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Mechanism of Gap-Filling During Postreplication Repair of Ultraviolet Damage in *Haemophilus influenzae*

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Deoxyribonucleic acid (DNA), pulse labeled after ultraviolet irradiation of excision-defective mutants of *Haemophilus influenzae*, is of lower single strand molecular weight than that of unirradiated cells but approaches the size of DNA from unirradiated cells upon further incubation in growth medium. This gap-filling process is controlled by the *rec-1* gene. Gap-filling occurs normally in a temperature-sensitive DNA synthesis mutant at the restrictive temperature showing that normal semiconservative DNA synthesis is not necessary for gap-filling. To test for recombinational events after irradiation, the DNA synthesized after irradiation was radioactively labeled for a short time in medium containing 5-bromodeoxyuridine followed by incubation for various times in nonradioactive, 5-bromodeoxyuridine-containing medium. The DNA was denatured and analyzed isopycnically. The labeled DNA was initially "heavy," but later shifted toward lighter densities. This shift occurred in the temperature-sensitive DNA synthesis mutant at the restrictive temperature and in the recombination-defective mutant *rec-2*, but was not seen in the *rec-1* mutant. The density shift can be interpreted as evidence that rather extensive exchanges occurred between parental DNA and the DNA made after irradiation. These results suggest that such exchanges are important for gap-filling in *H. influenzae*.

The deoxyribonucleic acid (DNA) synthesized immediately after ultraviolet (UV) irradiation in both bacteria (11, 16) and mammalian cells (3-5, 7, 8, 14, 15) is of lower single-strand molecular weight than that of unirradiated cells. This DNA contains gaps that have been estimated to be about 1,000 nucleotides in size (3, 10, 14). Upon further incubation in appropriate media, these gaps are filled and the size of the DNA approaches that of unirradiated cells. In *Escherichia coli*, postreplication repair is controlled by the *recA* gene product (20) and evidence has been presented that the gaps are filled in by genetic exchanges between sister chromosomes (9, 17). Evidence for gap-filling by de novo synthesis has been obtained with mammalian cells (3, 14).

Two recombination-deficient mutants of *Haemophilus influenzae* have been isolated (18): *rec-1*, which has a residual transformation frequency about 10^{-6} that of the wild type, is UV sensitive and unable to carry out postreplication repair as measured in alkaline sucrose gradients (11); *rec-2*, which has a residual transformation frequency about 10^{-7} that of the wild type, is UV resistant and carries out postreplication repair normally (18). The existence of *rec-2* suggests that the complete machinery for recombination is not necessary for the gap-filling that occurs during postreplication repair and raises the question of whether gap-filling in *H. influenzae* occurs by genetic exchanges or by de novo synthesis.

I find that genetic exchanges do take place and that normal DNA replication is not necessary for gap-filling.

MATERIALS AND METHODS

Microorganisms. *H. influenzae* strains *Rd* (wild type), *rec-1*, and *rec-2* have been described (18). The temperature-sensitive DNA synthesis mutant, *dna-9*, incorporates radioactive thymidine into DNA normally at 37 C but stops within 3 min after transfer to the restrictive temperature, 41 C (13). The excision-defective mutant, *uvr-2*, was obtained by transforming *Rd* with DNA from strain DB116 (19). Construction of the double mutants *dna-9uvr-2* and *rec-luvr-2* have been described (1, 12).

Sedimentation of DNA synthesized after irradiation. The size of the DNA synthesized after irradiation was determined by sedimentation in alkaline sucrose gradients as described previously (11). The 5 and 20% alkaline sucrose contained 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and

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0.2 N NaOH. The DNA of T4 and T7 bacteriophages were used as molecular weight markers.

