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UV-Induced Mutation in Bacteriophage T4

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Two late gene *am* mutants of bacteriophage T4 that can be induced to revert by UV were crossed to a temperature-sensitive ligase mutant. In the double mutants, UV-induced reversion was eliminated at a semirestrictive temperature. When the single *am* mutants were irradiated and then allowed a single passage in a permissive host, the UV-induced reversion frequency was increased by 15- to 25-fold. This increased mutagenesis was also abolished by the presence of the ligase allele. When the UV-irradiated single *am* mutants multiply infected a permissive host, allowing multiplicity reactivation to occur, the induced reversion frequency was reduced similarly to the reduction in lethality. The mutagenesis that remained was again abolished by the presence of the ligase allele. It is concluded that UV induces mutations in phage T4 through the action of a pathway that includes polynucleotide ligase. The increase in mutation frequency after growth in a permissive host implies that mutagenesis can occur at more than one stage of the infection rather than only in an early stage before expression of the mutant genome. The process of multiplicity reactivation appears to be error-free since it overcomes lethal lesions without inducing new mutations.

Error-prone repair (EPR) is a mechanism that mediates the conversion of DNA lesions to mutations. Evidence has been accumulating that UV induces mutations through EPR in *Escherichia coli* and other organisms (for review, see 13, 42). In phage T4, thymine dimers produced in DNA by UV irradiation can be rendered nonmutagenic by exposure to photoreactivating light (18, 32), indicating that dimers are premutational lesions. The transition mutations that arise from non-photoreactivated UV lesions are mainly G-C to A-T transitions and thus do not occur at the site of the thymine dimer (32). This implicates EPR in UV mutation induction in phage T4. The gene functions required for EPR can be defined by mutants that have increased sensitivity to lethal agents and reduced or eliminated inducible mutagenesis. Mutations in genes *x*, *y*, and 1206 of phage T4 are UV sensitive and reduce UV mutagenesis (22). In a similar manner, UV-sensitive mutants of other organisms show reduced or abolished UV mutagenesis. In phage λ, UV mutagenesis is reduced by the host *recA* and *lexA* mutations and by the phage *red* mutation (11, 15). In *E. coli*, *recA*, *lexA*, and *polC* mutants have reduced mutagenesis (8, 9, 25, 34, 41, 42). In *Bacillus subtilis*, mutants showing reduced UV mutagenesis are HA106, HA118, and HA101/17 (23). In yeast, *rev1*, *rev2*, *rad6*, *rad8*, *rad9*, and *rad18* mutants show this effect (3, 26-28, 37). In *Neurospora*, *uvs* and *upr* mutations (16), and in *A. nidulans*, the *uvs* mutants (12) have reduced UV mutagenesis. The enzymatic defects in most of these mutants remain unknown. The broad range of organisms in which such mutants have been isolated, however, implies that UV mutagenesis through EPR is probably a general mechanism. A knowledge of the enzymatic steps of EPR seems critical to understanding the mutagenic effects of UV.

The results presented here implicate polynucleotide ligase in the EPR of UV-induced lesions in phage T4. The temperature-sensitive ligase allele used, *tsB20*, has been previously shown to be UV sensitive (4) and to be defective in the conversion of nitrous acid-induced lesions to mutations (7). The experiments reported here show that under varying conditions of growth and multiplicity of infection (MOI), this allele eliminated UV-induced reversion of *am* mutations.

MATERIALS AND METHODS

Phage and bacteria. Phage mutants are denoted by their allele designation followed by the gene number in parentheses. Two amber mutants defective in late function genes were used. *am*N52(37) is from the California Institute of Technology collection and is defective in tail fiber formation; *am*S1(51) is a spontaneous mutant isolated in this laboratory and is defective in the central plug. *E. coli* CR63 (*su-1*$^+$) was used as the permissive host, and *E. coli* S/6/5 (*su*$^-$) was the restrictive host. The two *am* mutants were crossed to the temperature-sensitive ligase allele *tsB20*(30), from the California Institute of Technology collection, and double mutants were isolated on the basis of their inability to grow on *E. coli* CR63 (*su*$^+$)

at 40°C or on *E. coli* S/6/5 at 25°C, but their ability to grow on *E. coli* CR63 at 25°C. The identities of the *am* mutations in the double mutants were confirmed by complementation tests against the parent *am* mutants and, in the case of *am*N52(37)*tsB*20(30), by complementation tests against other gene 37 mutants as well. The identity of the *tsB*20(30) allele in the double mutant was confirmed by complementation tests against the original *tsB*20(30) parent.

