Carcinogenesis vol.7 no.3 pp.389–392, 1986

A112

Postreplication repair in ultraviolet-irradiated human fibroblasts: formation and repair of DNA double-strand breaks

Tzu-chien V.Wang and Kendric C.Smith

Department of Radiology, Stanford University School of Medicine, Stanford, CA 94305, USA

A neutral filter elution assay was used to determine if the post-replicational formation and repair of DNA double-strand breaks (DSB) occurs in u.v.-irradiated human cells. Excision-deficient XP12 cells were pulse-labeled with [^{3}H]thymidine after u.v. irradiation (1.5–3 J/m^2), and the nascent DNA was followed during repair incubation. With increasing u.v. radiation fluences, an increasing fraction of DNA was eluted at a fast rate, indicating that DSB were produced. The maximum yield of DSB was observed after about 24 h of post-irradiation incubation at 37°C. Similar results were also obtained with repair-proficient VA13 cells when irradiated at much higher fluences (7.5–15 J/m^2). It is concluded that, at the u.v. radiation fluences used in this work, the DSB produced in u.v.-irradiated human cells are the result of post-replication repair events, and at incubation times >24 h some of these DSB are repaired.

Introduction

Our current understanding of the major 'dark' repair systems for the processing and repair of u.v. radiation-damaged DNA in *Escherichia coli* can be summarized as follows. The pyrimidine dimers produced in DNA by u.v. irradiation can be removed from the DNA by an efficient *uvrABC*-dependent excision repair process (1,2). If these dimers (primary lesions) are not excised from the DNA and replication proceeds past them, DNA daughter-strand gaps (secondary lesions) will be formed and a dimer will reside in the single-stranded DNA that is opposite a gap in the newly synthesized daughter-strand DNA. *E. coli* cells possess efficient recombination systems for the repair of these DNA daughter-strand gaps (3). Finally, a daughter-strand gap can be converted to a tertiary lesion, a DNA double-strand break (DSB)*. Evidence for the postreplicational formation and repair of DSB has been obtained (4,5).

Analogous to bacteria, human cells in culture possess excision-repair systems for the removal of pyrimidine dimers, and they also exhibit a response resembling daughter-strand gap repair (reviewed in 6). It is not known, however, whether the post-replicational formation and repair of DSB that occurs in u.v.-irradiated *E. coli* (4,5) also occurs in u.v.-irradiated human cells. In this work, we employed a neutral filter elution assay (7) to test this possibility. Our results indicate that the postreplicational formation and repair of DNA DSB also occurs in human cells.

*Abbreviations: DSB, double-stranded breaks; XPA, xeroderma pigmentosum group A; MEM, minimal essential medium; PBS, phosphate-buffered saline; CMFBSSE, calcium- and magnesium-free Hank's balanced saline solution containing EDTA at 0.2%; TCA, trichloroacetic acid.

Materials and methods

Cell strains and cultures

Simian virus 40 (SV40)-transformed human cells VA13 and XP12 were kindly provided by Dr A.K.Ganesan. The VA13 cells were derived from normal human fibroblasts WI38 and are proficient in the excision repair of u.v. radiation-induced pyrimidine dimers. XP12 cells were derived from skin fibroblasts (XP12RO) of a xeroderma pigmentosum group A (XPA) patient, and are deficient in excision repair (8,9). Cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal calf serum. Cultures were incubated at 37°C in 5% CO_2 and 100% humidity.

U.v. irradiation

The source (254 nm) and measurement of the fluence rate for u.v. irradiation have been described (10). Cells were seeded at 0.6–2.5 × 10^5 cells per 60 mm Petri dish in 5 ml of medium containing, when needed, [^{14}C]thymidine at 0.02 μCi/ml (56 Ci/mol, ICN Radiochemicals). After 2–5 days of growth, the medium was removed and the cells were irradiated in 2 ml of phosphate-buffered saline (PBS). The PBS was replaced with 3 ml of prewarmed growth medium, and incubation was continued for 30 min at 37°C before 30 μCi of [^{3}H]thymidine (64 Ci/mmol, New England Nuclear Corp.) was added. After incubation for another 30 min at 37°C, the radioactive medium was removed, the cells were washed three times with PBS and were then incubated in fresh medium for the desired time. The irradiation times were staggered so that the appropriate repair interval was completed at the same time for simultaneous cell harvest.

