

Ultraviolet-Induced Decrease in Integration of *Haemophilus influenzae* Transforming Deoxyribonucleic Acid in Sensitive and Resistant Cells

AMIR MUHAMMED¹ AND JANE K. SETLOW

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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The decrease in integration of transforming deoxyribonucleic acid (DNA) caused by ultraviolet irradiation of the DNA was found to be independent of the presence or absence of excision repair in the recipient cell. Much of the ultraviolet-induced inhibition of integration resulted from the presence in the transforming DNA of pyrimidine dimers, as judged by the photoreactivability of the inhibition with yeast photoreactivating enzyme. The inhibition of integration made only a small contribution to the inactivation of transforming ability of the DNA by ultraviolet radiation.

Ultraviolet irradiation of deoxyribonucleic acid (DNA) in solution decreases its transforming ability and, except at very large doses, most of this biological effect is caused by formation of pyrimidine dimers (13). To understand the mechanism of this inactivation, we must know how ultraviolet irradiation affects the various steps in transformation, such as DNA penetration and integration. DNA penetration is relatively resistant to radiation (6). Notani and Goodgal (6) have, however, obtained evidence that ultraviolet irradiation decreases integration of transforming DNA into wild-type *Haemophilus influenzae*. They measured integration by observing the increase in amount of radioactivity from labeled transforming DNA that sedimented with the recipient DNA, as a function of the time of incubation of transforming DNA and competent cells. Since it is known that ultraviolet-induced pyrimidine dimers in transforming DNA can be integrated into the recipient genome (1, 11), and since excision repair of *H. influenzae* transforming DNA takes place after integration (Setlow and Beattie, unpublished experiments), some or all of the effect observed by Notani and Goodgal as inhibition of integration may actually have been loss of integrated material resulting from the excision repair. To test this possibility, we measured integration of ultraviolet-irradiated DNA into wild-type *H. influenzae* and into two ultra-

violet-sensitive mutants, one of which cannot carry out excision (11). We found that inhibition of integration is indeed induced by ultraviolet irradiation, but this inhibition makes only a small contribution to the inactivation of transforming DNA. Furthermore, the presence or absence of a repair mechanism has no effect on the magnitude of the ultraviolet-induced decrease in integration, although it has a profound effect on the survival of transforming ability (10). Integration of irradiated transforming DNA may be increased by photoreactivation with yeast photoreactivating enzyme. Since the biological effect is known to result from the monomerization of pyrimidine dimers (9), such dimers are clearly responsible for some of the observed loss of integration.

Steinhart and Herriott (15) have developed a new method of measuring DNA integration based on the loss of radioactive label from fully labeled competent cells after these cells are exposed to transforming DNA. They showed that the recipient DNA lost to the medium during transformation is equivalent to the amount of donor DNA integrated (15, 16). Their method is particularly suitable for measuring the effect of ultraviolet irradiation on integration, since the ultraviolet lesions are not in the labeled material. If a considerable amount of unlabeled integrated DNA containing dimers were excised and replaced specifically by the labeled material previously displaced in integration, the integration

¹ Present address: Radiation Genetics Institute, Lyallpur, Pakistan.

of DNA would appear more sensitive to ultraviolet radiation than it really is. If, however, excision of dimers in unlabeled integrated DNA were accompanied by breakdown of the DNA in the labeled unirradiated DNA region, the ultraviolet-induced decrease in integration would appear less sensitive than it really is.

MATERIALS AND METHODS

Wild-type or mutant *H. influenzae* cells were grown in Brain Heart Infusion growth medium (10) with vigorous aeration at 37 C to an optical density at 675 nm of 0.4. The medium also contained 1.8 μg of tritiated thymidine per ml (specific activity, 6.7 c/mmole). Cells were centrifuged, washed once with MII salts (14) and once with MIV medium (15), and finally resuspended at a concentration of about 8×10^8 /ml in 2 ml of MIV medium. Washed cells were incubated for 100 min at 37 C with vigorous aeration. The resulting competent culture was centrifuged and washed once with MII medium (14), and finally was resuspended at a concentration of 4×10^8 to 8×10^8 /ml in MII medium. The cells were then incubated at 37 C with irradiated or nonirradiated DNA (containing a streptomycin marker) at a final concentration of 1 μg /ml in a total volume of 0.5 to 0.6 ml. Control cells were incubated without DNA. Deoxyribonuclease at a concentration of 1 μg /ml was added to all samples after 9.5 min of incubation. After various times, 0.1-ml samples were added to 0.4 ml of cold 0.067 M phosphate buffer, pH 7.0, and immediately filtered through a 13-mm membrane filter (0.45- μm pore size; Millipore Corp., Bedford, Mass.). A sample of the filtrate was counted in a scintillation counter after addition of dioxane-naphthalene-2,5-bis-[2-(*t*-tert-butylbenzoxazolyl)]thiophane scintillation fluid. Total radioactivity in the cell suspension was also measured. After 30 min of incubation, a sample of the suspension was removed for measurement [by the agar overlay technique (3)] of the fraction of the cells transformed to streptomycin resistance.

Ultraviolet irradiation and photoreactivation of the DNA were carried out as previously described (10). Photoreactivating enzyme from yeast (about 0.3 μg /ml) prepared according to Muhammed (5) was added to irradiated DNA at a final concentration of 6.6 μg /ml. The time of photoreactivation was about 2 hr at 37 C, more than enough to monomerize all the dimers in the DNA. Samples were also incubated in the dark for the same length of time, as a control.

