

## X-ray Induced Activity of *N*-Ethylmaleimide-sensitive DNA Polymerase in Chinese Hamster Cells

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### Abstract

Chinese hamster DDN cells have been exposed to low doses of X-irradiation (< 15 Gy). These doses have been shown to induce significant levels of repair replication. Levels of activity of DNA polymerase were subsequently measured in cells exposed in G<sub>1</sub> phase of mitosis to 7.5 Gy of X-rays. Following exposure, the activity of this enzyme was observed to rise to a peak within 15 min post-irradiation, then decline and then rise again over a long period. The initial rise at least was found to be sensitive to inhibition by *N*-ethylmaleimide, a known inhibitor of DNA polymerase- $\alpha$  and DNA polymerase- $\gamma$  at the concentration used. It is therefore suggested that repair of X-ray-induced damage in these DON cells was at least in part dependent upon induced DNA polymerase activity and this may involve DNA polymerase- $\alpha$  or DNA polymerase- $\gamma$ , or both.

### Introduction

Exposure of eukaryote cells to ionizing radiation is known to induce a variety of types of damage in the DNA including base damage, and single- or double-strand breakage (Okada 1970; Lehmann 1978). There is now abundant evidence that much of this damage can be repaired in eukaryotes, as in prokaryotes, by means of a number of different enzyme-mediated repair pathways (for reviews see Cerutti 1974; Lehmann and Bridges 1977; Cleaver 1978). A large percentage of these lesions are repaired fairly rapidly, others are repaired more slowly over a longer time period. One step common to many of these pathways is the involvement of DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) activity. In fact several different DNA polymerases have been described from eukaryote cells and these have been extensively characterized (Bollum 1975; Hecht 1975; Weissbach 1977; Dube *et al.* 1978). Functions have been ascribed to some of these enzymes. Thus DNA polymerase- $\alpha$  is said to be involved in semiconservative replication (see Weissbach 1977 and Falaschi and Spadari 1978 for reviews) as is DNA polymerase- $\gamma$ . In contrast it has been claimed that DNA polymerase- $\beta$  is involved in the repair of damaged DNA (Bertazzoni *et al.* 1976; Hubscher *et al.* 1979). The evidence to date is by no means clear-cut, however. In view of the possible involvement of more than one DNA polymerase in repair in eukaryote cells it was decided to study the patterns of DNA polymerase activity in Chinese hamster cells exposed to relatively low doses of X-irradiation.

### Materials and Methods

#### *Cell Line and Culture*

Chinese hamster DON cells were used in all experiments. Cultures were routinely grown as monolayers in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% (w/w) foetal

calf serum, 60 µg/ml penicillin, 10 µg/ml kanamycin (Commonwealth Serum Laboratories, Parkville, Vic.) and 50 µg/ml streptomycin (Glaxo Ltd, Boronia, Vic.). Cultures were gassed with 10% (v/v) CO<sub>2</sub>-air mixture and passaged three times weekly. Cells were considered mycoplasma-free following routine examination with Hoechst 33258 staining (Hoechst Australia Ltd, Melbourne, Vic.) as described by Chen (1977).

Cells were detached from the culture bottles by means of trypsin digestion and harvested once they had reached stationary growth phase as reflected by cell confluency. In this way, all cells used in experiments were naturally synchronized in the G<sub>1</sub> or G<sub>0</sub> phase of the cell cycle. Use of cells at this stage removed the complication of endogenous DNA polymerase activity resulting from *de novo* DNA synthesis. Harvested cells suspended in conditioned medium were divided into aliquots of 5–7 ml each containing approximately  $1 \times 10^6$  cells/ml.

#### *Irradiation of Cell Suspensions*

All irradiations were performed at 4°C using 240 keV X-rays (1.0 mm Al filtration) at a dose rate of 120 rad/min. A total dose of 750 rad (7.5 Gy) was used. Irradiations were carried out at the Australian Atomic Energy Establishment at Lucas Heights, N.S.W. Controls were mock irradiated.

#### *Enzyme Extraction and Preparation*

Following irradiation, cell suspensions were incubated in a water-bath at 37°C for various time intervals (0–70 min) and then cooled on ice prior to centrifugation at 2000 *g* for 5 min at 4°C. After removal of the supernatant the pellet was washed three times in buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM NaCl and 20% w/v glycerol) and finally suspended in 2 ml of this buffer. Since no traces of the phenol red indicator remained after this washing it was assumed that all medium and trypsin had been removed.

