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Enzymatic induction of DNA double-strand breaks in γ-irradiated *Escherichia coli* K-12

(*polA1* mutant/repair/excision/degradation)

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ABSTRACT The *polA1* mutation increases the sensitivity of *E. coli* K-12 to killing by γ-irradiation in air by a factor of 2.9 and increases the yield of DNA double-strand breaks by a factor of 2.5. These additional DNA double-strand breaks appear to be due to the action of nucleases in the *polA1* strain rather than to the rejoining of radiation-induced double-strand breaks in the *pol*$^+$ strain. This conclusion is based upon the observation that γ-irradiation at 3° did not affect the yield of DNA double-strand breaks in the *pol*$^+$ strain, but decreased the yield in the *polA1* strain by a factor of 2.2. Irradiation of the *polA1* strain at 3° followed by incubation at 3° for 20 min before plating resulted in approximately a 1.5-fold increase in the D_0. The yield of DNA double-strand breaks was reduced by a factor of 1.5. The *pol*$^+$ strain, however, did not show the protective effect of the low temperature incubation upon either survival or DNA double-strand breakage. We suggest that the increased yield of DNA double-strand breaks in the *polA1* strain may be the result of the unsuccessful excision repair of ionizing radiation-induced DNA base damage.

Although the repair of DNA double-strand breaks appears to take place in eukaryotic cells (1), there are conflicting data for prokaryotic cells. A number of investigators have observed (2) or inferred (3, 4) the rejoining of double-strand breaks in *Micrococcus radiodurans*. Recently, Hariharan and Hutchinson (5) have suggested one or a very few double-strand breaks may be rejoined in *Bacillus subtilis;* however, their data are not conclusive. Experiments performed by Kaplan (6) did not show rejoining of these lesions in a strain of *Escherichia coli* K-12. Although most investigators have been concerned with testing the hypothesis that double-strand breaks may be enzymatically repaired upon post-irradiation incubation, it is also possible that such lesions may arise enzymatically after irradiation, thus exerting an effect deleterious to survival.

Recently, it has been shown that γ-rays introduce lesions in DNA that are not strand breaks but are sites susceptible to endonucleases prepared from *Micrococcus luteus* (7–9) and crude extracts of *E. coli* (10). Wilkins has found that these sites are rapidly removed during and immediately after irradiation of *E. coli* cells (9). Hariharan and Cerutti (11) have observed the disappearance of thymine radiolysis products from γ-irradiated *M. radiodurans* by an excision-type process. These data suggest that some bacteria may possess an excision repair system that recognizes and removes regions in the DNA that have been damaged by ionizing radiation. Such a repair system could give rise to the appearance of enzymatically induced DNA double-strand breaks as a result of overlapping excision breaks similar to those observed after ultraviolet irradiation (12). In this communication we provide evidence indicating that DNA double-strand breaks appear as a result of an enzymatic process in a γ-irradiated *E. coli* K-12 mutant deficient in DNA polymerase I (deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7).

MATERIALS AND METHODS

Bacterial Strains. Two strains of *E. coli* K-12 W3110, JG138 and JG139 (obtained from J. D. Gross), were used in this study. These strains are isogenic except that JG138 carries the amber *polA1* mutation.

Media and Culture Conditions. Minimal medium (13) was used, supplemented with thiamine (0.5 μg/ml) and thymine (10 μg/ml for overnight cultures). The overnight cultures were diluted 1:50 into fresh medium supplemented with [*methyl*-^{3}H]thymine (New England Nuclear Corp.; >12 Ci/mmol) at 200 μCi/ml and 2 μg/ml of unlabeled thymine. Cultures to be used for survival curves contained only unlabeled thymine at 10 μg/ml. Cells were grown at 37° for several generations in exponential phase to a final density of about 2×10^8 cells per ml.

