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Enzymatic Production of Deoxyribonucleic Acid Double-Strand Breaks After Ultraviolet Irradiation of *Escherichia coli* K-12

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We have observed the enzymatic production of deoxyribonucleic acid (DNA) double-strand breaks in *Escherichia coli* K-12 after ultraviolet irradiation. Double-strand breaks appeared in wild-type, *polA1*, *recB21*, *recA*, and *exrA* strains after incubation in minimal medium. The *uvrA6* strain showed no evidence of double-strand breakage under the same conditions. Our data suggest that *uvr*$^+$ cells, which are proficient in the incision step of excision repair, accumulate double-strand breaks in their DNA as a result of the excision repair process, i.e., arising from closely matched incisions, excision gaps, or incisions and gaps on opposite strands of the DNA twin helix. Furthermore, strains deficient in excision repair subsequent to the incision step (i.e., *polA*, *rec*, *exrA*) showed more double-strand breaks than the wild-type strain. The results raise the possibility that a significant fraction of the lethal events in ultraviolet-irradiated, repair-proficient (*uvr*$^+$) cells may be enzymatically-induced DNA double-strand breaks.

Single-strand breaks are introduced enzymatically into the deoxyribonucleic acid (DNA) of ultraviolet (UV) irradiated wild-type *Escherichia coli* K-12 cells as the first step in the *uvr* gene-dependent excision repair of photochemical lesions (8). As enzymatic repair proceeds, the photochemical lesions are excised and the resultant gaps are filled by one of the DNA polymerases (3, 10, 18) and joined to the remainder of the chromosome by polynucleotide ligase (12). By this mechanism of excision repair, wild-type *E. coli* cells are able to restore their UV-damaged DNA to the unirradiated state after low doses of UV light. As the dose increases, some of the excision gaps fail to be repaired (17). Furthermore, mutants which are deficient in steps of the excision repair process subsequent to the insertion of the first endonucleolytic nick accumulate unrepaired single-strand breaks after lower UV doses than do wild-type cells (17, 18).

It seems reasonable that as the frequency of unrepaired single-strand breaks increases, the probability of forming a double-strand break by the introduction of closely matched nicks or gaps should also increase. With this in mind, we have tested a number of strains for the enzymatic introduction of double-strand breaks as a result of excision repair. We have observed double-strand breaks only in strains which can insert nicks into their DNA (i.e., *uvr*$^+$). The number of DNA double-strand breaks observed was greatest for cells deficient in excision repair subsequent to the incision step (i.e., *polA*, *recA*, *recB*, *exrA*).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are given in Table 1 along with their genotypes and the sources from which they were obtained.

Media. Minimal medium (5) was used, supplemented with thiamine (0.5 μg/ml) and thymine (10 μg/ml for overnight cultures; 2 μg/ml for exponentially growing cells). Required amino acids were added appropriately (10^{-3} M).

UV irradiation. An overnight culture was diluted 1:50 into fresh medium, supplemented with [*methyl*-^{3}H]thymine (New England Nuclear Corp.; ~12 Ci/mmol) at 200 μCi/ml, and grown for several generations in exponential phase at 37 C to ~2×10^8 cells/ml. Deoxyguanosine at 100 μg/ml was added to the medium for the cells that did not require thymine. Cells were harvested on 0.45-μm membrane filters (Millipore Corp.), washed in DTM buffer (minimal medium without glucose or supplements), and resuspended in minimal medium at a cell density of about 4×10^6 cells/ml.

The suspension was then placed in a glass Petri dish on a rotary shaker and irradiated at room temperature with an 8-W General Electric germicidal lamp. The incident dose rate was determined with an International Light, Inc., germicidal photometer (no. Il-254).

