total RNA. The autoradiographic signals were quantitated by means of an imaging densitometer (BioRad, GS-670).

RESULTS AND DISCUSSION

The induction of DNA ligase I gene expression after UV irradiation is serum-independent

We previously reported that in confluent human primary fibroblasts grown in high (10%) serum, the level of DNA ligase I mRNA rises ~3-fold 24 h after a $UV_{254\ nm}$ irradiation dose of 20 J/m^2 (23). Since this irradiation dose has cytotoxic effects, it was conceivable to hypothesise that the late induction could be due to serum stimulation of the surviving no longer confluent cells.

We present here two experiments that rule out this possibility. First we determined on autoradiographic preparations the percentage of cells in S-phase and the level of UDS in confluent fibroblasts at different times after UV cell irradiation. As shown in Table 1, the number of replicating cells ranges between 1 and 2% in both untreated and treated fibroblasts, regardless of the time of post-UV cell incubation. Therefore, UV irradiation does not induce any substantial change in the percentage of S-phase cells in confluent fibroblast cultures. The small increase observed at the latest time after irradiation is not sufficient *per se* to explain the increase in DNA ligase I mRNA level. As expected, a high level of UDS is observed in the first 8 h after UV irradiation. At later times it progressively decreases and is no longer detectable 24 h after irradiation.

Table 1. Percentage of S-phase cells and level of UV-induced DNA repair synthesis (UDS) in confluent human fibroblasts irradiated with a UV dose of 20 J/m^2

Time after treatment (h)	Unirradiated cells S-phase (%)	UV irradiated cells S-phase (%)	UDS (grains/nucleus ± SEM)
8.0	1.37	0.80	43.3 ± 2.1
14.5	1.90	1.36	29.9 ± 1.4
24.0	1.20	1.00	13.7 ± 0.8
32.0	0.96	2.00	4.4 ± 0.3
36.0	1.00	1.87	3.2 ± 0.3
48.0	0.82	1.88	3.8 ± 0.3

Cells were labelled with [^{3}H]TdR for 8 h before processing.

In order to understand whether induction of DNA ligase I gene expression is nevertheless serum-dependent, we next verified its occurrence in cells starved for 5 days prior to UV irradiation. Therefore, confluent human primary fibroblasts were starved in low serum for 5 days, $UV_{254\ nm}$ irradiated with 20 J/m^2 and harvested at different time intervals to prepare both total RNA and protein extracts. The levels of DNA ligase I mRNA were determined by Northern blot analysis. Signals were quantitated by scanning the autoradiograph and normalised using 28 S rRNA levels revealed by ethidium bromide staining (see Materials and Methods). The β-actin mRNA level was also probed as an additional control. Under these conditions we observed that the DNA ligase I mRNA level increases ~6-fold (Fig. 1A and B). The kinetics of this induction parallels that previously obtained in the presence of high serum (23), thus ruling out a major role of serum in the response to UV irradiation. In this regard it is worth underlining that the extent of induction is higher than in the presence of serum, probably because the basal mRNA level achieved in starved cells is lower than in fed cells. Moreover, the increase in DNA ligase I mRNA level is accompanied by a proportional increase in enzymatic activity (Fig. 1C), measured in a poly(dA)–oligo(dT) assay (see Materials and Methods). In contrast, in the same experiment we found no increase in replicative DNA polymerase α activity (Fig. 1C). Thus UV induction of DNA ligase I gene expression seems to occur

