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Genetic Analysis of Repair of Ultraviolet Damage by Competent and Noncompetent Cells of *Bacillus subtilis*

CHARLES T. HADDEN AND DANIEL BILLEN

Division of Radiation Biology, Department of Radiology and Department of Immunology and Medical Microbiology, College of Medicine, University of Florida, Gainesville, Florida 32601

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The repair of ultraviolet (UV) damage in *Bacillus subtilis* W23T⁻ has been studied by transformation with deoxyribonucleic acid (DNA) extracted from irradiated cells before and after repair. The extent of repair of genetic markers by donor cells after low or moderate doses of UV was found to be related only to the initial degree of inactivation. After a very high dose, further inactivation occurred, also in proportion to initial damage. In addition, the competent recipient cells were shown to repair approximately 75% of the damage in transforming DNA. The sensitivities of markers irradiated either in vivo or in vitro appeared to be related to map position, the more proximal markers showing a greater resistance to UV inactivation.

It has been shown that in *Bacillus subtilis* (6) and in *Escherichia coli* (4) ultraviolet (UV) irradition of log-phase cells results in reinitiation of deoxyribonucleic acid (DNA) synthesis from the fixed origin. In contrast, X-ray treatment appears to cause initiation of replication from non-origin sites (4, 6; D. Billen, *unpublished observations*). To determine whether reinitiation at the origin in UV-treated cells of *B. subtilis* might be a result of polarity in the UV repair system, we have analyzed the inactivation and subsequent repair of several genetic markers.

Because inactivation of transforming DNA seems to be related to the distance between UV-induced lesions in the DNA (7), the transformation assay is a good indicator of the extent and general location of repair. We have found that repair of UV damage in *B. subtilis* W23T⁻, when measured with either Uvr⁺ or Uvr⁻ recipient cells, is independent of map position, as is degradation of heavily damaged DNA. In addition, when transforming DNA contains UV damage, the fraction of UV damage which is repaired by Uvr⁺ competent cells is independent of the marker tested and is constant, at about 75%. Of the markers tested for UV sensitivity, the two most proximal on the genetic map showed a markedly higher resistance to UV than the remainder of the markers.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Growth and irradiation of donor cells. Cells of *B. subtilis* W23T⁻ were grown at 37 C in a modified (5) Spizizen salts medium (SM) plus 0.5% glucose, 0.25% vitamin-free Casamino Acids (Difco), 40 μg of thymine/ml, and 2 μCi of ^{3}H-thymine/ml (thymine-*methyl*-^3H, Schwarz/Mann, Orangeburg, N.Y., 12.6 Ci/mmole). Cells in stationary phase were used to rule out any influence of any active replication fork on repair. Stationary-phase cultures were harvested by centrifugation, washed with one-half volume of saline citrate buffer (SSC, 0.15 M NaCl plus 0.015 M sodium citrate, *p*H 7.0), and resuspended in SSC at a cell density of around 2.5×10^8/ml. Purified DNA for in vitro inactivation experiments was irradiated in 0.1× SSC. Irradiation was carried out with two General Electric 15-w germicidal lamps at a dose rate of 444 ergs per mm^2 per min. Bacterial suspensions were stirred vigorously in a watch glass during irradiation and were kept cold by an ice bath.

In marker inactivation experiments, irradiation was interrupted at the appropriate doses, and samples were taken into tubes containing one-fortieth of the sample volume each of ice-cold 5×10^{-2} M NaCN and 10^{-1} M NaN_3. The samples were held in an ice bath until irradiation was complete and then harvested for extraction of DNA.