Neutral sucrose gradients. After irradiation and

TABLE 1. *List of strains*

Number	Genotype	Source or reference
JG137	F⁻ *thy rha str lacZ* (amb) *uvrA6*	J. D. Gross
JG139	F⁻ *thy rha str lacZ* (amb)	J. D. Gross
DY130	F⁻ *lacZ* (amb) *metE str recB21*	D. A. Youngs (7)
MM450	F⁻ *rha lacZ* (amb) *str recA56*	M. Monk
DY100	F⁻ *thy lacZ* (amb) *metE str polA1*	D. A. Youngs (7)
DY176	F⁻ *thy lacZ* (amb) *str exrA*	D. A. Youngs

incubation, 0.2 ml of the cell suspension was added to 0.3 ml of ice-cold 0.07 M tris(hydroxymethyl)aminomethane-0.017 M ethylenediaminetetraacetic acid at pH 7.6 containing lysozyme (Worthington Biochemicals) at 200 μg/ml. After 10 min on ice, 0.1 ml of the spheroplast suspension was layered on a 5 to 20% (wt/vol) neutral sucrose gradient containing 0.5% sodium dodecyl sulfate (recrystallized), 5×10^{-3} M tris(hydroxymethyl)aminomethane, 10^{-3} M sodium citrate, 10^{-2} M sodium chloride, and pronase at 0.1 mg/ml. The final gradient was 70% saturated in chloroform. After at least 90 min at room temperature, the gradients were centrifuged for 16 h at 8,000 rpm in a SW50.1 rotor in a Beckman model L2 or L265B ultracentrifuge. After centrifugation, the bottom of each tube was pierced and 12-drop fractions were collected onto Whatman no. 3MM filter paper disks, which were then dried, washed in 5% trichloroacetic acid, 95% ethanol, acetone, dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer. Further details of the neutral sucrose gradient method and molecular weight analysis have been described previously (T. Bonura, C. D. Town, K. C. Smith, and H. S. Kaplan, Radiat. Res., in press).

RESULTS

Wild-type *E. coli* K-12 cells are proficient in the excision repair of UV-induced photoproducts. After high incident UV doses, however, not all excised regions are repaired, and single-strand breaks or gaps accumulate (19). The possible accumulation of DNA double-strand breaks under these conditions was investigated using velocity sedimentation through neutral sucrose gradients.

Figure 1 shows the DNA sedimentation profiles for wild-type cells UV irradiated at room temperature. After irradiation the cells were allowed to complete excision repair in minimal medium, transformed to spheroplasts, and lysed on neutral sucrose gradients. The number average molecular weight of the unirradiated DNA relative to a T2 phage DNA marker was 4×10^8. Double-strand breaks began to appear in the wild-type strain after about 15 min of post-irradiation incubation at 37 C and reached a maximal number by 60 min. An unincubated sample irradiated with 100 J/m² showed no double-strand breaks (results not shown). The sedimentation distribution of the DNA broadened at doses up to about 60 J/m² after 80 min