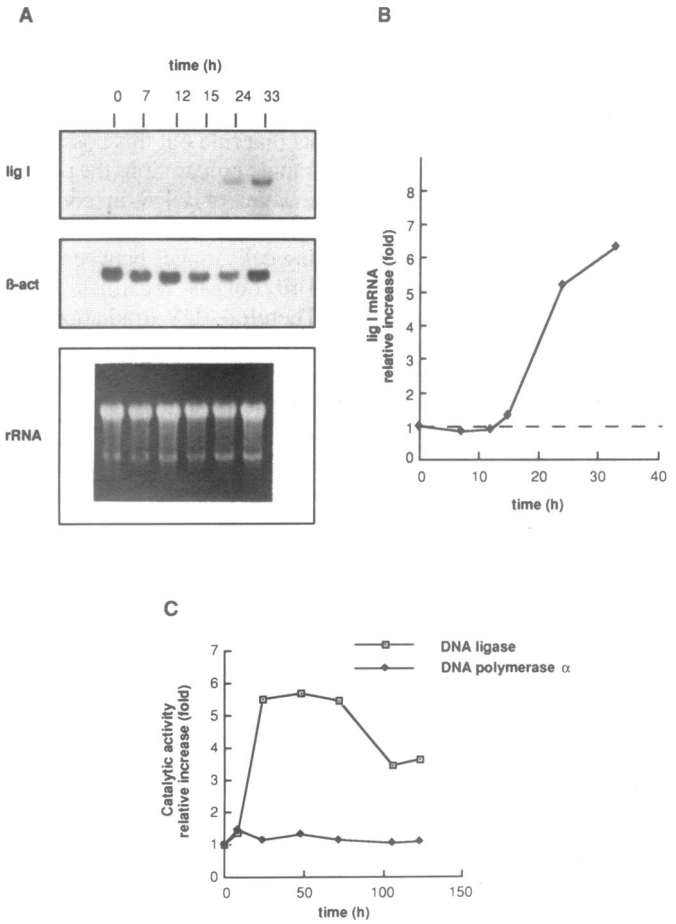

Figure 1. Effect of a 20 J/m^2 $UV_{254\ nm}$ dose irradiation on human primary fibroblasts. Confluent cells were starved for 5 days in low FCS medium and then UV irradiated with a dose of 20 J/m^2 as described in Materials and Methods. (**A**) Total RNA was extracted 0, 7, 12, 15, 24 and 33 h after treatment and probed in Northern blot hybridisations with the DNA ligase I-specific probe (lig I) and with a probe specific for human β-actin (β-act). rRNA: ethidium bromide staining of ribosomal RNA. (**B**) The autoradiograms were analysed as described in Materials and Methods. (**C**) Cell extracts were prepared 8, 24, 48, 72, 106 and 123 h after UV treatment (20 J/m^2) and assayed for DNA ligase and DNA polymerase α activity as described in Materials and Methods. Each point represents the average specific activity obtained in three independent experiments in which several concentrations of extract were assayed in the linear range of activity.

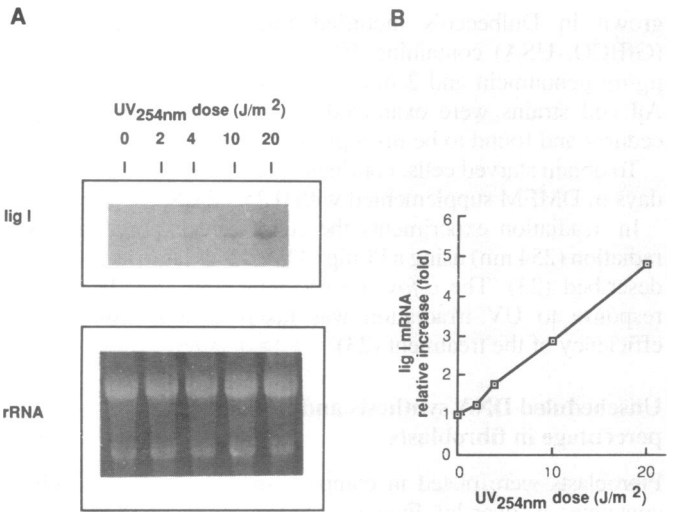

Figure 2. Effect of increasing $UV_{254\ nm}$ dose on DNA ligase I mRNA level. Confluent human fibroblasts were starved for 5 days in 0.25% FCS medium, then $UV_{254\ nm}$-irradiated at different UV doses (0, 2, 4, 10 and 20 J/m^2). Twenty four hours after treatment, total RNA was extracted. (**A**) Total RNA (10 µg) was probed in Northern blot hybridisations with the DNA ligase I-specific probe (lig I). rRNA: ethidium bromide staining of ribosomal RNA. (**B**) The autoradiograms were analysed as described in Materials and Methods.

through a pathway that differs from the one causing activation of the replication machinery.