In repair experiments, the irradiated cells were diluted 20-fold into warm medium (as described above, but lacking ^{3}H-thymine). Samples taken at the

TABLE 1. *Bacterial strains*

Strain designation	Prior designation	Genotype	Source
W23		Prototroph	S. Greer
W23T⁻	W23 Thy⁻	*thyA, thyB*	F. Rothman
BC 35		*purB6, leu-8, metB5*	J. Copeland
BC 36		*thr-5, leu-8, metB5*	J. Copeland
FB 6	Mu8u5u16	*purA16, leu-8, metB5*	A. K. Ganesan
FB 8	168 Trp⁻	*trpC2*	D. S. Nasser
SB 5		*hisA1, pyrA1, trpC2*	A. T. Ganesan
WB 2711		*purB6, metA3*	E. Nester
FB 13	168 UVR⁻ Trp⁻	*uvr, trpC2*	H. Reiter
FB 14		*uvr, purA16, metB5*	FB13-xFB6
FB 33		*uvr, purB6, metB5*	FB13-xBC35
FB 34		*uvr, hisA1, pyrA1*	FB14-xSB5

indicated times were harvested as in marker-inactivation experiments.

Extraction of DNA. DNA irradiated in vivo was extracted from cells by the "rapid-lysis" procedures described previously (6). For marker inactivation in vitro, DNA was extracted from strain W23 by a modification of the Marmur method (17), in which ribonuclease treatment was followed by phenol extraction and dialysis, as in the "rapid-lysis" procedure (6).

Transformation. Competent cells of *B. subtilis* 168 strains were prepared by a modification of the MM1/MM2 procedure of Dooley et al. (12), in which MM1 consisted of Spizizen salts (BSS) (2), 0.5% glucose, 50 μg/ml each of the amino acids described by Wilson and Bott (26) as being stimulatory to competence, and any other required supplements at 50 μg/ml (100 μg/ml for adenine). In MM2 the concentration of amino acids was reduced to 25 μg/ml, and 5 mM $MgSO_4$ was added. For strain FB 6, BSS was replaced by SM.

Competent cells were harvested by centrifugation and resuspended at 10 times their original concentration in BSS plus 10% glycerol. They were frozen in liquid nitrogen in 1-ml samples and stored at −76 C.

Transformations were done with limiting concentrations of DNA. For the transformation assay, rapidly thawed competent cells were diluted into BSS plus 0.5% glucose and 5 mM $MgSO_4$, and added to tubes containing DNA samples. Incubation with DNA was carried out for 20 to 60 min at 37 C and terminated by exposure to 10 μg of deoxyribonuclease per ml. Transformants were plated on the solid medium described previously (6).

The specific transforming activity of markers was calculated by normalization to DNA concentration, determined by the concentration of ^{3}H-thymine in DNA samples. The number of inactivating hits was calculated with the formula of Bresler et al. (7), in which the average number of hits per genetic target (Z) is given by $Z = 2(\sqrt{S_0/S} - 1)$, where S_0 is the specific transforming activity of a marker in unirradiated DNA and S is the specific transforming activity of that marker in the treated sample.

Dimer excision. Dimer analysis was carried out as described by Billen, Hadden, and Corrigan (*submitted for publication*). Exponentially growing cells of strain W23 or FB 8 were radioactively labeled in the presence of 20 μCi of carrier-free thymidine-*methyl*-^3H per ml (Schwarz/Mann, Orangeburg, N.Y., 14.4 Ci/mmole) plus 50 μg of deoxyadenosine per ml. Samples were prepared essentially by the procedure of Setlow and Carrier (25). About 4×10^8 irradiated cells were either frozen in liquid N_2 (time = 0) or centrifuged, resuspended, and incubated in 6 ml of growth medium at 37 C for 30 min. The culture was made 5% in trichloroacetic acid by the addition of 100% trichloroacetic acid, and about 5×10^8 unlabeled carrier cells were added. The zero-time sample was thawed and treated similarly. After 45 min in an ice bath, the acid-insoluble material was pelleted by centrifugation, washed once with 5% trichloroacetic acid, resuspended in 1 ml of 5% trichloroacetic acid, and hydrolyzed at 100 C for 30 min (Richard P. Boyce, *personal communication*). To the solubilized material was added 50 μg of calf thymus DNA per ml (Worthington Biochemical Corp., Freehold, N.J.). This mixture was lyophilized, resuspended in 150 μliters of 98% formic acid (Matheson, Coleman and Bell, Norwood, Ohio), and hydrolyzed at 175 C for 90 min. The samples were again lyophilized, resuspended in about 150 μliters of 0.1 N HCl, and chromatographed on Whatman no. 1 paper in a descending system, with *n*-butanol-water (86:14, v/v) as the solvent.