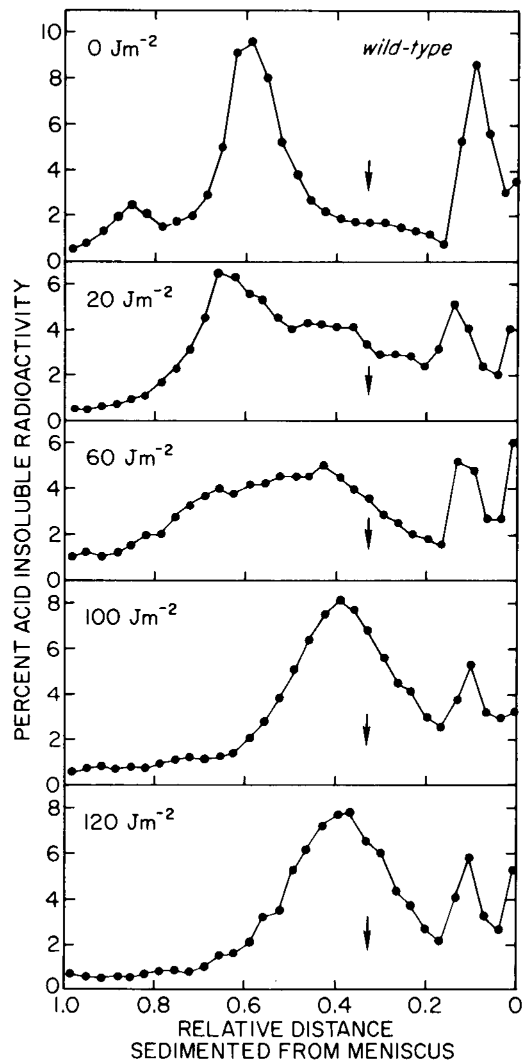

FIG. 1. *DNA sedimentation profiles for wild-type E. coli K-12 cells. After the UV irradiation of cells in minimal medium at room temperature, a portion of the cell suspension was incubated for 80 min at 37 C in a gyratory shaker. Cells were then transformed to spheroplasts, lysed on a 5 to 20% neutral sucrose gradient, and spun at 8,000 rpm for 16 h. The arrow indicates the position of a [¹⁴C]thymine-labeled phage T2 DNA marker. Incident UV doses are indicated on each profile.*

of incubation, indicating an increasing fraction of molecules containing double-strand breaks. Although at doses up to ~60 J/m² the heterogeneity of the DNA population made molecular weight measurements difficult, it is obvious that UV-irradiated, wild-type cells exhibit a dose-dependent increase in DNA double-strand breaks. At 100 J/m² where the sedimentation profile is more gaussian than at low doses, the number of double-strand breaks per genome is probably between 20 and 30.

If the double-strand breaks are the result of excision repair, strains deficient in the first step of excision repair should not show such breaks. We irradiated a strain carrying the *uvrA6* mutation (otherwise isogenic to the wild type) and incubated the cells in minimal medium for 80 min before analyzing for DNA double-strand breaks. The sedimentation profiles in Fig. 2 show no evidence of double-strand breaks at doses up to 160 J/m². To be certain that double-strand breaks were not inserted and repaired in the *uvrA* strain during the 80-min reincubation, we analyzed the DNA at various intermediate times after irradiation. At no time were double-strand breaks present in the irradiated sample (data not shown).

The sedimentation profiles for unirradiated cells were less reproducible than for irradiated cells, i.e., the amount of DNA in the peak and the position of the peak were variable. This can be seen in Fig. 2 where the irradiated DNA sedimented slightly faster than the unirradiated DNA. We have not yet established the cause of this inconsistency.

Cooper and Hanawalt (2) have reported that large repair patches are produced in the DNA of *rec*⁺ strains but not in *recA recB* mutants implicating the *recA* and/or *recB* gene product in the excision repair process. The *recA*, *recB*, and *exrA* gene products have also been implicated in a growth medium-dependent pathway of excision repair (19). If the *recB* nuclease contributed significantly to the expression of double-strand breaks as a consequence of excision repair, a *recB* mutant would be expected to show fewer breaks than the wild-type strain. This prediction is not in accord with the data in Fig. 3 where it can be seen that the DNA from the *recB21* strain sedimented more slowly than the wild-type strain (Fig. 1) at all doses. This suggests that exonucleolytic degradation by the *recB* nuclease is not essential for the appearance of the DNA double-strand breaks observed in our experiments.

Since the DNA of the *recB* strain shows more double-strand breaks than the wild-type strain and is also deficient in the growth medium-dependent branch of excision repair (19), we predicted that other mutants defective in steps of excision repair subsequent to the incision step should also show a greater yield of DNA double-strand breaks than the wild-type strain. The data given in Fig. 4 for the *polA1* strain substantiates this hypothesis. Similarly, the *recA* (Fig. 5) and *exrA* (Fig. 6) strains, which are deficient in the growth medium-dependent branch of excision repair (19), gave results similar to those of the *recB* strain.