The late induction of DNA ligase I gene expression is UV dose-dependent

We showed in the previous section that the DNA ligase I mRNA level in confluent resting fibroblasts increases 24 h after UV irradiation. Since under our experimental conditions ~99% of cells are in G_0 (Table 1), no interference between the response to UV stimulus and cell cycle should occur, making the results more easily interpretable. To further characterise this induction, we measured the steady-state level of DNA ligase I mRNA as a function of $UV_{254\ nm}$ dose. To this end, we treated serum-starved, confluent human primary fibroblasts with $UV_{254\ nm}$ doses ranging from 0 to 20 J/m^2. After 24 h, total RNAs were extracted and the level of DNA ligase I mRNA was determined by Northern blots. As shown in Figure 2, the DNA ligase I mRNA level increases in a dose–response manner and, when the signal is normalised to 28S rRNA, the curve shown in Figure 2B can be drawn. The mRNA level rises proportionally in the range 0–20 J/m^2. The dose–response correlation in DNA ligase I gene induction observed 24 h after UV irradiation suggests that this gene could be activated by DNA damage not removed by NER.

Induction of DNA ligase I gene expression in DNA repair-deficient cells

The data presented above suggest that DNA ligase I gene expression is activated by UV-induced DNA damage. To further support this hypothesis, we analysed gene induction in primary fibroblasts characterised by hypersensitivity to UV light as a consequence of defects in two different repair pathways, namely NER and post-replication repair. Cells from two patients affected by the classic or variant form of XP were analysed (33). In order to obtain comparable results between normal and XP cells we used a UV dose (2 J/m^2) with little cytotoxic activity on resting XP fibroblasts. The steady-state level of DNA ligase I mRNA was determined by Northern blot analysis of RNAs extracted at different times during post-UV cell incubation. Figures 3 and 4 show that in XP9PV (XP-C) and XP14PV (XP-V) cells, the increase in DNA ligase I mRNA at a UV dose of 2 J/m^2 is comparable with that observed on irradiating normal cells with a UV dose of 20 J/m^2. Therefore, the induction of DNA ligase I in cells with increased UV sensitivity occurs at lower doses than in normal cells. Interestingly, XP-V cells show a peculiarity in the kinetics of DNA ligase I induction: the increase in DNA ligase I mRNA is transient and drops 48 h after UV irradiation (Fig. 4). The same pattern was observed in cells from another XP-V