RESULTS

Repair of markers. In these experiments post-repair samples were taken after 20 to 30 min of incubation in growth medium. Essentially all of the repair which occurs appears to be completed by this time (9; *unpublished observations*). Three doses of UV were used: 400, 2,000, and 5,000 ergs/mm^2. Figure 1 shows the inactivation and repair of markers after a dose of 2,000 ergs/mm^2, when transforming activity was assayed in Uvr$^+$ recipient cells. The solid bars show the activity remaining immediately after irradiation, and the open bars indicate post-repair activity (the differ-

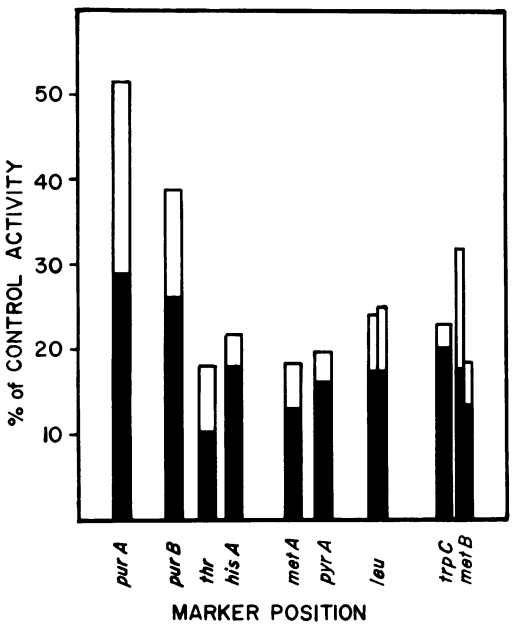

FIG. 1. *Inactivation and repair of markers at 2,000 ergs/mm². Cells of W23T⁻ were treated and analyzed as described in Materials and Methods, with Uvr⁺ recipients. Bars are spaced along the abscissa in proportion to the corresponding map position (13) of the markers. Closed bars, initial postirradiation activity; open bars, activity after 30 min of postirradiation incubation.*

ence between open and closed bars represents repair). Split bars represent assays of the same marker in two different recipient strains. Since Uvr⁺ recipient cells were used, transforming activity reflects repair carried out by both donor and recipient cells. Figure 2 gives results from an experiment in which the UV dose was 5,000 ergs/mm². In this experiment transforming activity was further reduced postirradiation, rather than repaired (the difference between open and closed bars indicates additional inactivation).

Number of inactivating hits. It appears from Fig. 1 and 2 that there might be a correlation between the extent of repair or degradation and map position, with the two proximal markers affected more than the markers in the distal three-fourths of the genome. However, when the number of inactivating hits per target was calculated, a more meaningful relationship emerged.

According to Bresler et al. (7), transforming DNA is inactivated when the integrated segment of donor DNA bearing the marker selected for also contains an inactivating hit. The average number of hits (Z) per genetic target can be calculated from the transformation data: $Z = 2(\sqrt{S_0/S} - 1)$. The initial damage to markers was calculated from transformation into Uvr⁻ recipients, and the extent of repair was calculated by the difference between that activity and activity of the DNA in Uvr⁻ and Uvr⁺ recipient cells after the repair period.