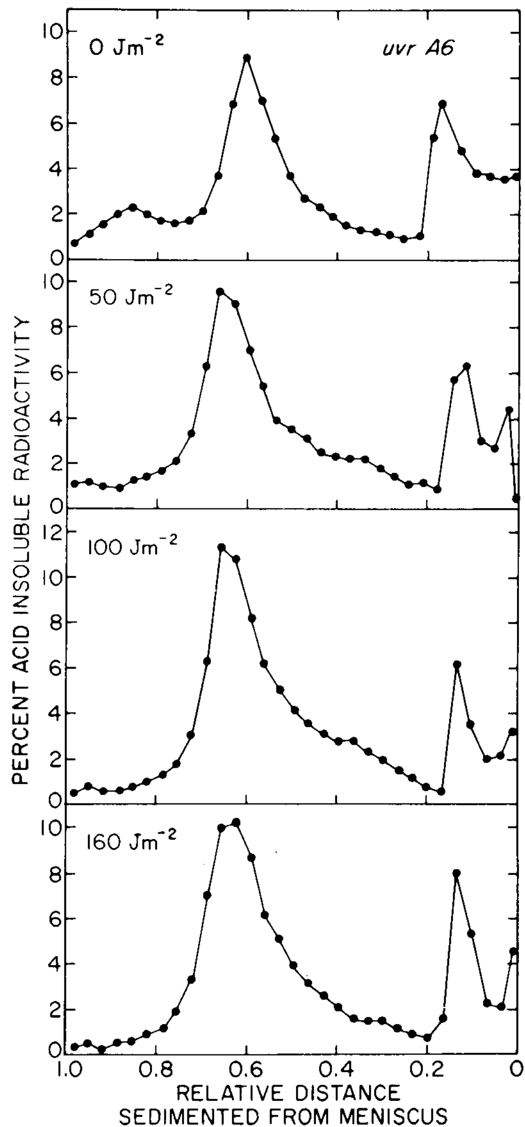

FIG. 2. *DNA sedimentation profiles for the uvrA6 strain (JG137). Conditions are identical to those given in the legend to Fig. 1.*

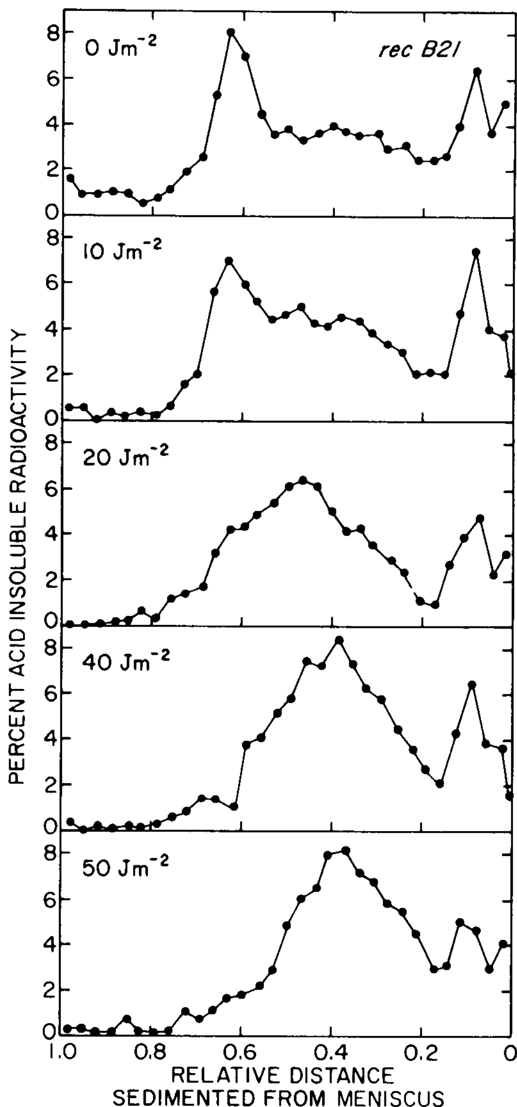

FIG. 3. *DNA sedimentation profiles for the recB21 strain (DY130). Conditions are identical to those given in the legend of Fig. 1.*

DISCUSSION

Incision breaks are introduced into the DNA of *E. coli* K-12 uvr^+ cells after UV irradiation as the first step in excision repair (9). Youngs et al. (17, 19) have shown that in wild-type *E. coli* K-12 nearly all incision breaks produced after UV doses up to 50 J/m^2 are subsequently rejoined during reincubation in growth medium. At higher doses single-strand breaks remain unrepaired. After a dose of 100 J/m^2, approximately 100 single-strand breaks remain unrepaired in wild-type *E. coli* K-12 (19). From our data, we have calculated that 20 to 30 DNA double-strand breaks are present under these same conditions, indicating that a significant fraction of unrepaired single-strand breaks may in fact be double-strand breaks. A double-strand break in DNA has been implicated as a lethal lesion in bacteriophage (4) and in cells of *E. coli* (11) after ionizing irradiation. Our results suggest that DNA double-strand breaks may also constitute a significant portion of the lethal events after UV irradiation in uvr^+ cells.