Irradiation. Cells were harvested by filtration on 0.45 μm membrane filters (Millipore Corp.), washed, and resuspended to approximately 2 to 4×10^7 cells per ml in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.6. The suspension was bubbled with air in a temperature-controlled, water jacketed glass chamber and irradiated using a Mark I ^{137}Cs irradiator (J. L. Shepard and Associates). The dose rate was determined by ferrous sulfate dosimetry to be 5.26 krad/min.

Survival Curves. After irradiation, an aliquot of the cell suspension was removed and diluted in 67 mM phosphate buffer, pH 7.0. Diluted samples were plated on minimal medium plates (supplemented with 10 μg/ml of thymine and solidified with 1.6% Difco Noble agar). The plates were incubated for 48–72 hr at 37° for colony formation.

Neutral Sucrose Gradient Sedimentation. After irradiation, 0.2 ml of the cell suspension was added to 0.3 ml of ice-cold 0.07 M Tris–0.17 M EDTA, pH 7.6, containing 200 μg/ml of lysozyme (Worthington Biochemicals). After 10 min on ice, 0.1 ml of the spheroplast suspension was layered on a 5–20% (weight/volume) neutral sucrose gradient containing 0.5% sodium dodecyl sulfate (recrystallized), 5 mM Tris·HCl, pH 7.6, 1 mM sodium citrate, 10 mM sodium chloride, and 1 mg/ml of Pronase (Calbiochem). To aid in the delipidization of the DNA, stock solutions of 10 and 40% sucrose, 10 mM sodium citrate, and 0.1 M sodium chloride were saturated with chloroform and diluted to the final concentration described. The resulting gradient was about 70% saturated in chloroform. After 90 min at room temperature, the gradients were centrifuged for 16 hr at 8000 rpm in a SW50.1 rotor in a Beckman model L, L2, or L265B ultra-

Abbreviation: D_0, the dose (in krad) required to reduce the survival to e^{-1} on the exponential portion of the survival curve.

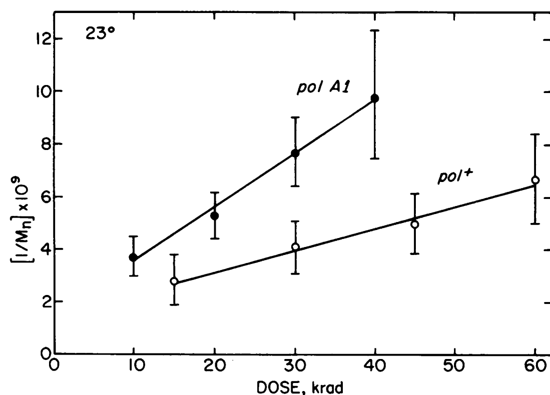

FIG. 1. Increase in reciprocal number average molecular weights ($1/M_n$) as a function of dose for *E. coli* K-12 *polA1* (●) and the *pol*+ strains (O) irradiated with ^{137}Cs γ-rays at 23°. The points shown represent the mean of four experiments for the *polA1* strain and five for the *pol*+ strain. The straight lines were fitted through the data by the method of least squares linear regression analysis. The vertical bars represent the standard deviation.

centrifuge. After centrifugation, the bottom of each tube was pierced and the gradient was fractionated by pumping 0.16-ml aliquots onto Whatman 3MM paper discs. The discs were dried and washed in 5% trichloroacetic acid, ethanol, and acetone. Radioactivity was determined in a liquid scintillation spectrometer. Further details of the neutral sucrose gradient method and molecular weight analyses have been reported (14).