Determination of genetic exchanges by equilibrium density gradient centrifugation. The general experimental design for detecting UV-induced genetic exchanges is outlined in Fig. 1. The *uvr-2* strain of *H. influenzae* was grown in brain heart infusion (BHI) growth medium in the presence of 0.06 μCi of [^{14}C]-thymidine per ml (49.7 mCi/mmol, Schwarz/Mann) for 90 min at 37 C to a final cell density of 5×10^8 to 8×10^8 cells/ml. The cells were shaken at 37 C for 10 min in nonradioactive BHI to deplete the cells of ^{14}C-labeled precursors. The cells were centrifuged, washed with BHI, and suspended in M9 salts to a concentration of 2.5×10^8 cells/ml. Six milliliters were irradiated in a 9-cm petri dish under a germicidal lamp (fluence of 0.7 J/m^2 per s). The cells were centrifuged, suspended in 1.0 ml of BHI containing 100 μg of 5-bromodeoxyuridine (BrdU) per ml and 50 μCi of [*methyl*-^{3}H]thymidine per ml (14 to 20 Ci/mmol, Schwarz/Mann), and incubated at 37 C for 10 min. The cells were centrifuged, washed once with 2 ml of BHI containing 100 μg of BrdU, resuspended in 1 ml of BHI containing 100 μg of BrdU per ml, and incubated at 37 C. At various times, 0.25 ml was removed, centrifuged, and suspended in 0.3 ml SSC (0.15 M NaCl-0.015 M sodium citrate) containing 0.5% Sarkosyl. The cells were lysed, and the DNA was denatured by heating at 100 C for 8 min followed by cooling on ice. The denatured samples were diluted to 0.7 ml with SSC and mixed with 1.5 ml of CsCl of refractive index 1.4004 and layered over 1.87 ml of CsCl of refractive index 1.4162. The tubes were filled with paraffin oil and centrifuged for at least 17 h at 32,000 rpm in a SW50.1 rotor at 20 C. Fifty to sixty fractions were collected on paper strips and processed as described (6). The DNA had an weight average molecular weight of about 5×10^6 after denaturation.

RESULTS

Detection of UV light-induced chromosomal exchanges. The experimental design to detect UV-induced chromosomal exchanges is outlined in Fig. 1 and described in Materials and Methods. The DNA synthesized before irradiation was of light density and labeled with ^{14}C. The DNA pulse labeled after irradiation was labeled with ^{3}H and banded at a heavy density. If the gaps are filled in by a process of recombination, one should see a shift of the ^{3}H toward a lighter density during the subsequent chase. However, if the gaps are filled by de novo synthesis then no density shift would be expected because BrdU is still present during the chase period. Figure 2 shows the result of such an experiment. Figure 2A and C represent an unirradiated control. After a 5-min pulse, the newly synthesized [^{3}H]DNA is well separated from the light-density [^{14}C]DNA synthesized before irradiation. Removal of the [^{3}H]deoxyribosylthymine and incubation for 40 min in the presence of cold BrdU-containing medium results in no significant shift in density of the DNA. Figure 2B and D are the results obtained from cells irradiated with 2.8 J/m^2 of 254-nm light. After a 10-min pulse, the newly synthesized DNA is again clearly separated from the light [^{14}C]DNA. However, during the 40-min chase in cold BrdU-containing medium, a significant shift in density occurs. Incubation for 60 and 80 min results in no further change in density from the 40-min incubation. The same shift occurs if alkali rather than heat is used to denature the DNA.

Figure 3 shows that the amount of density shift increases with the dose of UV up to 2.8 J/m^2.

Strand joining and DNA density shift in a temperature-sensitive DNA synthesis mutant. To determine whether normal semiconservative DNA replication is required for the density shift seen in irradiated cells, a mutant temperature sensitive for DNA synthesis was used. In addition to the *dna-9* mutation, the