The correlation between repair and initial damage is shown in Fig. 3, in which complete repair is represented by the diagonal. The data for Uvr⁻ recipients are assumed to indicate repair due solely to the W23T⁻ donor cells. From Fig. 3 it is clear that the amount of repair of UV-inactivated markers is strictly related to the initial damage. The intersection of the experimental curve with the diagonal implies that the irradiated donor cells repair completely an average of about 0.7 inactivating hit per target. Above that level of damage, the slope indicates that about 48% of additional hits are repaired. The data for Uvr⁺ recipients represent the combined repair carried out by both the donor and recipient cells. Beyond about 1.25 hits per target, about 85% of additional damage is repaired.

Use of the transformation assay involves the assumption that the donor cells are not grossly heterogeneous in their response to UV (e.g., dead cells responding differently from survivors). The fact that in Fig. 3 data taken from experiments using doses of 400 ergs/mm² (around 30% colony survival) and 2,000 ergs/

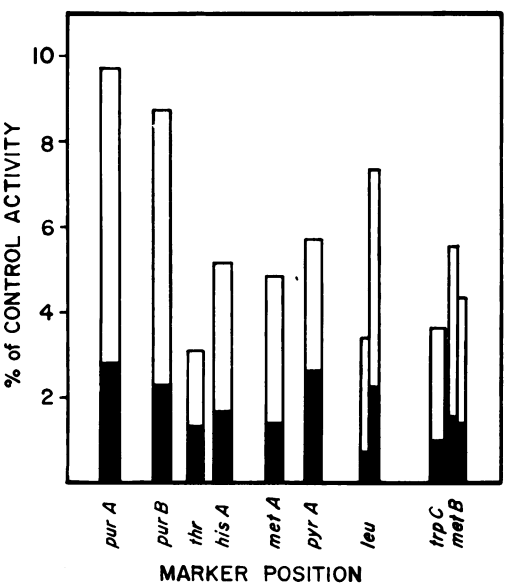

FIG. 2. *Inactivation of markers after a dose of 5,000 ergs/mm². Details similar to Fig. 1, except open bars indicate initial postirradiation activity and closed bars indicate activity after 30 min of postirradiation incubation.*

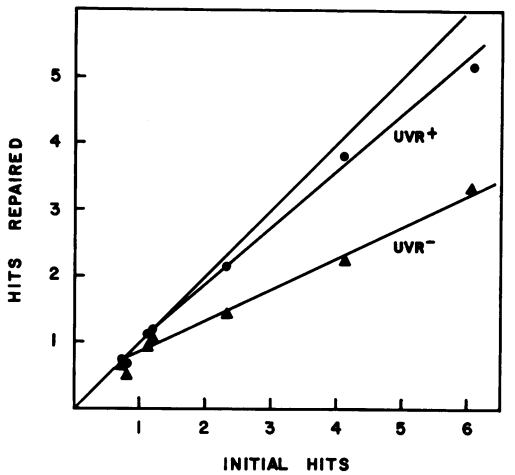

FIG. 3. *Relation of repair to initial UV damage. Data for several markers were taken from experiments at 400 ergs/mm² (20 min of incubation) and 2,000 ergs/mm² (30 min of incubation). Initial hits were calculated from initial postirradiation activity in Uvr⁻ recipients. Hits repaired were calculated by difference from postincubation samples assayed with either Uvr⁺ or Uvr⁻ recipients (hits repaired = initial hits − hits remaining). Closed circles, Uvr⁺ recipients; closed triangles, Uvr⁻ recipients.*

mm² (less than 1% colony survival) fit the same curves supports the validity of this assumption.

The data from Fig. 2 were also replotted in terms of inactivating hits (Fig. 4). In this case, the number of both initial hits and hits due to degradation were based on transformation of Uvr⁺ recipients. It appears that at very high doses of UV, at which degradation is more extensive than repair, the extent of further inactivation of markers is also dependent on the initial level of damage to the marker.