Neutral filter elution

The neutral filter elution method described by Bradley and Kohn (7) was followed. At the end of repair incubation, the cells were rinsed twice with 5 ml of calcium- and magnesium-free Hank's balanced saline solution containing 0.2% EDTA (CMFBSSE). One ml of ice-cold trypsin (0.05% in CMFBSSE) was added to cells (on ice) and removed after 45 s. The cells were held on ice for another 1.5 min before 5 ml of ice-cold PBS were added. The cells were dispersed by pipetting, and the number of cells was counted in a haemocytometer. The cells in cold PBS (<6 × 10^5 cells) were collected on a Unipore 25 mm, 2.0 μm-pore-size polycarbonate filter (Nucleopore) by gravity-filtration, washed with 15 ml of cold PBS, and lysed in the dark with 3 ml of a room-temperature solution of 0.05 M Tris, 0.05 M glycine, 0.025 M Na_2 EDTA, 2% (w/v) sodium lauryl sulfate, adjusted to pH 9.6 with NaOH, and containing freshly dissolved proteinase K at 0.5 mg/ml. After the samples had been lysed (30–45 min at room temperature), the elution pump was run at 1.9 ml/h. After 1 h, 30 ml of elution buffer (same as the above solution but without proteinase K) was gently added on top of the remaining lysis solution. Fractions (2.85 ml) were collected every 90 min for 15 h. At the end of the experiment, the DNA in the pump line and on the filter was recovered and treated as described (7). For samples that were incubated in medium for <12 h after pulse-labeling with [^{3}H]thymidine, the radioactivity of the eluted DNA was determined as follows: the eluate in each fraction (2.85 ml) was mixed with 0.15 ml of calf thymus DNA (1 mg/ml) and 0.5 ml of cold tricholoroacetic acid (TCA) (50% w/v). After sitting on ice for 45 min, the acid-precipitates were collected on Millipore HA membrane filters (0.45 μm pore size), washed with 10 ml of 0.1% cold TCA, and the filter was placed in a vial. Elution buffer (2.8 ml) was added to each vial, shaken to dissolve the precipitate, and the radioactivity was determined by liquid scintillation counting in 15 ml of Aquasol (New England Nuclear Corp.) or Instagel (Packard Instrument Co., Inc.) containing 0.5% acetic acid. For samples that were incubated in medium for >12 h the acid-soluble radioactive material in the eluate was negligible (data not shown), and the radioactivity of the eluted DNA was determined by directly counting the eluate in 15 ml of the counting solutions described above.

Elution profiles for DNA from bacteriophage T2 (mol. wt 1.1 × 10^8) and *E. coli* (intact genome mol. wt 2.8 × 10^9) were obtained to provide information about the elution properties of DNA with different molecular weights. The [^{14}C]-thymine-labeled T2 DNA was prepared as described (11). The *E. coli* cells were labeled with [^{3}H]thymidine at 37°C for 1 h, and were converted to spheroplasts by treatment with lysozyme (4). The T2 DNA was eluted from the filter without the lysis treatment described above. The *E. coli* spheroplasts were loaded on the filter, washed and lysed in the same manner as described above for human cells, and the DNA was eluted as described above.

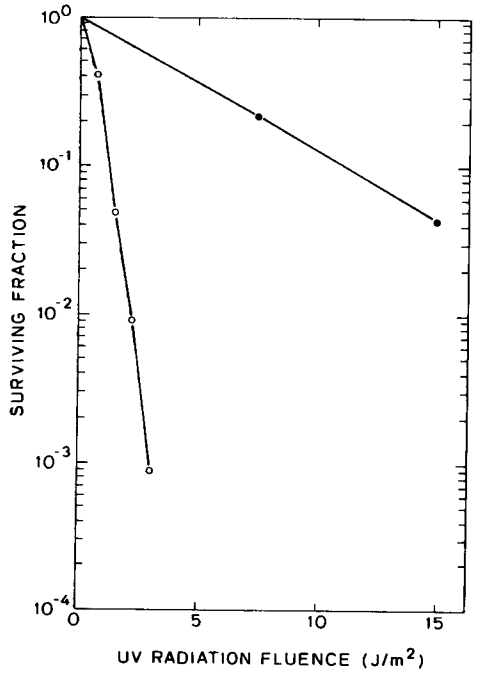

Fig. 1. Colony-forming ability of u.v.-irradiated human fibroblasts. Symbols: ●, VA13; ○, XP12. Data are the average of two experiments.

