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Single-Strand Breakage of DNA in UV-Irradiated *uvrA*, *uvrB*, and *uvrC* Mutants of *Escherichia coli*

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We transduced the *uvrA6*, *uvrB5*, *uvrC34*, and *uvrC56* markers from the original mutagenized strains into an HF4714 background. Although in the original mutagenized strains *uvrA6* cells are more UV sensitive than *uvrB5* and *uvrC34* cells, in the new background no significant difference in UV sensitivity is observed among *uvrA6*, *uvrB5*, and *uvrC34* cells. No DNA single-strand breaks are detected in UV-irradiated *uvrA6* or *uvrB5* cells, whereas in contrast a significant number of single-strand breaks are detected in both UV-irradiated *uvrC34* and *uvrC56* cells. The number of single-strand breaks in these cells reaches a plateau at 20-J/m^2 irradiation. Since these single-strand breaks can be detected by both alkaline sucrose and neutral formamide-sucrose gradient sedimentation, we concluded that the single-strand breaks observed in UV-irradiated *uvrC* cells are due to phosphodiester bond interruptions in DNA and are not due to apurinic/apyrimidinic sites.

In *Escherichia coli* cells *uvrA*, *uvrB*, and *uvrC* genes are involved in the initial stage of excision repair for DNA damage induced by UV radiation (2, 4, 17). All three genes are required for removal of dimers in vivo. The deficiency of *uvrA*, *uvrB*, and *uvrC* mutants in the repair of dimers can be complemented by T4 or *Micrococcus luteus* UV endonuclease (23). Both T4 and *M. luteus* UV endonucleases are bifunctional monomeric proteins which initiate the repair of dimers by the cleavage of the glycosyl bond of the 5′ pyrimidine moiety of the dimer and subsequent cleavage of the phosphodiester bond 3′ to the apyrimidinic site (1, 5, 11, 15). It is unclear whether the UVRABC enzymes function in vivo in the same fashion as T4 or *M. luteus* UV endonucleases. Seeberg et al (18) have shown that partially purified *uvrA*, *uvrB*, and *uvrC* proteins function collectively to produce single-strand breaks in UV-irradiated DNA. Sancar and Rupp (13) and Yeung et al. (29) recently found that the purified UVRABC proteins collectively excise 6 to 10 nucleotides of dimer-containing sequence and have proposed the term "excinuclease" to designate this particular function (13). Although the *uvrA* (14, 21), *uvrB* (7), and *uvrC* (12) gene products have all been demonstrated to be DNA-binding proteins, the individual roles of these proteins in the removal of dimers in vivo is unclear. The function of the *uvrC* gene product in the removal of dimers in vivo has been controversial. Although Kato (8), Seeberg and Rupp (19), and Sharma and Moses (22) have demonstrated that *uvrC* (but not *uvrA*) mutants are able to incise but cannot remove dimers in vivo, others have argued that the incision which occurs in UV-irradiated *uvrC* may not be dimer specific (20).

We have recently demonstrated that although the *uvrC* gene product appears to function collectively with the *uvrA* and *uvrB* gene products for the repair of UV and UV-like DNA damage, it may function independently in the repair of *N*-(deoxyguanosin-C8-yl)-2-aminofluorene adducts (24). These findings prompted us to reinvestigate the role of the *uvrC* gene in the repair of UV damage in vivo. We transduced two *uvrC* alleles into an *E. coli* C strain. Our alkaline sucrose gradient sedimentation results confirm those of Kato (8), Seeberg and Rupp (19), and Sharma and Moses (22), demonstrating that DNA single-strand breaks do occur in two different UV-irradiated *uvrC* strains, but not in *uvrA* or *uvrB* mutants. To determine whether the single-strand breaks observed in alkaline conditions are due to phosphodiester bond interruptions or to alkaline-labile bonds such as apurinic/apyridiminic (AP) sites, we developed a formamide-sucrose gradient sedimentation technique which denatures the DNA at neutral pH while preserving AP sites (J. Ross and M.-S. Tang, Anal. Biochem., in press). Based on data obtained by using these two techniques, we conclude that the single-strand breaks observed in UV-irradiated *uvrC* cells are not the result of depyrimidination, but are due to the breakage of phosphodiester bonds.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains are shown in Table 1. The *uvrA6* and *uvrB5* markers were introduced into HF4717 (MST1) by P1 transduction (9). The *uvrC56* and *uvrC34* markers were introduced into HF4717 by T4GT7 transduction as described by Wilson et al. (27).