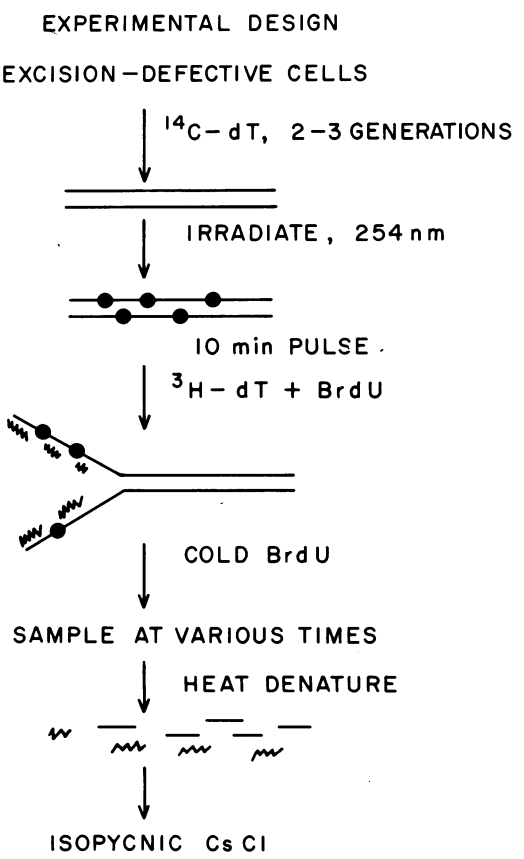

FIG. 1. *Experimental procedure for measuring UV-induced chromosomal exchanges.*

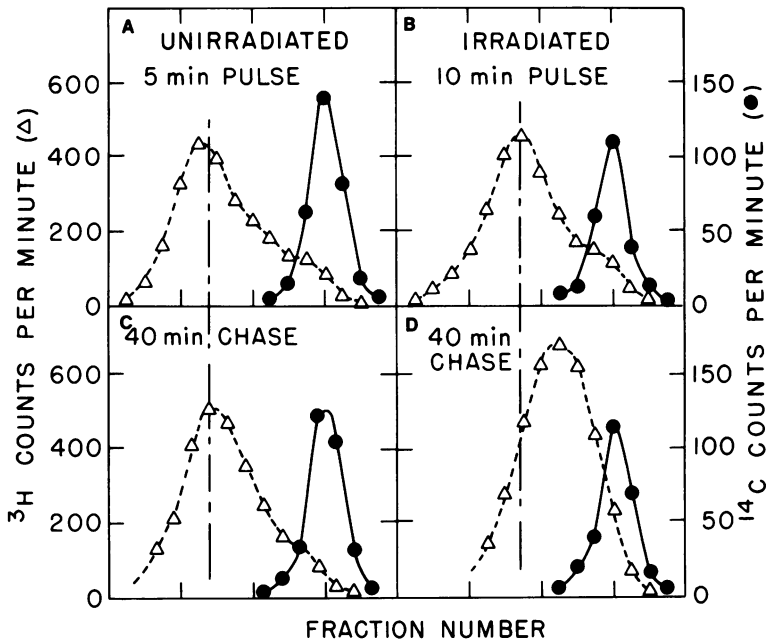

FIG. 2. *Distribution in CsCl density gradients of ^{14}C-labeled light DNA and DNA pulse labeled with ^{3}H after UV irradiation of uvr-2 cells. The experimental procedure was as outlined in Fig. 1. The cells were irradiated with a dose of 2.8 J/m^2. The fraction numbers were normalized and plotted so that the ^{14}C peaks were aligned.*

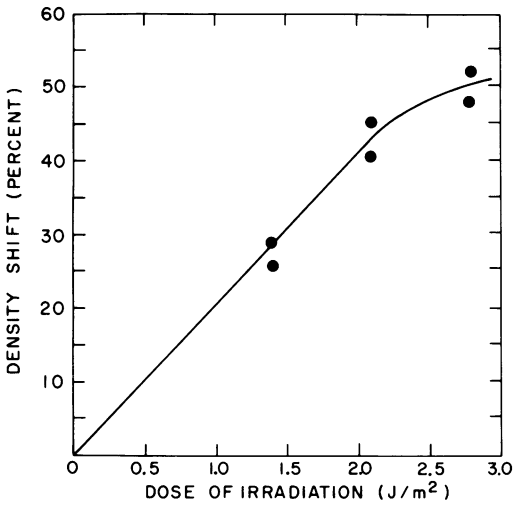

FIG. 3. *Dose dependence of the density shift observed for the DNA pulse labeled after UV irradiation. The experimental procedure was as outlined in Fig. 1 using the excision-defective (uvr-2) strain. The chase was for 40 min. The results are expressed as a percentage of the distance between the [^{3}H]DNA synthesized during a 10-min pulse after irradiation and the light density [^{14}C]DNA.*