Colony survival assay

Cells were trypsinized as described above, resuspended in CMFBSSE, and the number of cells counted in a haemocytometer. The single-cell suspension ($1-2 \times 10^5$ cells/ml) was u.v.-irradiated, diluted in medium, and the appropriate numbers of cells were seeded into 60 mm Petri dishes in 5 ml of medium. After 12 days of incubation, the dishes were fixed and stained with 0.25% crystal violet (in 100 ml formaldehyde and 900 ml methanol). Colonies were defined as containing >32 cells. The cloning efficiencies for unirradiated VA13 and XP12 cells were 50% and 17%, respectively.

Results

The u.v. radiation sensitivities of the VA13 and XP12 cells (Figure 1) used in this work are comparable with those obtained for other normal and XPA cells (12).

To determine whether the postreplicational formation and repair of DNA DSB occurs in human cells, XP12 cells were pulse-labeled with [^{3}H]thymidine after u.v. irradiation, and the fate of nascent DNA was followed by neutral filter elution. When the irradiated cells were lysed immediately after pulse-labeling, the nascent DNA eluted slowly, as did the DNA from unirradiated cells (not shown). After a 24-h incubation, however, the [^{3}H]DNA from irradiated cells eluted with biphasic kinetics; a fraction of the DNA eluted at a very fast rate, and the remaining DNA eluted at a much slower rate (Figure 2A). With increasing radiation fluences, not only was there an increasing fraction of [^{3}H]DNA that eluted at a fast rate, but there was also an increase in the elution rate of the remaining DNA, indicating that DNA DSB were produced. A similar result was also obtained for VA13 cells irradiated at much higher fluences (Figure 2B).

We have obtained elution profiles of DNA from bacteriophage T2 and *E. coli*. The elution rates of these DNAs (Figure 2A) was faster than that for the DNA from irradiated XP12 cells, indicating that the DNA from irradiated (3 J/m^2) XP12 cells was larger than *E. coli* DNA.

To further characterize the nature of the DSB produced and to determine whether these DSB can be repaired, [^{14}C]thymidine-prelabeled XP12 cells were u.v.-irradiated, pulse-labeled with [^{3}H]thymidine, and the repair of the DNA was followed over

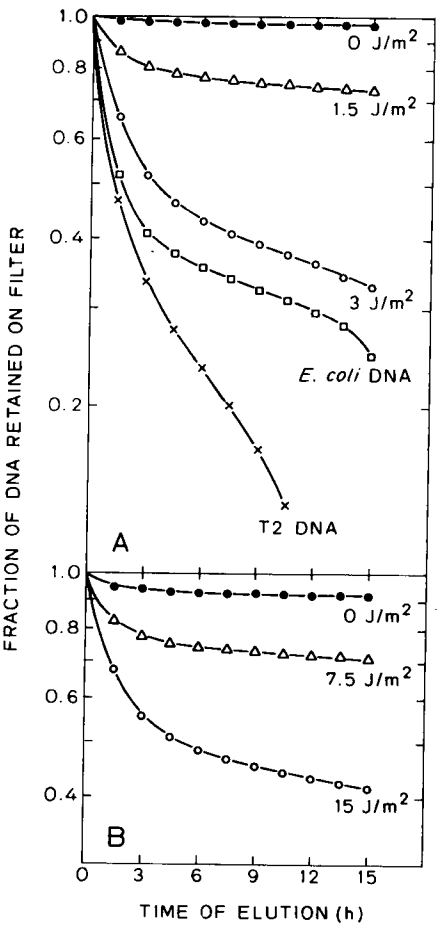

Fig. 2. Formation of DSB in the DNA synthesized after u.v. irradiation. Human fibroblasts XP12 (A) and VA13 (B) were cultured, u.v.-irradiated, and pulse-labeled with [^{3}H]thymidine. After 24 h of repair incubation at 37°C, the cells were lysed on a filter and the DNA was eluted as described in Materials and methods. The elution curves shown for *E. coli* DNA and bacteriophage T2 DNA were obtained by loading spheroplasts of [^{3}H]thymidine-labeled *E. coli* cells or purified [^{14}C]thymine-labeled T2 DNA on a filter, and the DNA was then eluted as described in Materials and methods.