Bacterial growth and UV irradiation. Bacteria were grown in L broth (10 g of tryptone, 5 g of yeast extract, and 15 g of NaCl in 1 liter of water) at 37°C in a shaking water bath overnight and then were diluted (1:500) with fresh medium. At a density of ca. 10^8 cells per ml, the cells were filtered, washed, and suspended in M9 medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 1 g of NH_4Cl, 0.12 g of $MgSO_4$, and 0.011 g of $CaCl_2$ in 1 liter of water) for UV irradiation with a General Electric germicidal lamp. After UV irradiation, the bacterial cells were diluted with M9 medium and plated on LB plates (L broth with 12 g of Bacto-Agar [Difco Laboratories] per liter).

To examine DNA strand breakage, *E. coli* cells were grown exponentially for at least three generations to a concentration of ca. 10^8 cells per ml in M9 medium with deoxyadenosine (200 μg/ml), glucose (0.4%), and Casamino Acids (0.25%). The cells were then labeled with [^{3}H]thymidine (10 μCi/ml) for 1 h, and cold thymidine (100 μg/ml) was added for another 1-h incubation period. The excess [^{3}H]thymidine was removed by filtration, and the cells were resuspended in M9 medium for UV irradiation. After UV irradiation, deoxyadenosine (200 μg/ml), glucose (0.4%), and

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TABLE 1. Bacterial strains

Strain	Repair-associated marker	Other markers	Source
MST1	Wt	*thr-1 leu-6 proA2 his-4 argE3* or *arg-49 lacY1 galK2 rpsL31* or *rpsL154 supE44*; an *E. coli* K-12 × *E. coli* C hybrid; φX sensitive	HF4714; T. Kunkel
MST3	*uvrB5*	Same as MST1 except Gal$^+$	This laboratory
MST8	*uvrC34*	Same as MST1 except His$^+$	This laboratory
MST13	*uvrA6*	Same as MST1 except Arg$^+$	This laboratory
MST14	*uvrC56*	Same as MST1 except His$^+$	This laboratory
SR23	*uvrA6*	*thr-1 leu-6 proA2 his-4 argE3 lacY1 galK2 rpsL31 thyA15 deoB2 ara-14 mtl-1 xyl-5 thi-1 tsx-33 supE44*	N. Sargentini
SR387	*uvrB5*	Same as SR23	N. Sargentini
SR231	*uvrC34*	Same as SR23	N. Sargentini
SR713	wt	Same as SR23	N. Sargentini

Casamino Acids (0.25%) were added to the cell suspension, and the cells were incubated at 37°C in a shaking water bath. These experiments were performed under General Electric golden fluorescent light to avoid photoreactivation.

Alkaline sucrose gradient sedimentation. After different times of incubation at 37°C, 0.2 ml of cells was treated with 0.1 ml of spheroplast mixture (50 mM Tris, 52 mM EDTA [pH 8.5], 15% sucrose, 9 mg of lysozyme per ml) at 0°C for 10 to 15 min. The spheroplasts were layered into 0.2 ml of Sarkosyl detergent mixture (0.5% Sarkosyl, 0.1 N NaOH, 10 mM EDTA, 0.1 M NaCl) which had been layered on top of 4.5 ml of preformed to 5 to 20 alkaline sucrose gradients (0.1 N NaOH, 10 mM EDTA, 0.1 M NaCl). The sedimentations were performed at 3.5×10^4 rpm for 100 min at 18°C in an SW50.1 rotor. Twenty-five fractions were collected from each gradient, the DNA in each fraction was precipitated with trichroloacetic acid (10%) and filtered onto Whatman GF/C filters, and the amount of radioactivity was counted in 3A70 (Research Products International) scintillation fluid. The M_n was calculated according to the method of Tang and Patrick (25), with T4 DNA as a molecular weight standard. The value of $10^8/M_n$ represents the number of breaks per 10^8 atomic mass units of DNA.