cells also carried the *uvr-2* mutation making them excision defective. Figure 4 shows the results of an experiment to determine if strand joining, as measured in alkaline sucrose gradients, is normal in these cells at the restrictive temperature. The cells were pulse labeled for 10 min in the presence of [^{3}H]deoxyribosylthymine plus 100 μg of BrdU per ml and then chased for 40 min in nonradioactive BrdU-containing medium at either 37 C or 41 C. Clearly, strand joining occurs as well at 41 C (in fact somewhat faster) as at 37 C even though semiconservative DNA synthesis is inhibited at the higher temperature.

Figure 5 shows the distribution of radioactivity on isopycnic CsCl density gradients of the DNA from a pulse-chase experiment like that described in Fig. 1 with the *dna-9uvr-2* double mutant. The top two panels show the distribution of radioactivity in the DNA after a 10-min pulse at 37 C. The bottom left and right panels show the distribution of radioactivity after a 40-min chase in cold BrdU-containing medium at 37 and 41 C, respectively. In both cases, the DNA synthesized after irradiation undergoes a significant shift toward a lighter density. Thus, semiconservative DNA replication is not required for the density shift shown by the DNA synthesized after irradiation.

DNA density shift in recombination defective mutants. Figure 6 shows the distribution of radioactivity on isopycnic CsCl density gradients of the DNA from a pulse-chase experiment

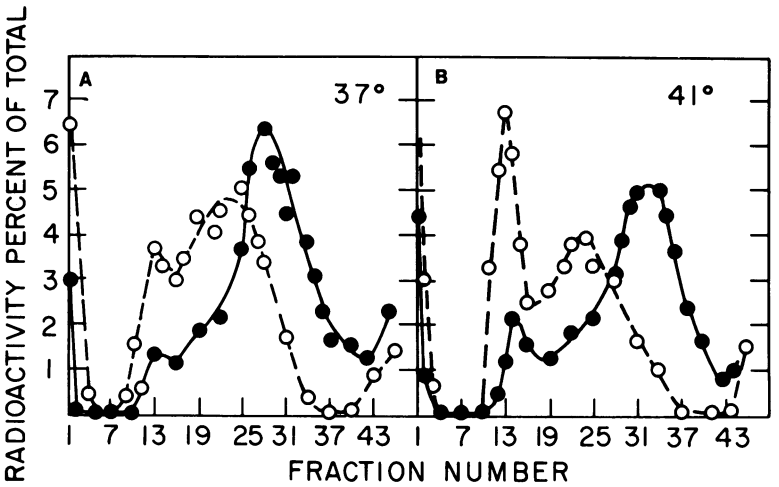

FIG. 4. *Distribution of radioactivity on an alkaline sucrose gradient of DNA pulse labeled after UV irradiation and chased at the permissive and restrictive temperatures in a temperature-sensitive DNA synthesis mutant. The double mutant dna-9uvr-2 was irradiated with a dose of 2.1 J/m² and pulse labeled for 10 min at 37 C (●). (A) The radioactivity was removed, and half the culture was incubated in nonradioactive BHI growth medium for 40 min at 37 C (○), and (B) half at 41 C (○). Sedimentation was from right to left.*