Media. Hershey broth (38) was used as the liquid growth medium. Phage stocks were routinely centrifuged and resuspended in adsorption medium consisting of (in grams per liter of distilled water): NH_4Cl, 1.0 g; KH_2PO_4, 3.0 g; and Na_2HPO_4, 6.0 g. Adsorption medium was used for adsorption in single-passage and multiple-infection experiments. The phage were diluted into a solution consisting of 0.1 M NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$ for UV treatment. Growth of phage on agar plates was by the agar overlay method (1).

UV treatment. Before irradiation, the phage were adjusted to titers of 2×10^{10}/ml for multiple-infection, 2×10^8/ml for single-infection, and 2×10^9/ml for direct plating experiments. Three milliliters of the diluted phage was placed in a plastic petri dish for each dose in each experiment. A 15-W General Electric G15T8 germicidal lamp was used at a distance of 40 cm, delivering either 13 ergs/mm^2 per s at full lamp or 1 erg/mm^2 per s with a 4.5- by 8.5-cm aperture. The petri dish was placed under the lamp, vigorously shaken during exposure, and immediately removed after the appropriate dose.

Direct plating. The phage were immediately diluted after UV treatment, plated with *E. coli* CR63, and incubated in the dark at 25 or 31°C, to determine survival of total phage. To determine survival of *am*$^+$ revertants, samples were also plated with *E. coli* S/6/5 and incubated in the dark at 25 or 31°C. High concentrations of *E. coli* S/6/5 were used to ensure low MOIs (<0.05) in order to avoid multiplicity reactivation (MR) on the plate. These precautions should be sufficient to avoid any complications of MR (5, 7). Because mutagenic events will occur during the first cycle of growth on the plates, the plates used in the *am tsB*20(30) double-mutant experiments to test the effect of the *ts* allele on reversion were prewarmed to 31°C overnight when growth was to be at 31°C.

Single passage: single and multiple infections. The phage, after UV treatment, were added to *E. coli* CR63 suspended at 2 to 6×10^8 cells/ml and starved for 30 min in adsorption medium. After 10 min of adsorption at 31°C, the infected cells were separated from the unadsorbed phage by centrifugation at 5,000 rpm for 10 min, resuspended in adsorption medium, and then diluted to 2 to 6×10^7 cells/ml in Hershey broth. The infected cells were incubated in the dark at 31°C for 60 min. Initial cell concentrations during adsorption higher than 6×10^8 cells/ml and subsequent incubation of cells at concentrations higher than 6×10^7 resulted in low burst sizes and elevated spontaneous reversion rates. Therefore, high cell concentrations were avoided. The number of infecting phage was adjusted to achieve an MOI of <0.05 for single infections and >2 for multiple infections. These precautions should be sufficient to avoid MR in the growth step of the single-infection experiments (5, 7). After incubation, chloroform was added and the phage were diluted and plated on *E. coli* CR63 for total phage survivors and on *E. coli* S/6/5 for revertant survivors.

Calculation of frequency of induced revertants. For each dose of UV, the frequency of newly induced revertants was taken as the frequency of revertants in the population minus the initial spontaneous frequency of revertants measured in the same experiment. In all cases, experiments were repeated at least once, and the frequencies of induced revertants obtained in the parallel experiments at a given dose were averaged.