Formamide-sucrose gradient sedimentations. Formamide-sucrose solutions (5 and 20% [wt/vol]) were prepared by directly dissolving sucrose in formamide containing 200 mM NaCl and 1 mM disodium EDTA. Sucrose gradients (5 to 20%) were prepared by using a Buchler gradient maker. Eastman Spectro Grade formamide was deionized before use by mixing with 2% (wt/vol) Bio-Rad AG501-X8(D) (20-50 mesh) and then filtered through Whatman no. 1 filter paper. To prevent aggregation of DNA with cellular components in the formamide gradient, nucleoids were prepared by the method of Worcel and Burgi (28) instead of lysing the spheroplasts on the top of the gradient. The nucleoids were prepared as follows. The cells were harvested by centrifugation and washed with M9 medium three times after various periods of incubation at 37°C. The pellets were resuspended to one-half the original volume of M9 medium. Spheroplasts were prepared by the addition of one-third volume of spheroplast mixture (50 mM Tris, 52 mM EDTA [pH 7.0], 15% sucrose, 9 mg of lysozyme per ml) and were incubated for 20

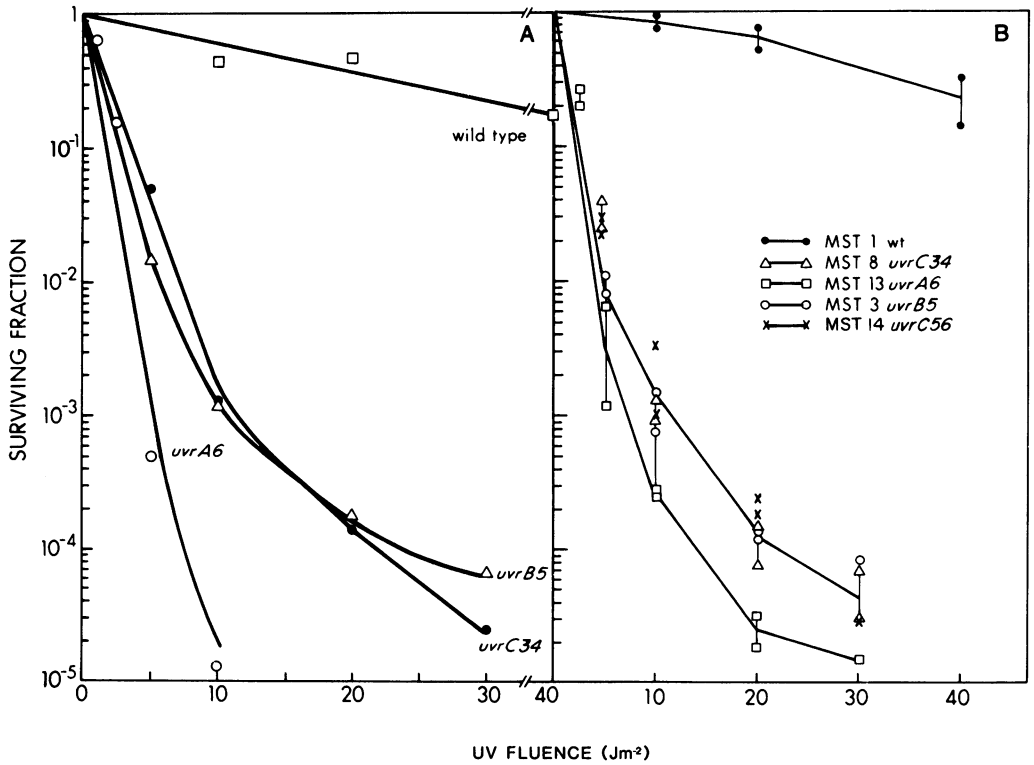

FIG. 1. The UV irradiation survival curves for *E. coli* cells. (A) *E. coli* K-12 wt, *uvrA6*, *uvrB5*, and *uvrC34* cells; (B) *E. coli* C wt, *uvrA6*, *uvrB5*, *uvrC34*, and *uvrC56* cells.