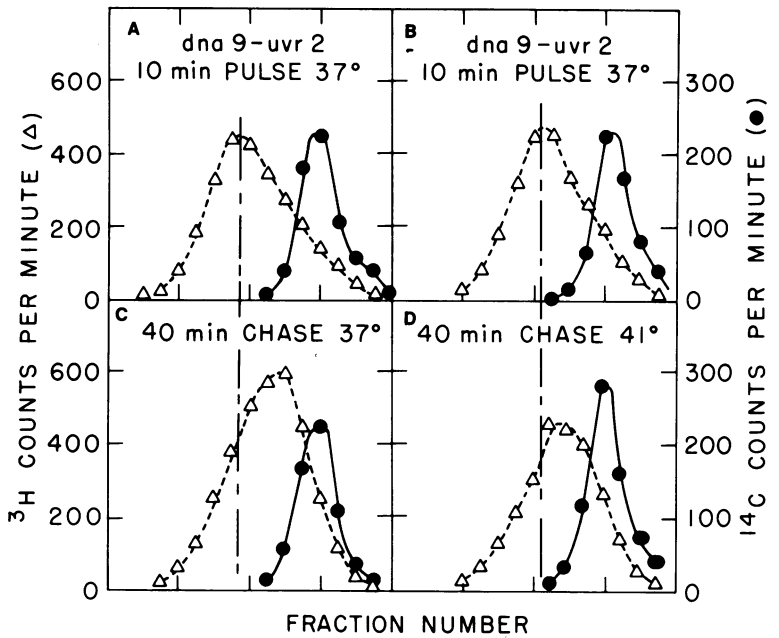

FIG. 5. *Density shift of the DNA pulse labeled after UV irradiation and chased at the permissive and restrictive temperatures in a temperature-sensitive DNA synthesis mutant. The experimental procedure was as outlined in Fig. 1, except the dna-9uvr-2 double mutant was used and the chase was for 40 min at either 37 C (C) or at 41 C (D). The cells were irradiated with a dose of 2.1 J/m². The fraction numbers were normalized and plotted so that the ¹⁴C peaks were aligned. Sedimentation was from right to left.*

as described in Fig. 1 with the two recombination deficient mutants *rec-1* and *rec-2*. Since a strain carrying the genes for *rec-2* that is also defective in the excision of pyrimidine dimers has not been constructed, strains of *rec-1* and *rec-2* that are excision proficient were used. No significant density shift is observed after the 40-min chase in cold BrdU-containing medium with the *rec-1* strain (Fig. 6C), which is unable to carry out postreplication repair. The same

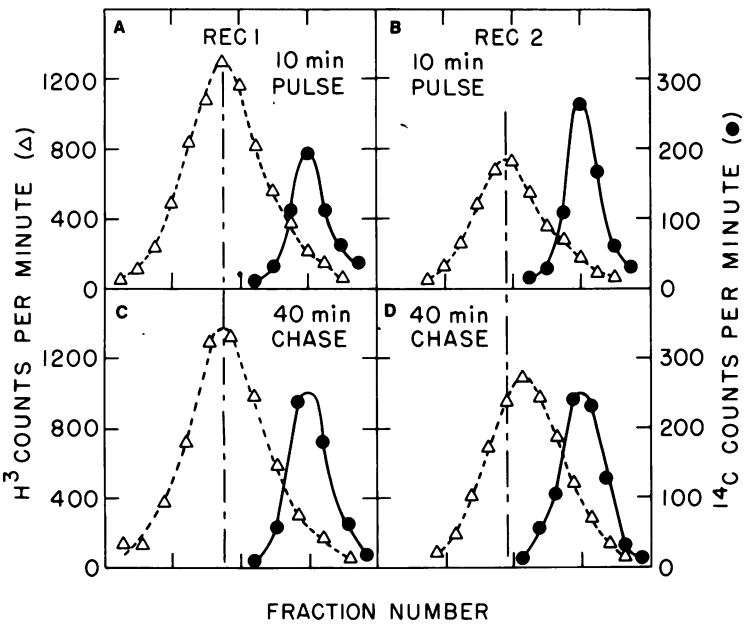

FIG. 6. *Distribution of CsCl density gradients of ^{14}C-labeled light DNA and DNA pulse labeled with ^{3}H after UV irradiation of rec-1 and rec-2 cells. The experimental procedure was as outlined in Fig. 1. The cells were irradiated with a dose of 4.2 J/m². The fraction numbers were normalized and plotted so that the ^{14}C peaks were aligned. Sedimentation was from right to left.*

experiment was done using the *rec-luvr-2* double mutant with similar results (data not shown). This result strengthens the idea that the density shift that is observed in other strains is indeed the result of genetic recombination between sister strands. Figure 6D shows that with *rec-2* there is a significant shift in density after the 40-min chase.