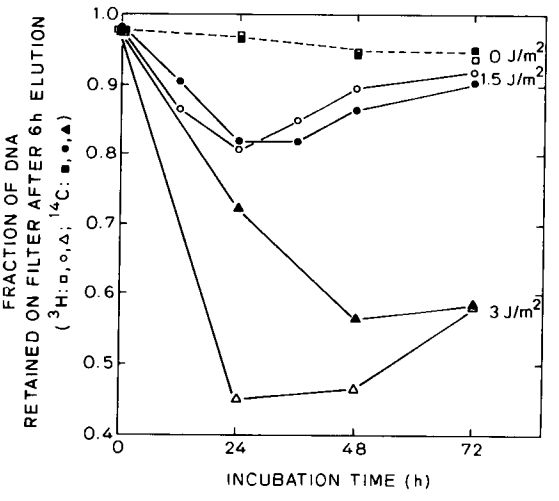

Fig. 3. Kinetics of the formation and repair of DNA DSB in u.v.-irradiated human XP12 cells. [^{14}C]thymidine-prelabeled XP12 cells were u.v.-irradiated, pulse-labeled with [^{3}H]thymidine and incubated in growth medium for different lengths of time. The cells were then lysed and the DNA eluted as described in Materials and methods. The fraction of the DNA that was retained on the filter after 6 h of elution was plotted as a function of the time of repair incubation.

a period of 72 h. The data are plotted as the percent of DNA retained on the filter after 6 h of elution as a function of incubation time after pulse labeling (Figure 3). At 1.5 J/m^2, both the [^{3}H]DNA and [^{14}C]DNA reached their fastest elution rates after about 24 h of repair incubation, and then slowly returned towards slower elution rates after longer incubation times, suggesting that some DSB were repaired. At a higher u.v. radiation fluence (3 J/m^2), the formation of DSB was much faster in the [^{3}H]DNA than in the [^{14}C]DNA, and the data suggest that few DSB were repaired at the later times. However, cells irradiated with this fluence tend to detach from the dish after long incubation times ($\geq$48 h), which might contribute an artifact to the analysis.

Discussion

Models for DNA repair processes in mammalian cells have evolved from our understanding of the repair systems discovered in prokaryotes. The recent finding that one of the two major pathways of postreplication repair in *E. coli* is responsible for the postreplicational formation and repair of DNA DSB (4) has prompted us to investigate whether such a repair process also occurs in u.v.-irradiated human cells.

The neutral filter elution assay developed by Bradley and Kohn (7) can detect small numbers of DNA DSB in mammalian DNA. Using this assay, we have demonstrated that DNA DSB were produced in XPA cells during repair incubation after low fluences of u.v. radiation (1.5–3 J/m^2) (Figure 2). Since the XPA cells are deficient in the excision of pyrimidine dimers from their DNA (8,9), the DSB produced in u.v.-irradiated XPA cells probably arise from events that occur after the replication of dimer-containing DNA. Consistent with this notion is the fact that the population of XPA cells that incorporated [^{3}H]thymidine after u.v. irradiation (i.e. those in S phase) exhibited a faster rate of DSB formation than did the total cell population (Figure 3).

We have shown that some of the DSB produced in u.v.-irradiated XPA cells can be repaired after long incubation times (Figure 3). The repair of DSB is rather slow and is u.v. radiation fluence-dependent: at u.v. radiation fluences of $\geq$3 J/m^2 very little repair of DSB can be detected in XPA cells (Figure 3, and unpublished data). This is reminiscent of the results for u.v.-irradiated excision-deficient cells of *E. coli*, which also exhibits the repair of DSB over a limited range of u.v. radiation fluences (unpublished data). Why these cells should exhibit such a u.v.radiation fluence-dependent repair of DSB is not known.

Bradley and Taylor (13) reported that DSB were produced in normal human fibroblasts, but not in XPA cells following 100–300 J/m^2 of u.v. radiation. These workers suggested that, at the high u.v. irradiation fluence used in their experiments, the formation of DSB arose from excision–repair events, possibly by the overlapping excision of two pyrimidine dimers that occur in close proximity but on the opposite strands of a DNA duplex, as was originally suggested for u.v.-irradiated *E. coli* cells (14). The failure by Bradley and Taylor (13) to detect DSB in u.v.-irradiated XPA cells can be attributed to the enormously high u.v. radiation fluence (100 J/m^2) given to their cells, which should inhibit DNA replication. In the absence of DNA replication, the postreplicational formation of DSB should not occur. This prediction has been shown to be true for excision-deficient *E. coli* cells (5).