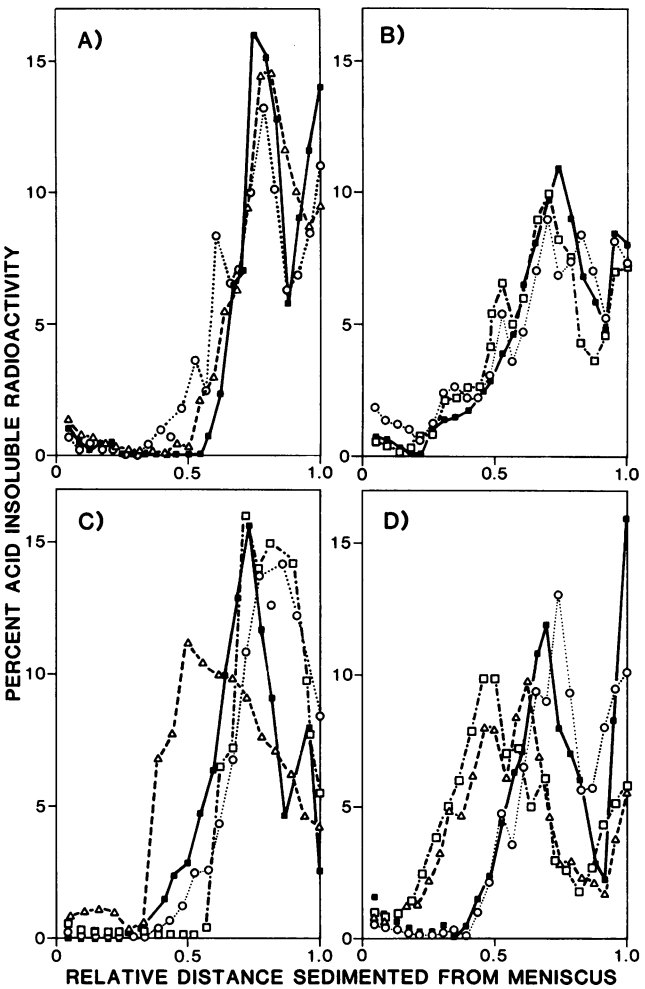

FIG. 2. Typical alkaline sucrose gradient sedimentation profile of UV-irradiated *E. coli* cells after different times of incubation. The spheroplasts were prepared and layered on top of preformed 5 to 20% alkaline sucrose gradients. The gradients wre centrifuged at 3.5 × 10^4 rpm for 100 min in an SW50.1 rotor at 18°C. (A) MST13 *uvrA6* (20 J/m^2); (B) MST3 *uvrB5* (20 J/m^2); (C) MST1 wt (60 J/m^2); (D) MST8 *uvrC34* (20 J/m^2). Symbols: ●, no UV control; ○, UV at zero time incubation; △, UV with 60 min of incubation; and □, UV with 120 min of incubation.

min at room temperature. An equal volume of lysis mixture (1% Brij-58, 0.4% deoxycholic acid, 2 M NaCl, 0.01 M EDTA) was then added to each sample. After the lysis solution was allowed to stand at room temperature for 20 min, the cell debris was pelleted by centrifugation at 13,000 × *g* for 30 s, and the resulting nucleoid suspension was carefully layered onto the gradient. The gradients were centrifuged at 1.6 × 10^4 rpm for the appropriate period of time at 18°C in a Beckman SW50.1 rotor. Methods used for fractionating the gradients, precipitating the DNA, and counting the radioactivity of DNA are the same as those described for the alkaline sucrose gradient sedimentation. The M_n was calculated according to the method of Ross and Tang (in press) by using T4 DNA as a molecular weight standard.

RESULTS

UV sensitivities of *E. coli* K-12 or C cells with mutations in *uvrA6*, *uvrB5*, *uvrC34*, or *uvrC56*. Among the original mutagenized *E. coli* K-12 *uvr* strains, the *uvrA6* cells are significantly more sensitive to UV than *uvrB5* and *uvrC34* cells (Fig. 1A). This observation is consistent with the results obtained by Ogawa et al. (10) and Howard-Flanders et al. (6). Since *uvrA*, *uvrB*, and *uvrC* gene products have been shown to be required for the removal of pyrimidine dimers (6, 10), the higher UV sensitivity of strain SR23 (*uvrA6*) compared with the other strains could be due either to a concealed mutation in the SR23 strain or to leakiness of the *uvrB5* and *uvrC34* mutations. To distinguish between these two possibilities, we transduced these *uvr* markers from the original mutagenized strains into *E. coli* C cells and tested their effects on UV sensitivities in the new background. No significant differences in UV sensitivity were observed among *E. coli* C strains with mutations in *uvrA6*, *uvrB5*, *uvrC34*, or *uvrC56* (Fig. 1B). These results are consistent with a model in which all three *uvr* gene products (UvrA, UvrB, and UvrC) are required for the repair of pyrimidine dimers. These results also suggest that the SR23 (*uvrA6*) cells may have a second unidentified mutation which contributes to the higher UV sensitivity of these cells relative to other Uvr$^-$ strains.