RESULTS

Direct-plating mutagenesis. Mutants *amS*1(51) and *amN*52(37) were selected for this study because of their low spontaneous reversion frequency combined with their high UV reversion frequency per lethal hit. Figure 1 shows the survival of these two mutants as a function of dose. The curve shows a small shoulder at low doses, as has been reported earlier (32). These two *am* mutants were then crossed with the temperature-sensitive ligase mutant *tsB*20(30), and the double mutants *amN*52(37)*tsB*20(30) and *amS*1(51)*tsB*20(30) were isolated. Baldy (4) had shown that this ligase mutant had progressively increasing sensitivity to UV at increasing growth temperatures when *E. coli* S/6/5 was used as the host, suggesting that ligase is involved in the repair of UV lesions. However, at 31°C, *am tsB*20(30) double mutants, grown on

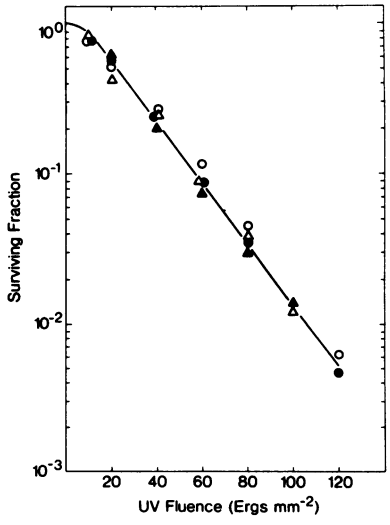

FIG. 1. *Survival of single and double mutants as a function of UV dose at 31°C. Single infection (MOI, <0.05). The strain, the symbol, and the number of experiments used in obtaining each set of points are: amN52(37), ●, 2; amN52(37)tsB20(30), ▲, 2; amS1(51), ○, 3; amS1(51)tsB20(30), △, 2.*

E. coli CR63 as the host bacterium, did not have measurably greater sensitivity than the *am* mutants alone (Fig. 1). This may be related to the fact that *tsB*20(30) mutants show a much greater temperature sensitivity on *E. coli* S/6/5 than they do on *E. coli* CR63 (C. Bernstein, personal communication).

At 31°C, UV-induced mutagenesis, as determined by direct plating, was eliminated by the ligase deficiency. In Fig. 2a, the frequencies of newly induced revertants of *amN*52(37) to am^+ and of *amN*52(37)*tsB*20(30) to am^+ *tsB*20(30) are compared. The *amN*52(37) mutation alone underwent a clear rise in reversion with UV dose. However, in the presence of the *tsB*20(30) allele, the UV-induced mutagenesis was abolished. In Fig. 2b, similar results are shown for the other *am* mutation. The single mutant *amS*1(51) could be induced to revert by UV. The ligase allele again eliminated UV mutagenesis in the double mutant.

Figure 2c shows the frequency of newly induced revertants in the *am tsB*20(30) double mutants at 25°C. *amN*52(37)*tsB*20(30) failed to show UV-induced mutagenesis at 25°C, whereas *amS*1(51)*tsB*20(30) did show a complete restoration of induced mutagenesis at this semipermissive temperature. The reason for this difference is not understood. These results, however, are similar to those reported for nitrous acid-induced mutagenesis (7). *amS*1(51)-*tsB*20(30) showed no nitrous acid-induced mutagenesis at 31°C and restored induced mutagenesis at 25°C. Another *am tsB*20(30) double mutant showed no induced mutagenesis at 31°C and only a very low level of mutagenesis at 25°C.

Single-passage mutagenesis. In the direct-plating assay for revertants, the irradiated phage were allowed to immediately infect the plating indicators *E. coli* CR63 and *E. coli* S/6/5. In the single-passage technique for measuring reversion frequency, the irradiated phage were allowed 60 min of growth at 31°C in the permissive host *E. coli* CR63 at low MOI (<0.05) before they were plated on the indicators *E. coli* CR63 and *E. coli* S/6/5. This passage increased the frequency of newly induced revertants detected (Fig. 3). The reversion frequency of *amN*52(37) was much greater when measured after a single passage than after direct plating (Fig. 3a). The *amS*1(51) mutant also showed a higher frequency of newly induced revertants after single passage than after direct plating (Fig. 3b). The increase was considerable: for *amN*52(37), the