It seems unlikely that these additional hits result from extension of only the sites of damage assayed in Uvr⁺ recipients immediately postirradiation. They might result from endonucleolytic action at undamaged sites, or they might represent a decreased probability of recipient-specific repair, for instance by degradation beginning at lesions which were initially repairable. A decrease in the probability of recipient-specific repair should show up if Uvr⁺ and Uvr⁻ recipients are compared. Recipient-specific repair by definition makes sites which are inactive in Uvr⁻ recipients active in Uvr⁺ recipients. A decrease in probability of repair would not decrease activity in Uvr⁻ recipients, but would decrease activity in Uvr⁺ recipients. That is, during postirradiation incubation after a very high UV dose, the number of hits assayed in Uvr⁻ recipients would not increase, but the number of hits assayed in Uvr⁺ recipients would increase.

Table 2 shows that, after 60 min of incubation following a dose of 5,000 ergs/mm², there is a 100% increase in number of hits measured in the Uvr⁻ recipients, compared with a 160% increase in hits measured in the Uvr⁺ recipients, and in fact, there are more new hits assayed in Uvr⁻ than in Uvr⁺ recipients. Table 3 shows that the Uvr⁺ recipient repaired 60% of the initial damage, and 49% of the postincubation damage. From these results we conclude that, unless the initial activity includes a considerable fraction (50% in this case, Table 2) of damage which can be repaired by Uvr⁻ recipi-

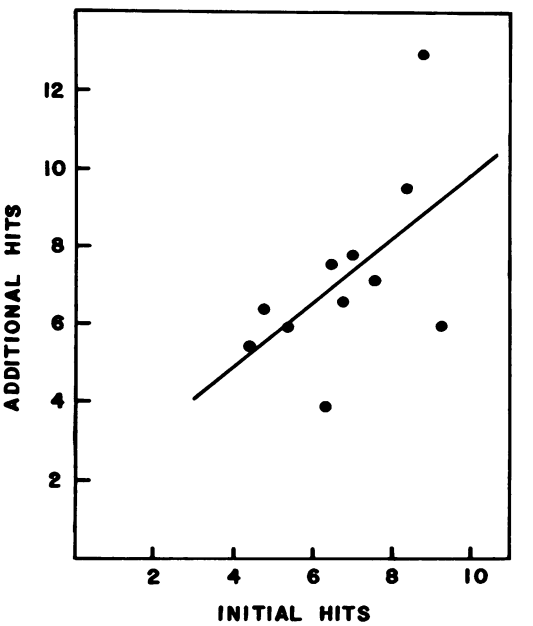

FIG. 4. *Relation of hits added during incubation to initial damage. Data were calculated from the data of Fig. 2. Initial hits were measured in Uvr⁺ recipients (additional hits = postincubation hits − initial hits).*

TABLE 2. *Number of hits in purB6 assayed with Uvr⁺ and Uvr⁻ recipients*[a]

Recipient	Time (min)	No. of hits	Hits added	Additional hits/initial hits
Uvr⁺ (WB 2711)	0	4.8		
	60	12.3	7.5	1.6
Uvr⁻ (FB 33)	0	11.9		
	60	24.0	12.1	1.0

[a] Samples used in transformation were from the same experiment as shown in Fig. 4.

ents, conversion of recipient-repairable to recipient-nonrepairable sites plays only a minor part in inactivation during postirradiation incubation.

Recipient-specific repair. There is clearly a contribution of the Uvr$^+$ recipient cells to the observed extent of repair of UV damage. That contribution is illustrated in more detail in Fig. 5, in which the repair of a sample by competent Uvr$^+$ recipient cells is plotted against the number of inactivating hits assayed in Uvr$^-$ recipients for the same sample. The figure includes data for several different markers and several different doses of UV, as well as unrepaired and post-repair samples. The slope of the curve implies that about 75% of the damage in the transforming DNA is repaired during the process of transformation. The close correlation of all points (r = 0.997) indicates that recipient-specific repair of these samples must not depend on any property of the donor DNA except the extent of UV damage in the molecule containing the marker being assayed.