One model for the formation of DSB in u.v.-irradiated excision-deficient *E. coli* cells is that a break occurs in the parental DNA strand opposite an unrepaired DNA daughter-strand gap, possibly through the attack of endonucleases that are specific for the single-stranded DNA (4,15). Such a model can also account for the DSB formed in u.v.-irradiated XPA cells. In fact, when u.v.-irradiated normal human cells were incubated in the presence of inhibitors of repair synthesis (e.g. aphidocolin, or hydroxyurea plus 1-β-D-arabinofuranosylcytosine) so that the excision gaps were held open for a longer period of time, the formation of DSB was detected at u.v. radiation fluences as low as 2.5 J/m^2 (16). These workers postulated that 'hot spots' of high lesion frequency occur, and that overlapping excision in these areas produces DSB. An alternative explanation of their results, which we prefer, is that the DSB occurs when a break is introduced in the single-stranded DNA opposite the excision gap, which is similar to the mechanism postulated above for the postreplicational formation of DSB at unrepaired DNA daughter-strand gaps in excision-deficient cells.

Our estimate of the number of DSB produced in u.v.-irradiated human cells suggests that this number is low compared with the number of pyrimidine dimers produced. The DNA of u.v.-irradiated (3 J/m^2) XPA cells incubated for 24 h eluted at a slower rate than that of *E. coli* DNA (Figure 2), suggesting that the size of these DNA fragments is $>2.8 \times 10^9$ daltons (assuming that the *E. coli* DNA is intact). The u.v. irradiation should have produced $\sim$2.3 dimers/10^8 dalton/J/m^2 (17,18). This suggests that, at most, there was one DSB formed during repair incubation for every 193 dimers produced in the DNA of XPA cells. This ratio is expected to be even smaller in excision-proficient VA13 cells, since it takes about a 5-fold higher fluence to observe the formation of the same level of DSB (Figure 2).

Little is known about the mechanism by which DNA DSB are repaired in human cells. If the repair involves recombination systems, as it does in *E. coli* (4,5,19) and in yeast (20), then reciprocal double-strand exchanges can occur during the repair process. This can result in the covalent joining of dimer-containing parental strands to daughter-strands synthesized after u.v. irradiation. Fornace (21) observed that about 1–3% of the pyrimidine dimers produced in the DNA of u.v.-irradiated human cells were found to be associated with newly synthesized DNA daughter-strands after 34–45 h of incubation after u.v. irradiation. It is not inconceivable that this limited transfer of dimer-containing parental strands into daughter-strands resulted from the postreplicational formation and repair of DNA DSB.

It is generally accepted that unrepaired DSB produce lethality and chromosome aberrations (22,23). Much less is known about the biological consequence of a repaired DSB. If such a repair event does not restitute the original DNA structure, it will lead to a mutation and/or transformation. Even if it restitutes an intact DNA structure, it may produce a phenomenon such as a sister-chromatid exchange. Whether there is any correlation between these events and the repair of DSB described here remains to be investigated.

Acknowledgements

We are grateful to Carmencita E.Nicolas for skillful technical assistance, to Drs N.J.Sargentini and R.C.Sharma for valuable discussions, and to Dr A.K.Ganesan for providing the human cell strains. This work was supported by Public Health Service research grant CA-02896 awarded by the National Cancer Institute, Department of Health and Human Services.

References

1. Boyce,R.P. and Howard-Flanders,P. (1964) Release of ultraviolet-light induced thymine dimers from DNA in *E. coli* K-12. *Proc. Natl. Acad. Sci. USA*, **51**, 293–300.
2. Setlow,R. and Carrier,W. (1964) The disappearance of thymine dimers from DNA: an error-correcting mechanism. *Proc. Natl. Acad. Sci. USA*, **51**, 226–231.