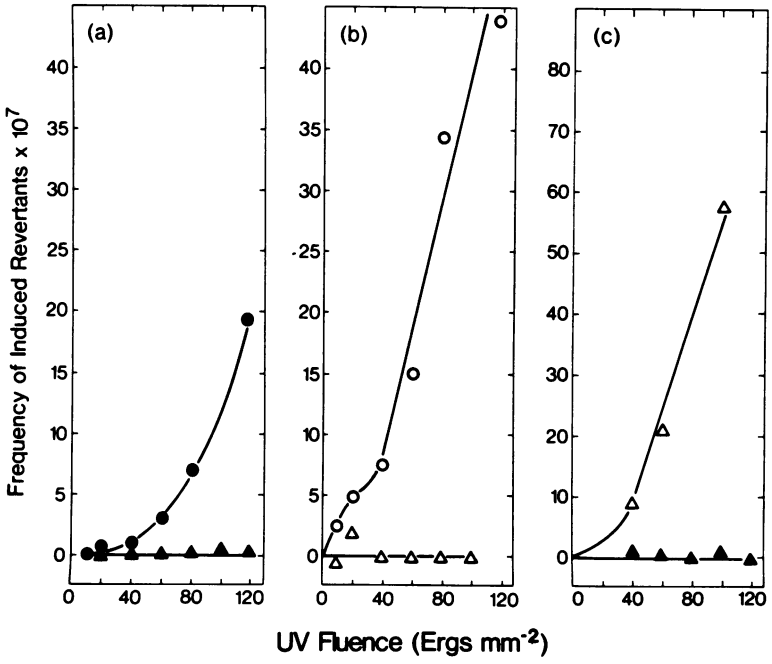

FIG. 2. *Frequency of newly induced revertants in single and double mutants as a function of UV dose. Assay by direct plating. Single infection (MOI, <0.05). The strain, the symbol, the number of experiments used in obtaining each set of points, and the temperatures at which growth occurred are: (a) amN52(37), ●, 2, 31°C; amN52(37)tsB20(30), ▲, 2, 31°C. (b) amS1(51), ○, 3, 31°C; amS1(51)tsB20(30), △, 2, 31°C. (c) amN52(37)tsB20(30), ▲, 2, 25°C; amS1(51)tsB20(30), △, 2, 25°C.*

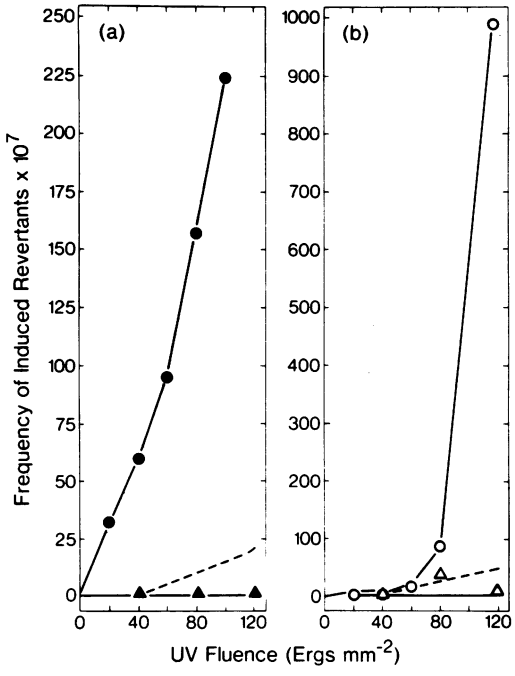

FIG. 3. *Frequency of newly induced revertants in single and double mutants as a function of UV dose. Assayed after passage of the phage through E. coli CR63 at 31°C. Single infection (MOI, <0.1). Dashed lines represent frequency of newly induced revertants observed after direct plating, taken from Fig. 2. The strain, the symbol, and the number of experiments used in obtaining each set of points are: (a) amN52(37), ●, 3; amN52(37)tsB20(30), ▲, 2. (b) amS1(51), ○, 2; amS1(51)tsB20(30), △, 2.*

frequency of newly induced revertants rose 15-fold with passage (from 15×10^{-7} to 225×10^{-7} at 100 ergs/mm^2); for *amS*1(51), the frequency of newly induced revertants rose 25-fold with passage (from 40×10^{-7} to $1{,}000 \times 10^{-7}$ at 120 ergs/mm^2) (Fig. 3). These frequencies of newly induced revertants were easy to measure, since the background frequency of spontaneous revertants averaged 6×10^{-7} for *amN*52(37) and 10×10^{-7} for *amS*1(51).