RESULTS

We have examined the dose response for DNA double-strand breakage in the *pol*+ and *polA1* strains of *E. coli* K-12 W3110 using a neutral sucrose gradient technique (14). Fig. 1 shows the reciprocal number average molecular weight plotted as a function of dose for the DNA liberated from spheroplasts prepared immediately after irradiation at 23°. The unirradiated points were excluded from the regression analyses because the DNA isolated from these cells is of lower molecular weight ($4.17 \pm 1.06 \times 10^8$) than the intact genome, and generally lower than that estimated by extrapolation of the linear region of the dose response curve shown in Fig. 1. Because these artifacts disappear when the DNA is sufficiently broken (14), only the data from irradiated cells were considered. It is evident that more DNA double-strand breaks exist in the *polA1* strain than in the *pol*+ strain immediately after irradiation at 23°.† The ratio of the slopes of the regression lines in Fig. 1 demonstrates a 2.5-fold increase in the yield of double-strand breaks in the *polA1* strain. An analysis of the survival curves (Fig. 2) gives D_0 values of 1.1 krad for the *polA1* strain and 3.2 krad for the *pol*+ strain. This 2.9-fold sensitization to killing is not significantly different ($p > 0.05$) from the 2.5-fold increase in the number of double-strand breaks observed in the *polA1* strain.

It has been observed in our laboratory that single-strand breaks measured immediately after x-irradiation at room temperature were more numerous in the *polA1* strain than in the *pol*+ strain (15). This difference was interpreted as being due to a rapid, DNA polymerase I dependent repair process present in *pol*+ cells. Our observation (Fig. 1) of an increased yield of DNA double-strand breaks in the *polA1* strain under similar conditions could be the result of a rapid DNA polymerase I dependent repair of double-strand breaks occurring in the *pol*+ cells. If this were the case, the yield of double-strand breaks in the wild-type strain should approach that observed with the *polA1* cells, provided repair is minimized by irradiation at low temperatures. However, Fig. 3 shows the opposite to be true. Irradiation of the *pol*+ strain at 3° showed a small but statistically insignificant decrease ($p > 0.05$) in the yield of DNA double-strand breaks compared to irradiation at 23°, whereas the extent of double-strand breakage in the *polA1* strain decreased by a factor of 2.2. The effect of temperature on the yields of DNA double-strand breaks is summarized in Table 1. These data are suggestive of an enzymatic process that occurs rapidly in the *polA1* strain during irradiation at 23°, and whose

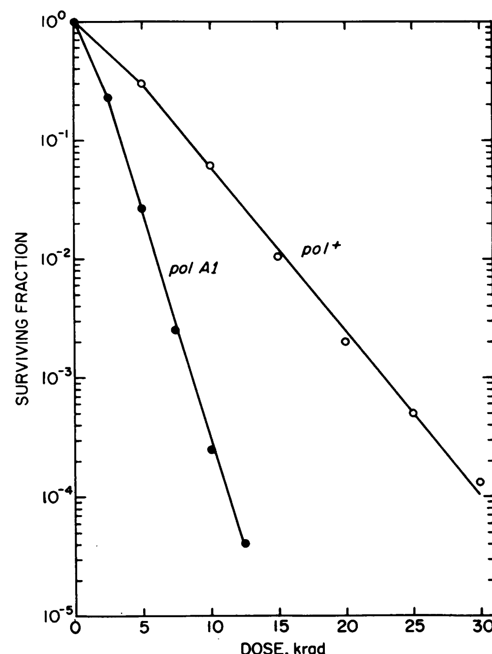

FIG. 2. Survival curves for the *polA1* (●) and *pol*+ (O) strains. Cells were γ-irradiated at 23° in 50 mM Tris buffer at pH 7.6 and continuously bubbled with air. Points represent the average of three experiments.

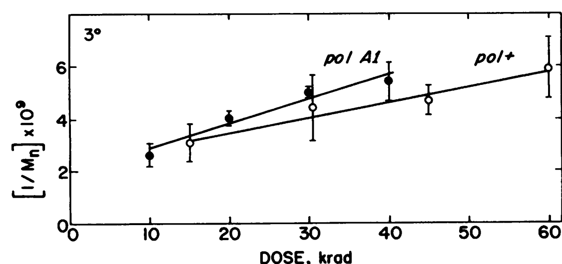

FIG. 3. Increase in reciprocal number average molecular weights as a function of dose for the *polA1* (●) and *pol*+ (O) strains irradiated at 3°. The points shown represent the mean of four experiments for the *polA1* strain and five for the *pol*+ strain. The straight lines were fitted through the data by the method of least squares linear regression analysis. The vertical bars represent the standard deviation. These data were gathered in conjunction with the experiments shown in Fig. 1.