Figure 7 shows several possible mechanisms

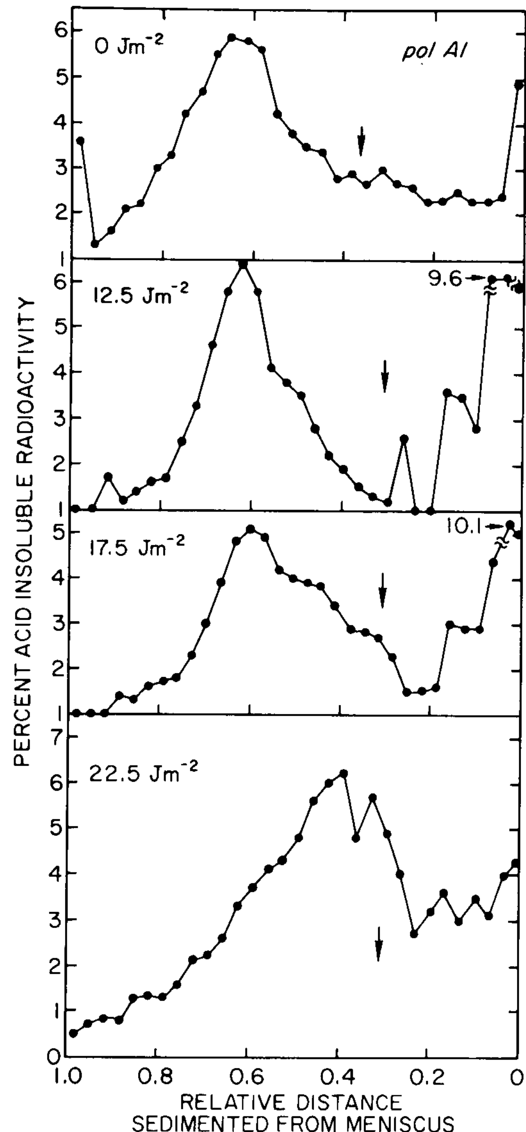

FIG. 4. *DNA sedimentation profiles for the polA1 strain (DY100). Conditions are identical to those given in the legend of Fig. 1.*

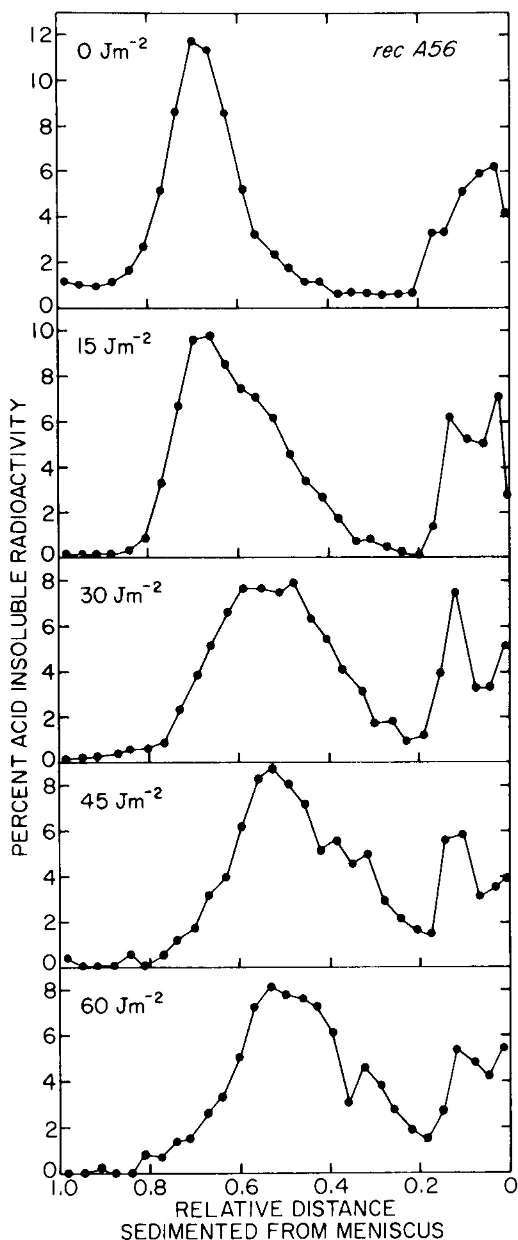

FIG. 5. *DNA sedimentation profiles for the recA56 strain (MM450). Conditions are identical to those given in the legend of Fig. 1.*