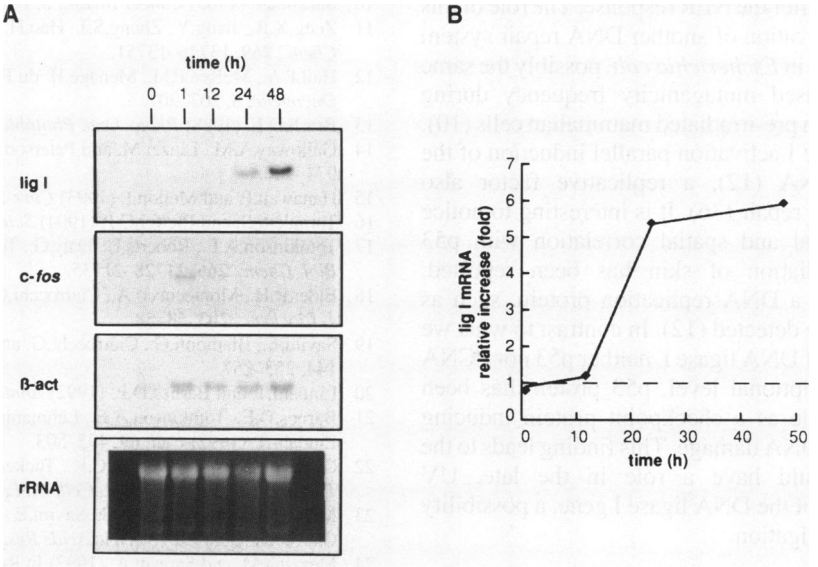

Figure 3. Effect of a 2 J/m^2 $UV_{254\ nm}$ dose on DNA ligase I mRNA level in XP-C fibroblasts. Confluent cells (XP9PV) were starved for 5 days in 0.25% FCS medium, then $UV_{254\ nm}$-irradiated with a 2 J/m^2 UV dose. RNA was extracted 0, 1, 12, 24 and 48 h after treatment. (**A**) Total RNA (10 µg) was probed in Northern blot hybridisations with the DNA ligase I-specific probe (lig I), with a probe specific for the c-*fos* proto-oncogene (c-*fos*) and with a probe specific for human β-actin (β-act). rRNA: ethidium bromide staining of ribosomal RNA. (**B**) The autoradiograms were analysed as described in Materials and Methods.

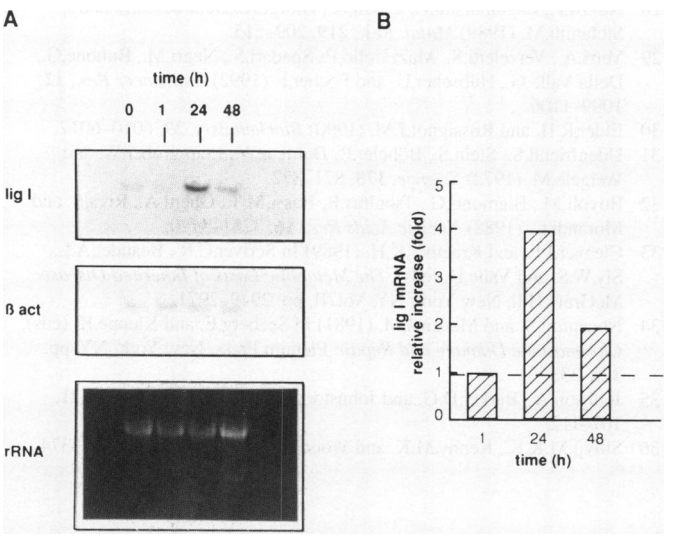

Figure 4. Effect of a 2 J/m^2 $UV_{254\ nm}$ dose on DNA ligase I mRNA level in XP variant fibroblasts. Confluent cells (XP14PV) were starved for 5 days in 0.25% FCS medium, then $UV_{254\ nm}$-irradiated with a 2 J/m^2 UV dose. RNA was extracted 0, 1, 24 and 48 h after treatment. (**A**) Total RNA (10 µg) was probed in Northern blot hybridisations with the DNA ligase I-specific probe (lig I) and with a probe specific for the human β-actin (β-act). rRNA: ethidium bromide staining of ribosomal RNA. (**B**) The autoradiograms were analysed as described in Materials and Methods.

patient (data not shown), making it likely that this is a typical feature of this form of XP.

CONCLUSIONS

In this paper we analysed in detail the late induction of DNA ligase I gene expression in response to UV irradiation.

Our results further support the concept that the enzyme plays a role in DNA repair, as previously suggested on the basis of several observations: (i) DNA ligase I, as well as its mRNA, is detectable in resting cells (neurones, peripheral lymphocytes, confluent primary fibroblasts, differentiated HL-60 cells) (23); (ii) both DNA ligase I activity and gene expression are induced, even if late, after UV irradiation of confluent primary fibroblasts (23,24,34); (iii) mutations in the DNA ligase I gene produce hypersensitivity to DNA damaging agents (21).