Cell suspensions on ice were then subjected to ultrasonic irradiation using an M.S.E. 150-W sonicator, for three 15-s bursts with 15-s cooling intervals. Crude extracts were then centrifuged at 32000 *g* for 20 min at 4°C and the resulting supernatant either assayed immediately or stored at –20°C. Little apparent loss of activity was noted following a single freeze-thaw of enzyme extracts.

#### *Polymerase Assay*

Gross DNA polymerase activity was measured using a method modified from Richardson *et al.* (1964). 0.3 ml of reaction mixture contained 10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 12 mM KCl, 0.4 mM KHPO<sub>4</sub>, 0.4 mM NaHPO<sub>4</sub>, 0.8 mM DTT; 4 µmol each of dCTP, dATP, dGTP, 40 µg of 'activated' calf thymus DNA and 0.3 µCi [*methyl*-<sup>3</sup>H]dTTP, specific activity 62.6 Ci/mmol (New England Nuclear, Boston, Mass.). 'Activated' DNA was prepared by the method of Richardson *et al.* 1964. This assay mixture was incubated with approximately 100 µg of cell protein for 30 min at 37°C and the reaction subsequently stopped by placing on ice and adding 0.5 ml ice-cold 1M HClO<sub>4</sub>. To this was added 0.5 ml DNA solution (Pollock Soft Roe Type VI, Sigma Co. Ltd, Clayton, Vic., 200 µg/ml) to act as a carrier DNA. Total acid-insoluble DNA was collected on pre-wetted Whatman GFC filter paper by gentle vacuum filtration after precipitation by trichloroacetic acid (TCA). The precipitate was washed twice with 7 ml of 5% (w/v) TCA, once with 7 ml 5% (w/v) TCA:5% (v/v) acetone and once with 7 ml 95% (v/v) acetone. Discs were then oven-dried for 30 min and counted in 5 ml scintillant [8 g/l 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophene (BBOT) in toluene] in a Hewlett Packard Prias liquid scintillation counter for 10 min.

#### *Protein Assay*

All cell-free enzyme extracts were assayed for protein concentration using the method of Bradford (1976). Protein determinations with Coomassie Brilliant Blue G250 (Sigma Co. Ltd) were unaffected by DTT or *N*-ethylmaleimide (Sigma Co. Ltd) present in the reaction medium. Protein standards were prepared using bovine serum albumin (Commonwealth Serum Laboratories, Parkville, Vic.) dissolved in homogenizing buffer.

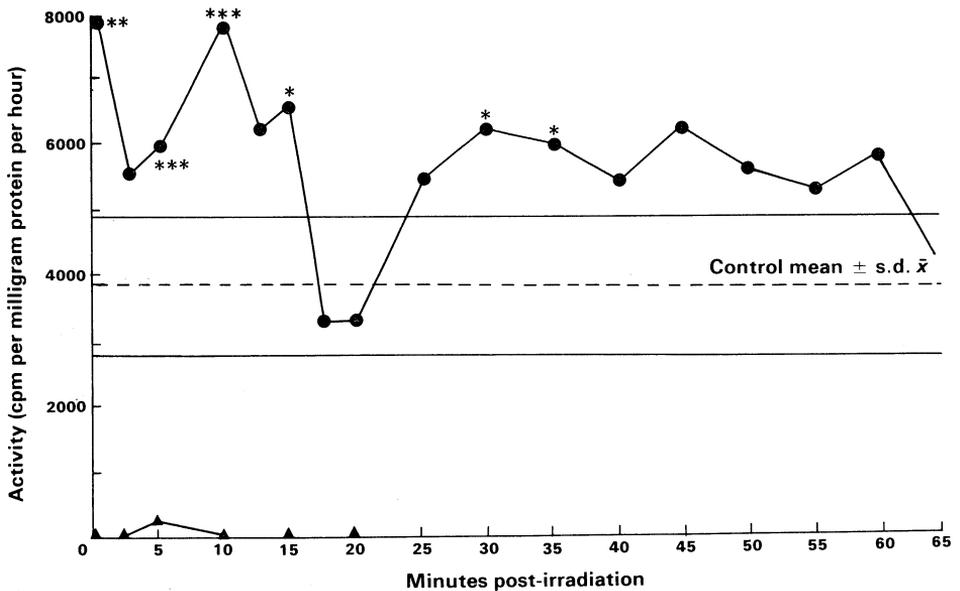
## **Results**

X-ray doses of less than 15 Gy were used in the present experiments so that any repair activity induced in the treated cells could be regarded as being biologically

meaningful as defined by Rasmussen *et al.* (1970). That Chinese hamster DON cells did in fact show repair activity following a dose of 7.5 Gy of X-rays was demonstrated by the incorporation of labelled DNA precursors into irradiated cells. Unirradiated control cells incorporated tritiated thymidine extensively (88 644 cpm/ $\mu\text{g}$  DNA). This incorporation was reduced in unirradiated cells treated with hydroxyurea (a potent inhibitor of semiconservative DNA synthesis) but the level of incorporation of tritiated thymidine was enhanced when cells treated with hydroxyurea at a final concentration of  $10^{-3}$  M were irradiated with low doses of X-rays as indicated in the following tabulation:

X-ray dose (Gy)	0	5.0	7.5	10	15
Tritiated thymidine incorporation (cpm/ $\mu\text{g}$ DNA)	7869	14 586	16 383	15 789	21 329

These results indicate an induction of DNA repair synthesis in these cells. The incorporation of labelled precursors into damaged DNA in irradiated cells also suggests that the cellular repair processes involved rather more than simple ligation of broken DNA chains.



**Fig. 1.** Activity levels of DNA polymerase following exposure of cells to 7.5 Gy X-rays. ● Assayed for total DNA polymerase. ▲ Total DNA polymerase assayed in the presence of *N*-ethylmaleimide. Asterisks indicate significant increases in activity over control values as shown by one-tailed *t*-test as follows: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

Since most of the proposed repair pathways in both prokaryotes and eukaryotes involve nucleotide insertion and therefore DNA polymerase activity at some point, activity of this enzyme was assayed in irradiated cells which had been incubated at  $37^{\circ}\text{C}$  for various time periods after exposure. The results of such an experiment are shown in Fig. 1—each point represents the mean of two or three replicates. It can

be seen that following X-irradiation there was a rapid increase in total DNA polymerase activity such that enzyme activity rose to a peak within 15 min. Activity then declined so that levels measured at 20 min post-irradiation were no greater than those seen in unirradiated control cells. This lower level of activity was, however, short-lived and a further increase followed with DNA polymerase activity remaining high for up to 1 h post-irradiation before declining again. Such a short-term response in total DNA polymerase activity has not been previously reported for eukaryote cells although somewhat similar patterns of response have been obtained following  $\gamma$ -irradiation of meiosis and pollen grain mitosis in lily (Hotta and Westerman, unpublished data).

In order to ascertain whether or not these changes in levels of DNA polymerase activity could be ascribed to the induction and/or activation of *particular* DNA polymerase moieties ( $\alpha$ ,  $\beta$  or  $\gamma$ ), *N*-ethylmaleimide (NEM) was added to the assay mixtures as a specific inhibitor of DNA polymerase- $\alpha$  (see Craig and Keir 1975; Roufa *et al.* 1975). The substitution of 1 mM NEM for DTT effectively eliminated DNA polymerase activity in the assays as can be seen in Fig. 1. This finding suggests that DNA polymerase- $\alpha$  is involved in the initial response. It is possible, however, that the NEM was also acting to inhibit DNA polymerase- $\gamma$  activity since it is known that 1 mM NEM may inhibit 50–80% of DNA polymerase- $\gamma$  activity (see Weissbach 1977).

## Discussion

We have shown that when Chinese hamster DON cells are irradiated in the  $G_1$ -phase of the cell cycle with doses of less than 15 Gy, measurable repair activity is induced. This incorporation of labelled precursors into DNA damaged by ionizing radiation has been suggested by Painter and Cleaver (1967, 1969) to be a manifestation of the repair capacity of the cell. Major X-ray-induced DNA lesions include single-strand breaks and base damage—both of which can be repaired in eukaryote cells (for references see Dugle *et al.* 1976). In many different cell types the number of radiation-induced breaks has been shown to decrease significantly during the first 40 min post-irradiation but a later, slower phase of rejoining often follows the initial rapid phase (Lett *et al.* 1967; Donlan and Norman 1971; Horikawa *et al.* 1972; Matsudaira and Furino 1972).

Using DON cells grown and irradiated under similar conditions to those used in the present experiments we have shown (J. Dempsey, personal communication) that single-strand breaks induced in the DNA are largely repaired within 30 min post-irradiation. This time-course for repair coincided with the initial rapid rise in DNA polymerase activity reported above. It is thus possible that this initial rise is associated with cellular repair activities—the repair of single-strand breaks and the incorporation of labelled nucleotides being related by common steps, one of which is DNA polymerase activity. Since we have already noted that repair activity in eukaryotes may well consist of an initial rapid and later slower phase, it is possible that the second more sustained rise in DNA polymerase activity seen may be related to the slower repair phase. Other explanations for the second rise are possible, however. A somewhat similar temporal pattern of DNA polymerase activity has been obtained following exposure of naturally synchronized lily cells to low doses of  $\gamma$ -rays (Hotta and Westerman, unpublished data). This was true for both meiotic prophase cells as well as for cells in  $G_1$  phase of the first mitosis in the pollen grain.