† It should be noted that the yield of DNA double-strand breaks for the *pol*+ strain (JG139) aerobically irradiated with ^{137}Cs γ-rays is 1.9 times less than the value we reported (14) for the same strain irradiated under the same conditions with 50 kVp x-rays. This difference is real and is reflected in a correspondingly higher relative biological effect for 50 kVp x-rays with respect to cell killing (Bonura, Youngs, and Smith, manuscript in preparation).

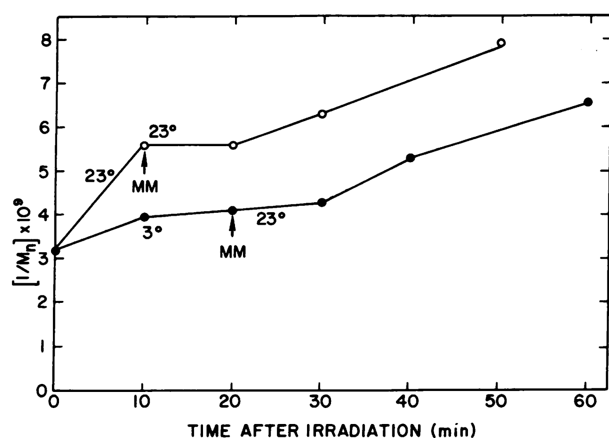

FIG. 4. Increase in reciprocal number average molecular weight of the *polA1* strain as a function of time after irradiation with 10 krad at 3° in 50 mM Tris, pH 7.6. Cells were held in buffer at 3° (●) for 20 min or 23° (○) for 10 min before being returned (arrows) to minimal medium (MM) at 23°. Points represent the average of two experiments.

rate is significantly diminished, but not totally inhibited, by irradiation at 3°. The yield of DNA double-strand breaks maximizes quite rapidly (within about 10 min) upon incubation of the *polA1* strain at 23°. Fewer DNA double-strand breaks appear when the *polA1* strain, irradiated at 3°, is held on ice, and it requires about 20 min to achieve the maximum yield of breaks (data not shown).

In the experiment shown in Fig. 4, DNA double-strand breaks were measured after irradiating *polA1* cells at 3° with 10 krad. Cells were then held in Tris buffer either at 3° or 23° (for 20 or 10 min, respectively) before being resuspended in minimal medium at 23°. More DNA double-strand breaks were introduced during the incubation at 23° in buffer than during the incubation at 3° in buffer, and this difference persisted even when the temperature was raised and the cells were further incubated in minimal medium.

Upon extended incubation (>80 min at 37°), >80% of the DNA becomes acid-soluble in the *polA1* strain, and estimates of double-strand breakage lose significance. In all experiments that we have described, the amount of DNA degraded at the time of sedimentation analysis was always 10–15% or less.

The effects of temperature on DNA double-strand breakage correlate with the effects of temperature on survival (Fig. 5). The *pol*$^+$ strain, which showed little or no differential effect of irradiation on DNA double-strand breakage at 3° or 23°, showed no change in survival when the cells were held at 3° or 23° for a short time before plating. The *polA1* strain, however, showed a 1.5-fold increase in the D_0 for cells held for 20 min at 3° before plating relative to cells held at 23° before plating, correlating with the decrease in DNA double-strand breakage under similar conditions.

Table 1. Effect of temperature on the yield of DNA double-strand breaks in *polA1* and *pol*$^+$ strains of *E. coli* K-12

Irradiation temperature	*pol*$^+$ (× 10^9·krad)	*polA1* (× 10^9·krad)
23°	0.082 ± 0.016*	0.205 ± 0.033
3°	0.058 ± 0.013	0.093 ± 0.011

Numbers represent the slopes of the regression lines shown in Figs. 1 and 3.