Sensitivity of markers to UV inactivation. Since donor-specific repair also depends only on initial damage, the initial observation that repair might be related to map position implies that the proximal markers might be more resistant to inactivation by UV. Therefore we determined the rates of inactivation of markers irradiated in vivo. Representative data are given in Fig. 6. The dose required for one inactivating hit per marker was taken as a measure of sensitivity of that marker. These one-hit doses are given in Fig. 7. The two proximal markers are clearly more resistant to inactivation by UV, and the more distal markers appear to be more nearly equal in sensitivity. Thus, any effect of map position on the extent of repair must be due solely to the apparent position dependence of marker sensitivity.

We considered the possibility that some aspect of the organization of the cell might be responsible for the apparent resistance of proximal markers to UV. For instance, perhaps the DNA is packaged so that the proximal section is surrounded by the rest, or perhaps some other cell structures protect this region. The ribosome cistrons are located between the two proximal

TABLE 3. *Repairability of pre- and postincubation hits by Uvr$^+$ recipients*[a]

Time (min)	Total hits[b]	Unrepaired hits[c]	Hits repaired by Uvr$^+$	Percent repaired
0	11.9	4.8	7.1	60
60	24.0	12.3	11.7	49

[a] Data are taken from Table 2.
[b] Assayed with Uvr$^-$ recipients (FB 33).
[c] Assayed with Uvr$^+$ recipients (WB 2711).

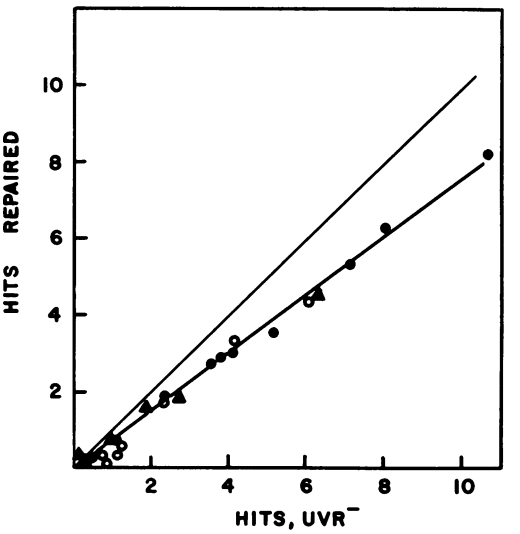

FIG. 5. *Recipient-specific repair of damaged transforming DNA. Each point represents transformation of a Uvr$^+$ and a Uvr$^-$ recipient for the same marker and with the same sample (hits repaired = Uvr$^-$ hits − Uvr$^+$ hits). Triangles, post-repair activities, Fig. 3; open circles, initial activities, Fig. 3; closed circles, marker inactivation (FB 6 and FB 14).*

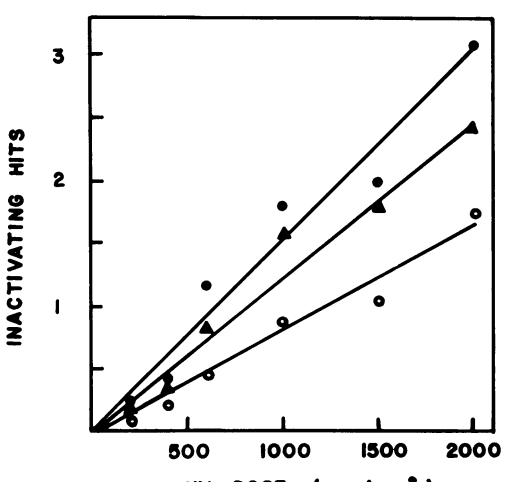

FIG. 6. *Representative data on marker inactivation. Samples were treated and analyzed as described in Materials and Methods, with Uvr$^+$ recipients. Open circles, purA16; closed circles, leu-8; triangles, metB5.*