whereby a double-strand break may result from excision repair if two pyrimidine dimers are sufficiently close together. A double-strand break can occur if two endonucleolytic nicks are inserted adjacent to closely opposed pyrimidine dimers such that the forces of hydrogen bonding and base stacking cannot maintain the integrity of the double-helical structure (Fig. 7A). A second possibility is that excision in one strand may proceed past an incision break on the opposite strand resulting in the formation of a double-strand break (Fig. 7B). Alternatively, excision may proceed past an opposing unincised pyrimidine dimer resulting in a gap opposite the dimer. If this pyrimidine dimer is subsequently recognized by the UV endonuclease, a double-strand break will occur. A third possibility is the formation of a double-strand break by the juxtaposition of excision gaps on opposing strands of DNA (Fig. 7C). The least probable of these possibilities is the formation of a double-strand break by opposing nicks

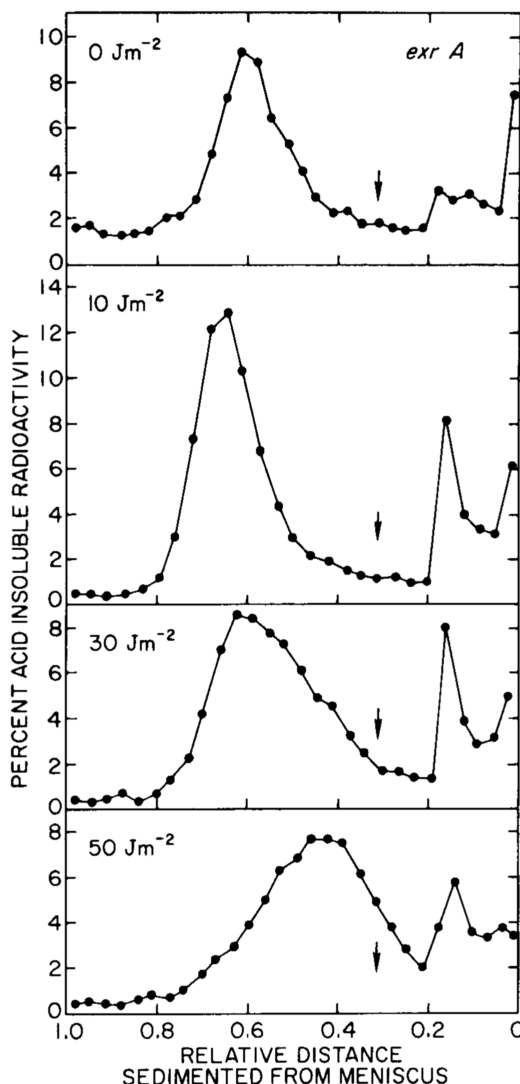

FIG. 6. *DNA sedimentation profiles for the exrA strain (DY176). Conditions are identical to those given in the legend of Fig. 1.*