Here we show that UV induction of DNA ligase I is serum-independent and is mainly due to an increase in the DNA ligase I mRNA level. In fact, after UV irradiation of starved confluent primary fibroblasts, both DNA ligase I activity and mRNA levels increase ~6-fold. In this respect the DNA ligase I gene differs from other genes encoding replicative enzymes, such as DNA polymerase α. A specific transcriptional induction of the DNA ligase I gene has also been observed after treatment of stationary phase cultures of either budding or fission yeasts with UV irradiation. However, in contrast to what we have observed in human cells, in yeast the up-regulation of mRNA steady-state level is not matched by a comparable increase in catalytic activity (35).

Under our experimental conditions DNA ligase I gene expression increases when UDS is no longer detectable. Thus, if the enzyme plays a role in NER, it is clear that the basal level detectable in starved confluent fibroblasts is sufficient to accomplish it. In contrast, our data support the hypothesis that late induction of the gene is correlated with a DNA repair system activated late after UV irradiation and probably as a consequence of the presence of unrepaired DNA damage. This conclusion is based on three observations: (i) induction occurs when repair synthesis is no longer occurring and is serum-independent; (ii) the extent of induction directly depends on the UV dose; (iii) induction occurs at lower doses in XP cells.

On the basis of the presented data we would like to speculate that DNA ligase I gene expression is under the control of a

'checkpoint' that operates after the NER response. The role of this checkpoint is probably activation of another DNA repair system similar to the SOS response in *Escherichia coli*, possibly the same as that causing an increased mutagenicity frequency during reactivation of viral DNA in pre-irradiated mammalian cells (10). The kinetics of DNA ligase I activation parallel induction of the immunoreactivity of PCNA (12), a replicative factor also required for DNA excision repair (36). It is interesting to notice that for PCNA a temporal and spatial correlation with p53 induction after UV irradiation of skin has been reported. However, no induction of a DNA replication protein, such as DNA polymerase α, can be detected (12). In contrast to what we have observed in the case of DNA ligase I, neither p53 nor PCNA are induced at the transcriptional level. p53 protein has been suggested as playing a role as a checkpoint protein inducing growth arrest in cells with DNA damage. This finding leads to the hypothesise that p53 could have a role in the late, UV dose-dependent induction of the DNA ligase I gene, a possibility that deserves further investigation.

ACKNOWLEDGEMENTS

The authors are grateful to M. T. Chiesa for her technical assistance. This work was supported by a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC) and by a grant from the PF 'Ingegneria Genetica' of the CNR. ES was supported by a fellowship from the PF 'Ingegneria Genetica' of the CNR.