Whether such increases in enzyme activity reflect *de novo* synthesis of DNA polymerase(s) or an activation of pre-existing molecules is not known at present but some results obtained in the lily system, as yet unconfirmed for the DON cells, may be pertinent. Irradiation of lily cells in the presence of the protein synthesis inhibitor cycloheximide led to a marked reduction in the levels of  $\gamma$ -ray-induced DNA polymerase activity. Similarly Chui and Baril (1975) have reported that activity of DNA polymerase- $\alpha$  and of DNA polymerase- $\beta$  can be greatly reduced within 60 min in Hela cells in late G<sub>1</sub> by addition of cycloheximide.

Methotextrate or high doses of X-rays or ultraviolet light have been reported to increase DNA polymerase levels in *Tetrahymena pyriformis* cells (Westergaard and Pearlman 1969; Keiding and Westergaard 1971). Although important differences (especially size of dose) exist between these latter experiments and the ones described above, it is of interest to note that Westergaard and Pearlman (1969) reported that the methotextrate-induced rise in DNA polymerase activity in *T. pyriformis* was probably *de novo* synthesis since it was abolished by addition of cycloheximide to the treated cell cultures.

In our initial experiments no attempt was made to distinguish whether *particular* DNA polymerase moieties were being induced. The finding that at least the initial phase of increased polymerase activity was sensitive to the presence of NEM would suggest that either DNA polymerase- $\alpha$  or DNA-polymerase- $\gamma$  or both are involved in this response, but *not* DNA polymerase- $\beta$ . Our finding is in apparent contradiction to much recent work which has suggested that DNA polymerase- $\beta$  is the major repair enzyme whilst DNA polymerase- $\alpha$  and DNA polymerase- $\gamma$  are involved in semi-conservative DNA synthesis (see Chang *et al.* 1973; Bertazzoni *et al.* 1976; Robison and Fansler 1978; Hübscher *et al.* 1979). However, some authors, for example Hübscher *et al.* (1979), have not ruled out the possibility of some minor involvement of DNA polymerase- $\alpha$  and perhaps DNA polymerase- $\gamma$  in repair. That this may indeed be the case is suggested by the findings of Das and Fujimura (1979) that DNA polymerase- $\alpha$  and DNA polymerase- $\beta$  from calf thymus are *both* 'quasi-processive', i.e. add 10–15 nucleotides before dissociating from the template-like *E. coli* DNA polymerase-I, a major repair enzyme.

The activity of DNA polymerase induced in *T. pyriformis* cells by very large doses of X-rays (reported by Westergaard *et al.* 1970) was correlated with the mitochondrial fraction. It is, however, possible that they were in fact measuring DNA-polymerase- $\gamma$  activity since there is still some confusion as to whether DNA polymerase- $\gamma$  and mitochondrial DNA polymerases are the same enzyme or different ones having similar properties (Bolden *et al.* 1977; Weissbach 1977; Tanaka *et al.* 1978; Hübscher *et al.* 1979). Involvement of DNA polymerase- $\gamma$  in repair of damaged DNA molecules may also explain the findings of Wolff and Luippold (1955) that addition of 1% MnCl<sub>2</sub> solution to cells in the interval between two halves of a split dose of X-rays led to a very marked drop in the amount of cytological damage manifested later in the cell cycle. Whilst all DNA polymerases have an *in vitro* requirement for divalent cations (Mg<sup>2+</sup> or Mn<sup>2+</sup>) it is noteworthy that Mn<sup>2+</sup> is an important cofactor for  $\gamma$ -polymerase (see Dube *et al.* 1978).

Thus whilst it would not be wise at this stage to suggest that only DNA polymerase- $\alpha$  or DNA polymerase- $\gamma$ , or both, are involved in repair of damaged DNA in X-irradiated Chinese hamster cells, it is clear from our results that, as Hübscher *et al.* (1979) point out, DNA polymerase- $\beta$  may not be the only eukaryote DNA

polymerase involved in repair processes. Further studies, using other specific inhibitors of DNA polymerases, are now in progress.

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