3. Rupp,W.D. and Howard-Flanders,P. (1968) Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. *J. Mol. Biol.*, **31**, 291–304.
4. Wang,T.V. and Smith,K.C. (1983) Mechanisms for *recF*-dependent and *recB*-dependent pathways of postreplication repair in UV-irradiated *Escherichia coli uvrB*. *J. Bacteriol.*, **156**, 1093–1098.
5. Wang,T.V. and Smith,K.C. (1986) Postreplicational formation and repair of DNA double-strand breaks in UV-irradiated *Escherichia coli* cells. *Mutat. Res.*, **165**, 39–44.
6. Hanawalt,P.C., Cooper,P.K., Ganesan,A.K. and Smith,C.A. (1979) DNA repair in bacteria and mammalian cells. *Annu. Rev. Biochem.*, **48**, 783–836.
7. Bradley,M.O. and Kohn,K.W. (1979) X-ray induced DNA double-strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic Acids Res.*, **7**, 793–804.
8. Cleaver,J.E. and Trosko,J.E. (1970) Absence of excision of ultraviolet-induced cyclobutane dimers in xeroderma pigmentosum. *Photochem. Photobiol.*, **11**, 547–550.
9. Setlow,R.B., Regan,J.D., German,J. and Carrier,W.L. (1969) Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA. *Proc. Natl. Acad. Sci. USA*, **64**, 1035–1041.
10. Wang,T.V. and Smith,K.C. (1981) Effect of *recB21, uvrD3, lexA101* and *recF143* mutations on ultraviolet radiation sensitivity and genetic recombination in Δ*uvrB* strains of *Escherichia coli* K-12. *Mol. Gen. Genet.*, **183**, 37–44.
11. Sargentini,N.J. and Smith,K.C. (1986) Characterization and quantitation of DNA strand breaks requiring *recA*-dependent repair in X-irradiated *Escherichia coli*. *Radiat. Res.*, in press.
12. Andrews,A.D., Barrett,S.F. and Robbins,J.H. (1978) Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet radiation. *Proc. Natl. Acad. Sci. USA*, **75**, 1984–1988.
13. Bradley,M.O. and Taylor,V.I. (1981) DNA double-strand breaks induced in normal human cells during the repair of ultraviolet light damage. *Proc. Natl. Acad. Sci. USA*, **78**, 3619–3623.
14. Bonura,T. and Smith,K.C. (1975) Enzymatic production of deoxyribonucleic acid double-strand breaks after ultraviolet irradiation of *Escherichia coli* K-12. *J. Bacteriol.*, **121**, 511-517.
15. Wang,T.V. and Smith,K.C. (1982) Effects of the *ssb-1* and *ssb-113* mutations on survival and DNA repair in UV-irradiated Δ*uvrB* strains of *Escherichia coli* K-12. *J. Bacteriol.*, **151**, 186–192.
16. Bradley,M.O. and Taylor,V.I. (1983) Repair-induced DNA double-strand breaks after ultraviolet-light and either aphidocolin or 1-β-D-arabinofuranosylcytosine/hydroxyurea. *Carcinogenesis*, **4**, 1513–1517.
17. Waters,R. and Regan,J.D. (1976) Recombination of UV-induced pyrimidine dimers in human fibroblasts. *Biochem. Biophys. Res. Commun.*, **72**, 803–807.
18. Lehmann,A.R. and Kirk-Bell,S. (1978) Pyrimidine dimer sites associated with the daughter DNA strands in UV-irradiated human fibroblasts. *Photochem. Photobiol.*, **27**, 297–307.
19. Krasin,F. and Hutchinson,F. (1977) Repair of DNA double-strand breaks in *Escherichia coli*, which requires *recA* function and the presence of a duplicate genome. *J. Mol. Biol.*, **116**, 81–98.
20. Resnick,M.A. and Martin,P. (1976) The repair of double-strand breaks in the nuclear DNA of *Saccharomyces cerevisiae* and its genetic control. *Mol. Gen. Genet.*, **143**, 119–129.
21. Fornace,A.T. (1983) Recombination of parent and daughter strand DNA after UV-irradiation in mammalian cells. *Nature*, **304**, 552–554.
22. Bonura,T. and Smith,K.C. (1975) Quantitative evidence for enzymatically-induced DNA double-strand breaks as lethal lesions in UV-irradiated *pol*$^+$ and *polA1* strains of *E. coli* K-12. *Photochem. Photobiol.*, **22**, 243–248.
23. Natarajan,A.T. and Zwanenburg,T.S.B. (1982) Mechanisms for chromosomal aberrations in mammalian cells. *Mutat. Res.*, **95**, 1–6.

Received on 16 July 1985; accepted on 17 December 1985