The *tsB*20(30) allele was effective in eliminating the UV mutagenesis measured by the single-passage method. Even at high doses, the *amN*52(37)*tsB*20(30) double mutant had no detectable UV mutagenesis (Fig. 3a). The same result was obtained for the *amS*1(51)*tsB*20(30) double mutant (Fig. 3b).

Multiple-infection mutagenesis. In the single-passage experiments, the phage were allowed to singly infect the permissive host *E. coli* CR63, and the progeny of this infection were plated on *E. coli* CR63 and *E. coli* S/6/5. Under conditions where more than one irradiated phage simultaneously infect a bacterium, MR occurs and the two genomes interact to efficiently eliminate lesions that would be lethal in a single infection (e.g., 19, 30). In Fig. 4a the survivals of *amN*52(37) and *amN*52(37)*tsB*20(30) are compared in single and multiple infections. The multiple-infection survival curve showed much higher values at all doses. One measure of the efficiency of MR is the ratio of the final slope of the multiple-infection curve compared with the final slope of the single-infection curve. In these experiments, the multiple-infection curve had a final slope one-seventh that of the single-infection curve. MR shown by the double mutant containing *tsB*20(30) is about the same as MR of the single *am* mutant and may in fact show an MR slope even less steep than that of the single *am* mutant. Thus, as in the case of MR of nitrous acid-induced lesions (35), the *tsB*20(30) defect in ligase does not decrease MR of UV-induced lesions. Figure 4b shows MR for *amS*1(51) and its double mutant. Here the multiple-infection survival curve had a final slope one-sixth that of the single-infection curve. Again, the presence of the *tsB*20(30) ligase allele did not decrease MR of UV-induced lesions.

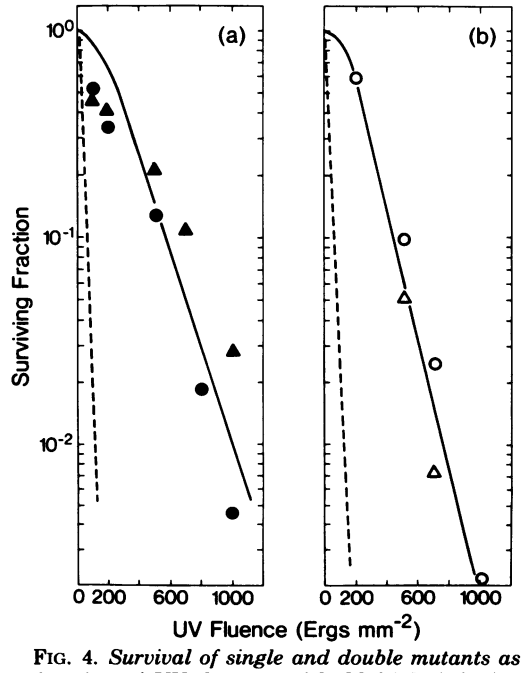

FIG. 4. *Survival of single and double mutants as a function of UV dose at 31°C. Multiple infection (MOI, >2). Dashed lines represent survival in single infection (MOI, <0.05), taken from Fig. 1. The strain, the symbol, and the number of experiments used in obtaining each set of points are: (a) amN52(37), ●, 5; amN52(37)tsB20(30), ▲, 4. (b) amS1(51), ○, 2; amS1(51)tsB20(30), △, 2.*

The induction of mutations can be followed in phage that have been repaired by MR. In Fig. 5a, the frequency of newly induced revertants is compared per unit dose after passage in *E. coli* CR63 for *amN*52(37) in multiple and single infections. MR reduced mutation induction per unit dose. Figure 5b shows similar results for *amS*1(51). The frequency of newly induced revertants after multiple infection was lower than after single infection per unit dose.

The *tsB*20(30) allele could eliminate the UV mutagenesis that occurred during MR. The *amN*52(37)*tsB*20(30) double mutant showed no UV-induced reversion among progeny of multiply infected cells (Fig. 5a). Similarly, no UV-induced reversion to am^+ was found in progeny of multiple infections of UV-treated *amS*1(51)*tsB*20(30) phage (Fig. 5b).