REFERENCES

1 Ames,B.N. (1989) *Environ. Mol. Mutagen.*, **16** (suppl.), 66–77.
2 Cerutti,P.A. (1991) *Cancer Cells*, **3**, 1–7.
3 Ciarrocchi,G., Montecucco,A., Pedrali-Noy,G. and Spadari,S. (1988) In Douglas,R.H., Moan,J. and Dell'Acqua,F. (eds), *Light in Biology and Medicine*. Plenum Publishing, New York, NY, Vol. 1, pp. 219–226
4 Friedberg,E.C. (1985) *DNA Repair*. Freeman, New York, NY.
5 Rowley,R., Hudson,J. and Young,P.G. (1992) *Nature*, **356**, 353–355.
6 Kuerbitz,S.J., Plunkett,B.S., Walsh,W.V. and Kostan,M.B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7491–7495.
7 Fornace,A.J. (1992) *Annu. Rev. Genet.*, **26**, 507–526.
8 Zhan,Q., Carrier,F. and Fornace,A.J., (1993) *Mol. Cell. Biol.*, **13**, 4242–4250.
9 Walker,G.C. (1984) *Microbiol. Rev.*, **48**, 60–93.
10 Sarasin,A. (1985) *Cancer Invest.*, **3**, 163–174.
11 Zeng,X.R., Jiang,Y., Zhang,S.J., Hao,H. and Lee,M.Y.W.T. (1994) *J. Biol. Chem.*, **269**, 13748–13751.
12 Hall,P.A., McKee,P.M., Menage,H. du P., Dover,R. and Lane,D.P. (1993) *Oncogene*, **8**, 203–207.
13 Brash,D.E. (1988) *Photochem. Photobiol.*, **48**, 59–66.
14 Galloway,A.M., Liuzzi,M. and Peterson,M.C. (1994) *J. Biol. Chem.*, **269**, 974–980.
15 Hanawalt,P. and Mellon,I. (1993) *Curr. Biol.*, **3**, 67–69.
16 Tornaletti,S. and Pfeifer,G.P. (1994) *Science*, **263**,1436–1438.
17 Tomkinson,A.E., Roberts,E., Daly,G., Totty,N.F. and Lindahl,T. (1991) *J. Biol. Chem.*, **266**, 21728–21735.
18 Elder,R.H., Montecucco,A., Ciarrocchi,G. and Rossignol,J.-M. (1992) *Eur. J. Biochem.*, **203**, 53–58.
19 Savini,E., Biamonti,G., Ciarrocchi,G. and Montecucco,A. (1994) *Gene*, **144**, 253–257.
20 Lindahl,T. and Barnes,D.E. (1992) *Annu. Rev. Biochem.*, **61**, 251–281.
21 Barnes,D.E., Tomkinson,A.E., Lehmann,A.R., Webster,A.D.B. and Lindahl,T. (1992) *Cell*, **69**, 495–503.
22 Caldecott,K.W., McKeown,C.K., Tucker,J.D., Ljungquist,S. and Thompson,L.H. (1994) *Mol. Cell. Biol.*, **14**, 68–76.
23 Montecucco,A., Biamonti,G., Savini,E., Focher,F., Spadari,S. and Ciarrocchi,G. (1992) *Nucleic Acids Res.*, **20**, 6209–6214.
24 Mezzina,M. and Sarasin,A. (1992) In Rossman,T. (ed.), *Induced Effects of Genotoxic Agents in Eukaryotic Cell*. Plenum Press, New York, NY, pp. 51–63.
25 Mellon,I., Spivak,G. and Hanawalt,P.C. (1987) *Cell*, **51**, 241–249.
26 Currant,T., MacConnel,W.P., Van-Straaken,F. and Verma,I.M. (1983) *Mol. Cell. Biol.*, **3**, 914–921.
27 Gunning,P., Ponte,P., Okayama,H., Engel,J., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 787–795.
28 Nuzzo,F., Lagomarsini,P., Casati,A., Giorgi,R., Berardesca,E. and Stefanini,M. (1989) *Mutat. Res.*, **219**, 209–215.
29 Verri,A., Verzeletti,S., Mazzarello,P., Spadari,S., Negri,M., Bunone,G., Della Valle,G., Hübscher,U. and Focher,F. (1992) *Anticancer Res.*, **12**, 1099–1106.
30 Elder,R.H. and Rossignol,J.M. (1990) *Biochemistry*, **29**, 6010–6017.
31 Udenfriend,S., Stein,S., Böheler,P., Daiman,W., Leimgruber,W. and Weigele,M. (1972) *Science*, **178**, 871–872.
32 Buvoli,M., Biamonti,G., Tsoulfas,P., Bassi,M.T., Ghetti,A., Riva,S. and Morandi,C. (1988) *Nucleic Acids Res.*, **16**, 3751–3770.
33 Cleaver,J.E. and Kraemer,K.H. (1989) In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds), *The Metabolic Basis of Inherited Disease*. McGraw Hill, New York, NY, Vol. II, pp. 2949–2971.
34 Nocentini,S. and Mezzina,M. (1981) In Seeberg,E. and Kleppe,K. (eds), *Chromosome Damage and Repair*. Plenum Press, New York, NY, pp. 329–333
35 Johnson,A., Barker,D.G. and Johnston,L.H. (1986) *Curr. Genet.*, **11**, 107–112.
36 Shivji,M.K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.