Alkaline sucrose gradient sedimentation. Seeberg (16) and Sancar and Rupp (13) have shown that *uvrA*, *uvrB*, and *uvrC* gene products function collectively in the excision of pyrimidine dimers in vitro, and neither the combination of any two of these gene products nor any single gene product is able to incise UV irradiated DNA. If *uvrA*, *uvrB*, and *uvrC* gene products function in vivo the same way as has been demonstrated in vitro, no incision or removal of pyrimidine dimers would be expected in any of these *uvr* strains after UV irradiation. To test this possibility, the size of DNA in UV-irradiated *uvrA*, *uvrB*, *uvrC*, and wild-type cells was measured by alkaline sucrose gradient sedimentation after

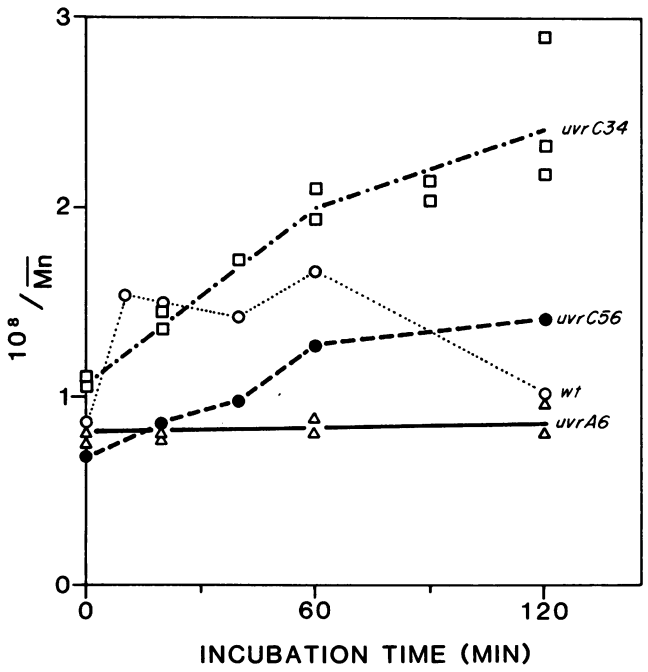

FIG. 3. DNA single-strand breaks measured in UV-irradiated *E. coli* C cells after different times of incubation at 37°C. The molecular weights were calculated by using T4 DNA as a standard. Symbols: □, MST8 *uvrC34* (20 J/m^2); ●, MST14 *uvrC56* (20 J/m^2); △, MST13 *uvrA6* (20 J/m^2); and ○, MST1 wt (60 J/m^2).

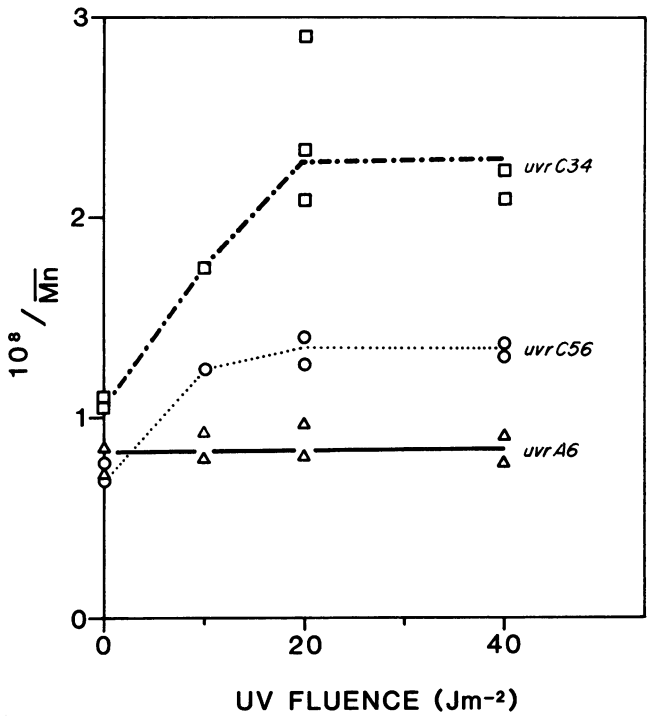

FIG. 4. DNA single-strand breaks measured in *E. coli* cells irradiated with different UV fluences. Cells were UV irradiated and incubated in media for 2 h at 37°C. The method of centrifugation is the same as described in the legend to Fig. 2.