These comparisons between single- and multiple-infection reversion frequencies were made per unit dose of UV. However, because MR is an efficient mode of lethal lesion repair, the level of survival was much higher per unit dose among the progeny of a multiple infection compared with a single infection. In Fig. 6a, the reversion frequencies of *amN*52(37) in single and multiple infections are compared per log of the surviving fraction. Progeny of multiple infections showed a significantly lower frequency of newly induced revertants than did progeny of single infections at equal survival levels. In Fig. 6b, *amS*1(51) also showed that the frequency of newly induced revertants among the progeny of multiple infections was consistently lower than the frequency of newly induced revertants among progeny of a single infection at equal levels of survival.

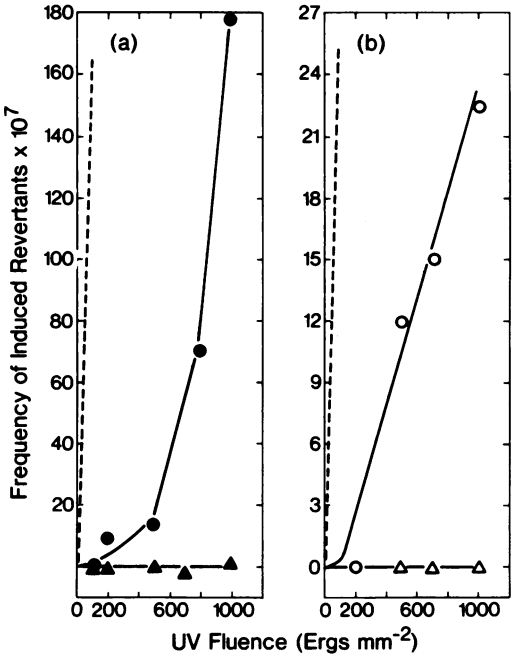

FIG. 5. *Frequency of newly induced revertants in single and double mutants as a function of UV dose. Assayed after multiple infection (MOI, >2) at 31°C. Dashed lines represent frequency of newly induced revertants observed in single passage with single infection, taken from Fig. 3. The strain, the symbol, and the number of experiments used in obtaining each set of points are: (a) amN52(37), ●, 5; amN52(37)tsB20(30), ▲, 4. (b) amS1(51), ○, 2; amS1(51)tsB20(30), △, 2.*

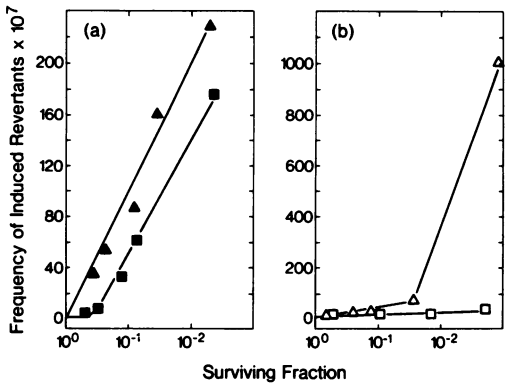

FIG. 6. *Frequency of newly induced revertants in single mutants as a function of surviving fraction. Assayed after single (MOI, <0.1) and multiple (MOI, >2) infections at 31°C. The strain, the condition of infection, the symbol, and the number of experiments averaged are: (a) amN52(37), single infection, ▲, 3; amN52(37), multiple infection, ■, 5. (b) amS1(51), single infection, △, 2; amS1(51), multiple infection, □, 2.*

DISCUSSION

Ligase in EPR pathway. The elimination of UV-induced mutagenesis in the presence of *tsB*20(30) at a semirestrictive temperature suggests that polynucleotide ligase is involved in the EPR pathway of repair of thymine dimers in phage T4. This finding supports previous work on phage T4 which implicated EPR in UV mutagenesis (22) and the gene 30 ligase in nitrous acid mutagenesis (7). The ligase deficiency eliminated mutagenesis under direct-plating, single-passage, and multiple-infection conditions. Because all measurable UV-induced reversion is eliminated in the presence of the *tsB*20(30) ligase allele, no other mechanism (in which the ligase is not involved) appears to contribute significantly to UV mutagenesis.