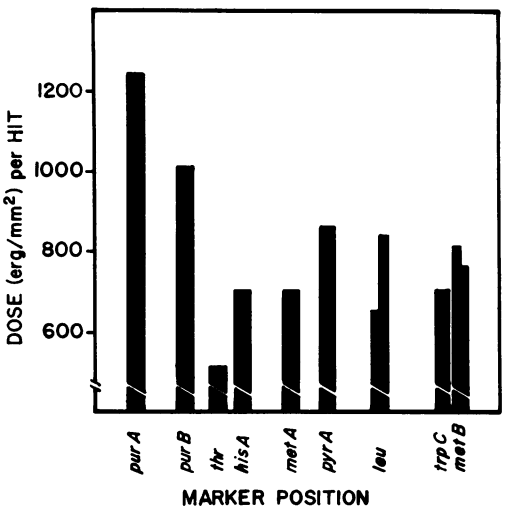

FIG. 7. *Sensitivity of markers to UV. Data from the experiment illustrated in Fig. 6 were used to determine marker sensitivities in ergs/millimeter², as described in the text.*

markers used in this study (13). A locally high concentration of ribosomal ribonucleic acid and ribosomal proteins might shield the DNA from UV. However, when the sensitivities of markers were determined by using purified DNA, so that no cell structure was involved, we found the same relative rates of inactivation as for irradiation in vivo (Table 4). We concluded that the sensitivities to UV we have observed are intrinsic in the markers.

DISCUSSION

There are a number of ways in which the UV repair system might be organized. For instance, there might be specific initiation sequences in the DNA, from which the repair enzymes would act in a polar or sequential fashion. If these initiation sites were concentrated in one or a few regions, such as at the origin of replication, then there would initially be selective repair of those regions. Alternatively, the repair enzymes might occur in conjunction with the replication complex, so that newly synthesized DNA is preferentially repaired. Or the repair enzymes might be distributed randomly about the cell, with a random probability of repair beginning at any given site of UV damage. Our data show clearly that there are no large regions preferentially repaired by W23T⁻, although we cannot rule out favored sites or polarity over very short regions. The rapidity of repair in stationary-phase cells rules out any dependence on an active replication complex; furthermore, even moderate doses of UV (400 ergs/mm²) cause a marked lag in DNA synthesis (6). The best interpretation of the data is that the probability of repair of any damage site is the same as for any other site, and independent of other factors such as local structure of the DNA, packaging of the DNA, etc.

The repair of about half of the UV damage of W23T⁻ is consistent with the results of Reiter and Strauss (23) with *B. subtilis* 168. Recalculation of their data shows that, during a 40-min postirradiation incubation, 57% of the inactivating hits were removed from the *trpC* marker of irradiated Uvr⁺ cells. Furthermore, dimer excision experiments show that cells of W23 and of 168 Trp⁻ (FB 8) excise dimers approximately equally (Table 5).

Munakata and Ikeda (19) and Bron and Venema (10) have shown that, when Uvr⁺ and Uvr⁻ competent cells are transformed with the same preparation of DNA irradiated in vitro,

TABLE 4. *Relative marker sensitivities of DNA irradiated in vivo and in vitro*[a]

Marker	Sensitivity[b]	
	In vivo	In vitro
purA	1.77	1.59
purB	1.47	1.37
thr	0.74	0.70
metA	1.00	1.00
leu[c]	0.93	0.96
leu[d]	1.28	1.08
metB[c]	1.18	0.99
metB[d]	1.11	1.00

[a] In vivo data are taken from Fig. 6. In vitro data were determined similarly on samples of DNA irradiated after purification.
[b] Ergs per hit, normalized to *metA* = 1.00.
[c] Assayed in FB6.
[d] Assayed in BC 36.