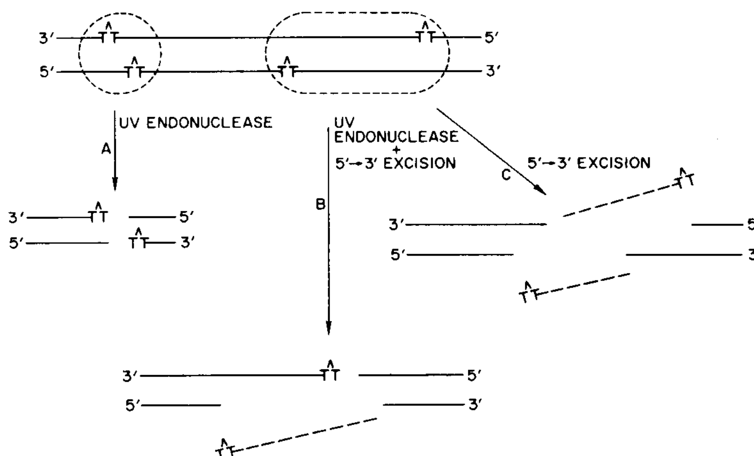

FIG. 7. *Possible mechanisms describing the formation of double-strand breaks in DNA during the course of excision repair. Dashed circles indicate the region of a potential double-strand break arising from closely opposed endonucleolytic nicks (A), an excised gap passing an opposed nick (B), or two opposed excised gaps (C). The symbol* $\hat{TT}$ *represents a thymine dimer but can be interpreted more broadly as any excisable UV photoproduct.*

since only a small fraction of all possible incision breaks exists at any time (14).

Those strains that are proficient in the excision of pyrimidine dimers, *recA*, *recB* (16), *polA1*, wild type (1), and presumably *exrA*, all exhibit double-strand breaks after some dose of UV radiation. Youngs et al. (19) have shown, with respect to the wild-type strain, that the *recA*, *recB*, and *exrA* strains are somewhat deficient in the gap-filling step of excision repair. Because of this deficiency it seems likely that excised regions are more susceptible to further enlargement by degradation. This coupled with the persistence of excised regions even at relatively low UV doses makes likely the formation of a double-strand break by overlapping excision. Youngs et al. (19) have also shown that the wild-type strain efficiently repairs UV-induced incision breaks after incident UV doses up to about 50 J/m^2. This observation correlates well with the dose at which we begin to detect double-strand breaks in this strain, i.e., ~60 J/m^2. Perhaps in the wild-type strain the amount of degradation at a pyrimidine dimer is so small and repair synthesis so efficient that only when dimers are very nearly opposed (i.e., within a few base pairs) will a double-strand break appear.

Our data concerning the *polA1* strain are interesting in that there was a more dramatic decrease in the DNA molecular weight after 20 J/m^2 than in any other strain examined. Indeed, the *polA1* strain has been shown to be extremely defective in the repair of UV-induced DNA single-strand breaks (19), attesting to the importance of DNA polymerase I in the excision repair process. Our data are consistent with the hypothesis that very long pieces of DNA are excised in the *polA1* stain increasing the probability of introducing a double-strand break by the excision processes shown in Fig. 7B, C. Experiments by Cooper and Hanawalt suggest that strains deficient in DNA polymerase I undergo "large patch" repair (2). We suggest that such repair is likely to result in double-strand breaks. In support of this, Paterson and co-workers (13) observed significantly more DNA degradation in a UV-irradiated *polA1* strain relative to the wild type. This increased DNA breakdown in the *polA1* mutant is believed to be caused by the 5′→3′ exonuclease associated with DNA polymerase I (6).

The possibility that such lesions as enzymatically induced double-strand breaks occur during excision repair has been suggested (7, 15) but not demonstrated. Indeed, Harm (7) proposed that the protection afforded to *E. coli* B/r (Hcr$^+$) by dose fractionation was a result of decreasing the probability for the existence of opposed excision gaps by distributing the UV damage over a considerable time period. In this way, Harm suggested, repair of one lesion could be complete before the introduction of the next lesion such that mutual interference in repair (i.e., the probability of forming a double-strand break) is diminished. His observation that the dose rate effect is small in the Hcr$^-$ strain, *E. coli* B_{s-1}, supports his hypothesis and is consist-

ent with our experiments with the *E. coli* K-12 *uvrA* strain.

Our data demonstrates that DNA double-strand breaks arise during the course of excision repair in *uvr*$^+$ strains. Preliminary calculations indicate that after a given UV dose the number of DNA double-strand breaks in *uvr*$^+$ strains was greatest in the *polA1* strain, least in the wild type with *recA*, *recB*, and *exrA* being intermediate. Further experiments are in progress to determine more precisely the yield of excision repair-induced DNA double-strand breaks as well as their contribution to cell killing.

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