Although the two late gene mutants, *amN*52(37) and *amS*1(51), behaved similarly in general, they showed somewhat different kinetics of revertant induction. The largest difference was shown in the single-passage experiments (Fig. 3), where *amN*52(37) showed an accumu-

lation of UV-induced revertants at low doses, whereas *amS1*(51) showed few induced revertants until after a 40-erg/mm^2 exposure. These variations suggest that the neighborhood of DNA in which the premutational lesion occurs influences the ability of EPR to convert the lesion to a mutation.

UV-induced mutations are eliminated by the presence of the *tsB*20(30) allele at 31°C, whereas the relative survival after UV irradiation is not noticeably affected. This may be due to action of the EPR pathway on only a small fraction of UV-induced lesions, substitution of another, more accurate repair pathway, or modification of the EPR pathway at 31°C by the *tsB*20(30) mutation in such a way as to increase its fidelity. We may note, however, that when much more restrictive temperatures are used and a temperature-sensitive ligase allele is present, replication is greatly reduced, both in phage T4 (4, 10) and in *E. coli* (33). Under these damaging conditions, the temperature-sensitive ligase deficiency seems to cause lesions leading to mutagenesis (10, 33).

Time course of mutation. It has previously been noted that hydrazine-induced mutagenesis in *Saccharomyces cerevisiae* (29), nitrous acid- and UV-induced mutagenesis in actinophages (2), and hydrazine mutagenesis in *Haemophilus influenzae* (24) are much greater after replication has been allowed to occur. In phage T4, reversion induced by nitrous acid (5) and ethyl methane sulfonate (21) is also increased by preliminary passage through a permissive host. These induced mutants appear in bursts with nonmutant phage. This suggests that mutagenesis increases upon replication. One interpretation offered for this has been that premutational lesions may occur on a nontranscribed strand and would not be expressed without permitting replication. Replication permits segregation and subsequent expression in progeny (6, 40). This would account, however, for only two-fold differences in mutation frequency before and after replication. Differences of 15- and 25-fold are noted here, and differences of 10-fold were found in yeast (29). This delayed appearance of mutations in phage T4 implies that a persistent lesion may generate mutations at late times in the infectious cycle of phage T4. These late-occurring mutations may arise too late in the first infectious cycle to be effectively expressed under direct-plating conditions and therefore can only be detected after passage through a permissive host. This has been proposed by Green and Krieg (21) for phage T4, and a similar scheme was proposed by Lemnott (29) for yeast.

Pawl et al. (36) have shown that about half of all thymine dimers induced by UV are not removed but persist during the course of infection. Drake (18) showed that the majority of lesions that lead to mutation can be removed by photoreactivation. Thus it is likely that persistent UV-induced dimers can account for the delayed appearance of UV-induced mutagenesis. Thymine dimers have been found to persist in *E. coli* after replication (20). In *E. coli,* however, all potentially mutagenic dimers are converted to mutations by EPR in the first postirradiation cell division, and those which persist are not premutational lesions (42).

Accuracy of recombinational repair. MR has long been thought to be a recombinational process (30). Genes essential to recombination have been shown to be necessary for MR (14, 35). For UV, two processes appear to cooperate to produce MR. One, governed by the host *polA* gene (31) and the phage *v* gene (39) and gene 47 (35), determines the final slope of the MR survival curve. The second process, governed by phage genes *x,* 1206 (39), 32, 44, 46 (14), and 47 (35), determines the extent of the shoulder of the survival curve. The question may be asked, when these recombinational processes are operating during MR, are they accurate?

When multiple infections are compared with single infections per unit dose of UV, the multiple infections show a lower frequency of newly induced mutations (Fig. 5). However, survival is higher per unit dose in the multiple infection than in the single infection due to MR. The data in Figure 6 demonstrate that the frequency of induced revertants per log of surviving fraction was somewhat higher in single infections than in multiple infections. It is clear that MR, which is very effective in eliminating lethal lesions, does not generate new mutations. In fact, the results suggest that in reaching the same level of survival, MR may have somewhat selectively eliminated lesions which in the single infection were converted into mutations. Thus MR appears to be an accurate repair process.

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