TABLE 5. *Dimer excision by cells of W23 and 168 Trp⁻*[a]

Strain	UV dose (ergs/mm²)	Time (min)	³H counts/min in dimer	³H counts/min in thymine	Percent ³H in dimer
W23	1,000	0	826	336,432	0.24
		30	352	283,550	0.15
168 Trp⁻	1,500	0	1566	678,044	0.23
		30	1329	769,468	0.17

[a] Dimer analysis was carried out as described in Materials and Methods. Data were chosen to give similar initial fractions of dimers.

the fraction of the damage repaired by Uvr$^+$ recipient cells varies from 74 to 88%, depending on the marker tested. We have found that repair of DNA irradiated in vivo is relatively independent of the marker assayed, whether or not the donor cells have been allowed to carry out partial repair. In contrast, recipient repair in *Haemophilus influenzae* shows striking selectivity for markers repaired if DNA is irradiated in vitro (21), but not if it is irradiated in vivo (11).

The direct dependence of recipient-specific repair in *B. subtilis* on the amount of damage in the DNA preparation shows that within the specificity of the recipient's repair system there is no change during the postirradiation incubation in the distribution of kinds of inactivating lesions. For example, a molecule containing a pyrimidine dimer would be inactive in the Uvr$^-$ recipient, but would have a high probability (75%) of being repaired in the Uvr$^+$ recipient. However, if the dimer had been removed, leaving a single-strand gap, the molecule would be equally active in both the Uvr$^+$ and the Uvr$^-$ recipient, since dimer excision is the function lacking in *uvr* strains (23). Thus single-strand nicks or gaps must not accumulate during repair of moderately or lightly damaged molecules. (This has been shown to be true also in *E. coli* [R. B. Setlow, W. L. Carrier, and R. W. Williams, Biophys. Soc. Abstr., p. 83, 1967].)

Our data (Fig. 5) show a consistent recipient-specific repair by competent Uvr$^+$ cells of 75% of the inactivation in DNA samples, compared to donor-specific repair of only around 50%. Since we have observed little additional repair after 30 min, the quantitative difference between donor-specific and recipient-specific repair is puzzling. Both *H. influenzae* (3) and *B. subtilis* (8) appear to repair UV-damaged transforming DNA after integration. Conceivably, competent cells might make DNA more susceptible to repair during the processing of exogenous DNA for transformation, or the physiological state of the competent cells might be sufficiently different from that of noncompetent cells to account for the difference in repair (transformants of *B. subtilis* are known to be less photoreactivable than the noncompetent cells [16]). Specifically, repair might continue for a loner time in competent cells, or there might be less degradation of irradiated DNA than in the irradiated donor cells. The differences between W23 and 168 derivatives of *B. subtilis* in DNA homology, modification, and restriction systems could also contribute to differences in repair.

When the DNA is damaged by an extremely high dose of UV, the repair system can no longer reactivate the DNA. A similar saturation of the repair system has been demonstrated in *E. coli* (1). Instead, additional inactivating hits are put into the DNA during postirradiation incubation (Fig. 4). These additional hits are put into stretches in the DNA which presumably do not contain pyrimidine dimers, since they are initially active in Uvr$^-$ recipient cells. It is possible that the additional hits reflect attack on photoproducts which are repairable in Uvr$^-$ cells.

Genetic markers are clearly heterogenous in their physical properties. They have markedly different buoyant densities (14), thermal inactivation temperatures (14, 20, 24), and sensitiviies to UV (15, 18, 19, 20, 27). It has generally been concluded that the UV sensitivity of a marker in DNA irradiated in vitro is correlated with the percentage of adenine plus thymine in the neighborhood of the markers (15, 18, 19, 20, 22, 27). The position effect we have observed is much more marked than the spectrum of buoyant densities of genetic markers reported for *B. subtilis* by Ganesan and Lederberg (14), or the T_m values given for thermal inactivation of markers (14, 20). However, these reported values are most likely influenced by base composition over a much larger distance than the inactivation of transforming markers by UV.

ACKNOWLEDGMENTS

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