* Standard deviation of the slope.

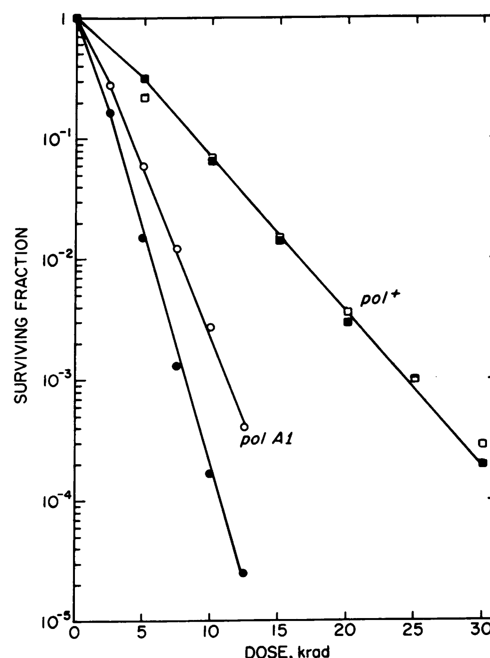

FIG. 5. Survival curves for the *polA1* (○, ●) and *pol*$^+$ (□, ■) strains irradiated at 3° and held for 20 min at 3° (○, □) or 10 min at 23° (●, ■) before plating. Points represent the average of two experiments.

DISCUSSION

The enzymatic induction of DNA double-strand breaks after UV irradiation due to overlapping regions of excision repair has been hypothesized (16, 17). We have recently demonstrated the occurrence of such breaks after UV irradiation in *uvr*$^+$ strains of *E. coli* K-12 (12). The question of whether a cell introduces double-strand breaks enzymatically after x- or γ-irradiation has not been previously addressed.

To test this hypothesis we have γ-irradiated the *polA1* strain at 23° and compared the yield of DNA double-strand breaks to that observed with the *pol*$^+$ strain at the same temperature. We observed a 2.5-fold higher yield of DNA double-strand breaks in the *polA1* strain. These breaks appear to be due to the action of nucleases in the *polA1* strain rather than to the rejoining of double-strand breaks in the *pol*$^+$ strain. This interpretation is based upon the observation that γ-irradiation at 3° did not affect the yield of DNA double-strand breaks in the *pol*$^+$ strain but decreased the yield in the *polA1* strain (Figs. 1 and 3). Furthermore, reincubation at 23° after irradiation at 3° did not change the DNA sedimentation profile for the *pol*$^+$ strain (data not shown), but did result in a shift to a lower molecular weight for the *polA1* strain (Fig. 4).

The yield of DNA double-strand breaks that appeared after the *polA1* cells were returned to 23° was approximately 1.5-fold less if cells were held on ice after irradiation than if held at 23° (Fig. 4). Assaying cell survival for the *polA1* strain after similar treatment showed a 1.5-fold increase in the D_0 dose. There was no such temperature effect on survival (Fig. 5) of DNA double-strand breakage in the *pol*$^+$ strain.

The beneficial effects on survival of post-irradiation incubation at temperatures suboptimal for growth is not in itself

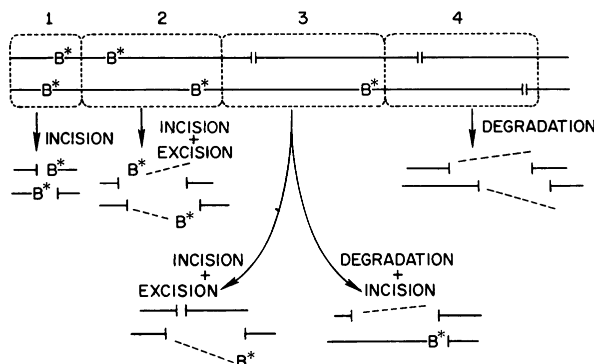

FIG. 6. Possible mechanisms for the formation of a DNA double-strand break by enzymatic processes after ionizing irradiation. The symbols B* and —| |— represent excisable damage (such as base damage) and single-strand breaks or gaps, respectively.