different times of incubation. The typical results of DNA sedimentation analyses are shown in Fig. 2. The size of DNA did not change in UV-irradiated *uvrA* and *uvrB* cells during post-irradiation incubation (Fig. 2A and B). In contrast, the size of DNA decreased progressively during post-irradiation incubation in *uvrC* cells after the same treatment. In wild-type cells, UV irradiation resulted in a decrease in the size of the DNA, which recovered to the original value by 120 min post-irradiation. Changes in DNA molecular weight as a function of post-irradiation incubation times for these mutants are summarized in Fig. 3. The appearance and disappearance of single-strand breaks in UV-irradiated wild-type cells during post-irradiation incubation was presumably due to excision repair of the pyrimidine dimers. Changes in DNA molecular weight as a function of UV fluence after 2 h post-irradiation incubation are shown in Fig. 4. In both UV-irradiated *uvrC34* and *uvrC56* cells, the number of single-strand breaks observed reached a plateau at 20 J/m^2. A similar result has been reported by Seeberg et al. (20).

Formamide-sucrose gradient sedimentation. Since in alkaline sucrose gradients DNA is denatured by high pH, the single-strand breaks observed under these conditions might have resulted either from alkaline-labile AP sites or from preexistent phosphodiester bond interruptions. To differentiate between these two possibilities, we developed a formamide-sucrose gradient sedimentation technique in which the DNA is denatured at neutral pH while the AP sites are preserved (Ross and Tang, in press). If the single-strand breaks observed in the UV-irradiated *uvrC* cells under alkaline sucrose gradient conditions are the result of AP sites, then no single-strand breaks should be observed in these cells under formamide-sucrose gradient sedimentation conditions. However, the size of DNA in UV-irradiated *uvrC34* cells after 2 h of incubation was significantly smaller than that in unirradiated cells (Fig. 5). The molecular weight detected by this method was slightly smaller than that detected under alkaline conditions (Table 2). Therefore, the single-strand breaks detected by alkaline sucrose sedimentation do not arise from alkaline-labile sites.

DISCUSSION

Using both alkaline sucrose and neutral formamide-sucrose gradient sedimentation, we demonstrated that a significant number of single-strand breaks occur in UV-irradiated *uvrC* cells. These single-strand breaks are probably not the result of DNA degradation, since we did not observe any difference in DNA degradation among UV-irradiated *uvrA*, *uvrB*, and *uvrC* cells (data not shown). We have previously shown that alkaline sucrose gradient conditions cause single-strand breaks in DNA containing AP sites and that neutral formamide-sucrose gradients can denature double-stranded DNA while preventing the breakage of phosphodiester bonds at AP sites (Ross and Tang, in press). Therefore, the single-strand breaks we detected in UV-irradiated *uvrC* cells are not due to AP sites. This conclusion is consistent with the finding of Demple and Linn (1) that *E. coli* cells do not excise pyrimidine dimers via a DNA glycosylase. There are at least two possibilities that can account for the generation of single-strand breaks in UV-irradiated *uvrC* cells. One is that these single-strand breaks are the result of incisions at pyrimidine dimers. Grossman et al. (3) have proposed that in *E. coli* cells UVRA and UVRB proteins can incise dimers, and that the incised interruptions can be ligated. The proposed function of the UVRC protein is to prevent these incised interruptions from being ligated back, thus permitting the excision and resynthesis to proceed. Under this hypothesis the single-strand breaks observed in UV-irradiated *uvrC* cells would presumably represent the