DISCUSSION

DNA that is pulse labeled in the presence of BrdU after UV irradiation can be clearly separated on isopycnic CsCl density gradients from the DNA made before irradiation. Upon subsequent chase in nonradioactive medium containing BrdU, the density of the DNA made during the pulse shifts toward a lighter density. The shift is approximately proportional to the dose of UV light at low doses. It is interpreted to be the result of recombinational events which result in covalent linkage of light parental DNA to the heavy DNA made after irradiation. The strongest support for this interpretation is the finding that *rec-1* (defective in gap joining) does not exhibit any shift in density during the chase period.

The *rec-2* strain is even more deficient in recombination, as measured by transformation frequencies than is the *rec-1* strain; yet the *rec-2* strain carries out the exchanges between parental DNA and the DNA made after irradiation in a normal fashion. The defect in the *rec-2* strain is believed to be the inability of competent cells to make DNA containing single-stranded tails which presumably is the locus of interaction with transforming DNA (13). The DNA that is made after UV irradiation contains gaps which would presumably be the locus of interaction with its sister strand. Such gaps have been shown to be "aggressive intermediates" in the recombination of bacteriophage ϕX174 (2).

Rupp et al. (17) calculated that in *E. coli* there occurred one exchange for every one or two pyrimidine dimers in the DNA. The following quantitative considerations led to the conclusion that in *H. influenzae* there are many more exchanges than pyrimidine dimers. *Haemophilus* cells irradiated with a dose of 2.8 J/m² synthesize DNA with a number average molecular weight of about 10×10^6 during the 10-min pulse labeling. Now, if the gaps opposite the pyrimidine dimers are the same size as found in *E. coli*, namely about 1,000 nucleotides, then an exchange of a piece of DNA of 5×10^5 or even 10^6 daltons should change the density of the DNA made after irradiation only 5 to 10% compared to the 40 to 50% that is observed. Since no significant breakdown of the DNA synthesized

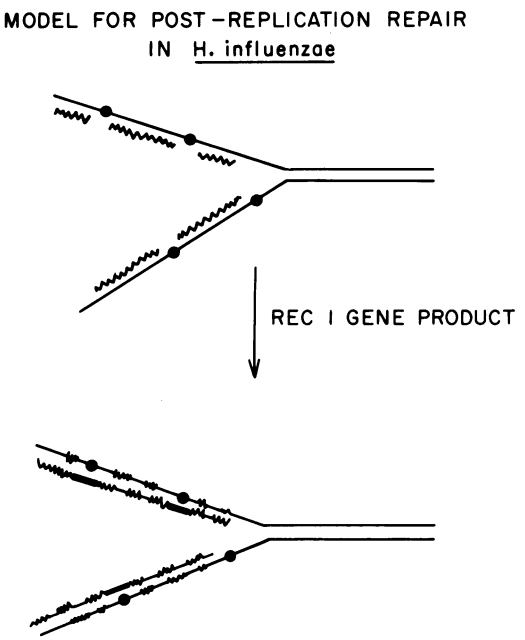

FIG. 7. *Model for postreplication repair in H. influenzae. The straight line represents the ^{14}C-labeled DNA, the circles represent pyrimidine dimers and the wavy line represents DNA pulse labeled with ^{3}H in the presence of BrdU after irradiation.*

either before or after irradiation is observed during the chase period, it is unlikely that the gaps are greatly enlarged. Thus, it would appear that a considerable number of exchanges occur in addition to the site of the gap (Fig. 7).

An attempt to estimate the size of the DNA exchanged by shearing the DNA before analysis on CsCl gradients was unsuccessful as shearing to a molecular weight of about 6×10^5 yielded a diffuse smear of radioactivity instead of a defined peak. This was true of the DNA made in unirradiated as well as irradiated cells. This phenomenon is presumably due to the heterogeneity in base composition along the *H. influenzae* chromosome.

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