new. Stapleton, Billen, and Hollaender (18) made the observation that holding *E. coli* B/r at 18° for a number of hours after irradiation resulted in a 1.5-fold increase in the D_0 relative to a 37° incubation. The authors postulated that the effect of holding at low temperature after irradiation was to alter the relationship between two competitive processes, one being synthesis and the other "destructive".

Dugle and Dugle (19) observed an enhancement in survival by holding γ-irradiated *B. subtilis* cells at 0° before plating. They did not measure changes in DNA molecular weight, however.

Since we find no effect of temperature on the yield of DNA double-strand breaks in the *pol*$^+$ strain, we may assume that no preferential inhibition of degradation and/or repair occurs. However, the *polA1* strain is deficient in DNA polymerase I, which is required both for the rapid repair of primary x-ray-induced breaks (15) and of excision gaps (at least after UV irradiation, refs. 20–22). A reduction in temperature would be expected to have a preferential effect on degradation and the excision process in the *polA1* strain.

Fig. 6 shows four conditions whereby DNA double-strand breaks could arise by enzymatic processes. By reducing the temperature, the rates of incision, excision, and degradation and the rate of resealing of radiation-induced breaks would be reduced. Ordinarily, DNA polymerase I would play a role in the repair of the radiation-induced DNA single-strand breaks (Conditions 3 and 4) and probably also in the repair of incision breaks produced as the first step in the repair of base damage (Conditions 1–3). Thus, in the repair proficient *pol*$^+$ strain, there would probably be no net effect of temperature on the appearance of DNA double-strand breaks by Conditions 1–4, consistent with our experimental data.

The *polA1* strain, however, excises longer pieces of DNA than *pol*$^+$ cells after UV irradiation (23). In addition, the *polA1* strain cannot rapidly rejoin radiation-induced DNA single-strand breaks (15) and is probably at least partially defective in the gap-filling step of excision repair after ionizing radiation as it is after UV irradiation (20–22). Since degradation and resynthesis are not well coordinated in the *polA1* strain, DNA double-strand breaks could occur by all four conditions. Because base damage is thought to be more abundant than single-strand breakage (8, 9, 11) and because clustering of x-ray damage of the sugar moiety (24) and thymine residues (25) has been suggested to occur in the proximity of a single-strand break, Conditions 2 and 3 may be more probable than Condition 4 in determining the appearance of enzymatically induced DNA double-strand breaks in the *polA1* strain. Condition 1 is unlikely because of the low probability of producing base damage at such close proximity by independent events at the doses used.

The yield of double-strand breaks was decreased in the *polA1* strain by a factor of about 1.5 and the survival was increased by a factor of 1.5 when the cells were held at 3° for 20 min before they were returned to 23°. This implies that some of the lesions or processes shown in Fig. 6 were altered during the 20-min incubation at 3°, resulting in a decrease in the length of the excised segment, a more efficient repair of direct single-strand breaks, and/or a chemical modification of some damaged bases such that they are no longer a substrate for the excision repair system.

In summary, we have shown that enzymatically induced DNA double-strand breaks appear rapidly after the γ-irradiation of *E. coli* K-12 *polA1*, a strain that is partially defective both in the repolymerization step in the excision repair of UV-induced damage (20–22) and in the repair of ionizing radiation-induced DNA single-strand breaks (15, 20). This suggests that enzymatically induced DNA double-strand breaks may arise in the *polA1* strain either as a consequence of overlapping excised regions of the DNA or the overlap of an excised region with a single-strand break induced directly by the radiation. Proof for this hypothesis must await the isolation of a mutant defective in the excision repair of ionizing radiation-induced DNA base damage.

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