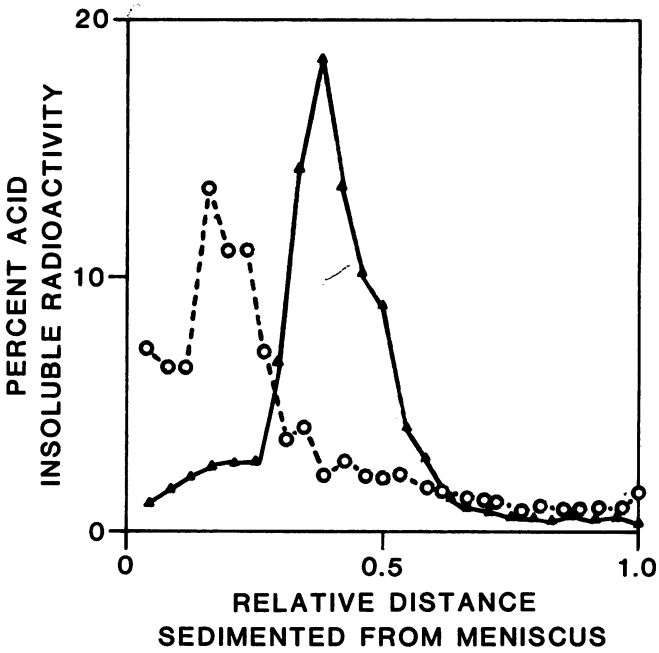

FIG. 5. Typical neutral formamide-sucrose gradient sedimentation profile of MST8 *uvrC34* cells. The preparation of DNA was described in the text. The gradients were centrifuged at 1.6 × 10^4 rpm at 20°C for 6 h in an SW50.1 rotor. Symbols: ▲, unirradiated control; ○, 20 J of UV irradiation per m^2 and 2 h of incubation.

TABLE 2. Comparison of M_ns calculated from alkaline sucrose gradient and formamide-sucrose gradient sedimentation in UV-irradiated (20 J/m^2) *uvrC34* cells after 2 h of incubation

Sucrose gradient	M_n[a] ($\times 10^7$)
Alkaline	4.3, 4.8, 3.4
Formamide	3.2, 3.5

[a] The M_n is calculated according to Veatch and Okada (26) equation $M_n = 0.6\ M_w$. Each value in this column represents a different experiment.

equilibrium between incision and ligation at the dimer sites. Using UvrA, UvrB, and UvrC proteins partially purified from cells containing plasmids encoding *uvrA*, *uvrB*, or *uvrC* genes, Sancar and Rupp (13) and Yeung et al. (29) have demonstrated that UvrA, UvrB, and UvrC proteins together function as an excinuclease, incising a few nucleotides both 5′ and 3′ from the dimer. However, they also have shown that singly, or in any pairwise combination, these proteins do not incise dimers in DNA. To reconcile all of these in vitro observations with our finding that single-strand breaks occur in UV-irradiated *uvrC* cells, one must postulate that the defective UvrC protein can function together with UvrA and UvrB proteins to produce an incision on one side of the dimer only, since no dimer excision has been observed in UV-irradiated *uvrC* cells (6).

In both UV-irradiated *uvrC34* and *uvrC56* cells, the number of single-strand breaks after 2 h reaches a plateau at 20 J/m^2. Due to the uncertainty in determining molecular weight of the *E. coli* chromosome by centrifugation, the number of single-strand breaks cannot be accurately calculated. This plateau level of single-strand breaks could reflect the limited number of defective UvrABC complexes, which bind to the dimer region and incise the phosphodiester bond at one side of the dimer, but are unable to complete the excision and thus are not released from the DNA. As a consequence the number of single-strand breaks produced in UV-irradiated *uvrC* cells would reflect to the total number of defective UvrABC complexes available in the cells. The reason that fewer single-strand breaks were observed in *uvrC56* cells than in *uvrC34* cells after UV treatment is unclear. It is possible that fewer UvrABC complexes are formed in *uvrC56* cells than in *uvrC34* cells due to a difference in the type of mutation in the two strains.

One other possibility which may account for the occurrence of single-strand breaks in UV-irradiated *uvrC* cells is that in *uvrC* cells, the UvrA and UvrB proteins alone may be able to incise photoproducts other than pyrimidine dimers. However, we observed no significant difference in UV sensitivities between *uvrC* cells and *uvrA* cells, despite the fact that *uvrA* (but not *uvrC*) cells are defective in the ability to produce single-strand breaks after UV irradiation. Whatever the mechanism associated with the production of single-strand breaks in UV-irradiated *uvrC* cells, it does not appear to play an important role in UV lethality.

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