RESULTS AND DISCUSSION

Results of a typical experiment with wild-type *H. influenzae* are shown in Fig. 1, in which the radioactivity released from the cells is plotted against time of incubation of cells with transforming DNA. Considerably more radioactive material was released from cells exposed to nonirradiated DNA than from cells not exposed to DNA; irradiated DNA caused less release than nonirradiated DNA. In the experiments of Stein-

hart and Herriott (15), the rate of radioactivity release from cells exposed to DNA decreased and became about the same as that of the control cells after about 40 min of incubation. We did not observe such a phenomenon, either in wild-type *H. influenzae* (Fig. 1) or in the mutant strains (Fig. 3). We are unable to explain these differences. We have concluded that we cannot explain the difference between our results and those of Steinhart and Herriott on the basis of continued entrance of DNA into the cells beyond the time (9.5 min) at which the enzyme was added to degrade the DNA that was outside the cells, because deoxyribonuclease at a concentration 100 times the usual concentration did not change the kinetics of the radioactivity release.

Figure 2 shows the results of a control experiment confirming the conclusion of Steinhart and Herriott that the additional release of radioactivity caused by exposure of competent cells to homologous DNA is closely involved in the transformation process. The recipient cell (Fig. 2) was DB117, in which the level of transformation is about 10^{-7} that of wild-type *H. influenzae*, although the cells take up DNA normally (11). Figure 2 shows clearly that addition of homologous DNA caused no increase in radioactivity in the filtrate of DB117. We conclude that DB117 is defective in integration of transforming DNA. This conclusion is in accord with previous work on this mutant, which has shown that there is no linkage of a marker on the transforming DNA with a marker in the recipient DB117 cell. DB117

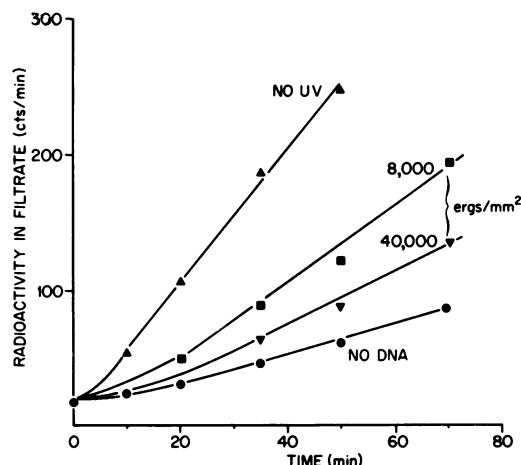


FIG. 1. Kinetics of release of radioactivity into the medium from wild-type *H. influenzae*. Ultraviolet irradiation of the DNA was at 254 nm. Total radioactivity in the solution was 6,100 counts/min, and percentage transformation from the unirradiated DNA was 2.8.

is also deficient in its ability to recombine phage DNA, in that multiplicity reactivation of ultraviolet-irradiated *H. influenzae* phage and vegetative recombination of temperature-sensitive *H. influenzae* phage (2) do not take place in DB117. Thus, DB117 apparently lacks some enzymatic activity associated with recombination of DNA molecules.

Some results of an experiment similar to those of Fig. 1 and 2 with the mutant DB116 are presented in Fig. 3. This mutant is unable to excise pyrimidine dimers from its DNA (11), although transformation is normal. As with wild-type *H.*

influenzae, ultraviolet radiation decreased the radioactivity release caused by exposure to transforming DNA. Figure 3 also illustrates the radioactivity release from exponentially growing DB116 cells that were or were not exposed to transforming DNA. The specific release of label assumed to be caused by integration of transforming DNA was not found in such cells, or in exponential wild-type cells, which agrees with the fact that almost no cells in the exponential phase of growth take up DNA (G. Price and J. K. Setlow, unpublished) or can be transformed (3).

Representative data on three strains of *H. influenzae* are shown in Fig. 4, where percentage integration is plotted against ultraviolet dose. The percentage integration was calculated in the following way from data such as those of Fig. 1 and 3. Radioactivity in the filtrate at 20 min was read off the whole curve (which was always a straight line starting at 20 min or earlier). This particular time was chosen because it is well within the time that Steinhart and Herriott found to be the duration of specific release resulting from exposure to homologous DNA. The difference between the radioactivity release in the experimental and control (no DNA) samples was taken as 100%, and the integration resulting from the irradiated DNA was compared with it.

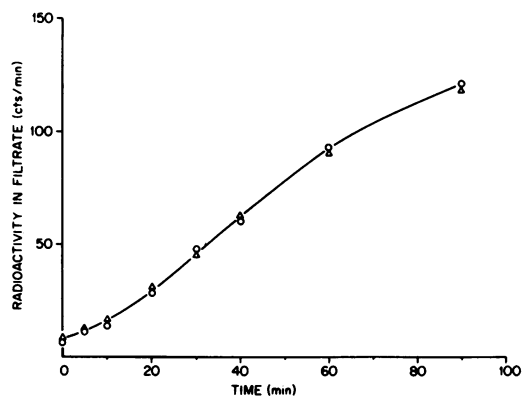


FIG. 2. Kinetics of release of radioactivity into the medium from *H. influenzae* strain DB117. Symbols: Δ , exposed to transforming DNA; \circ , control.

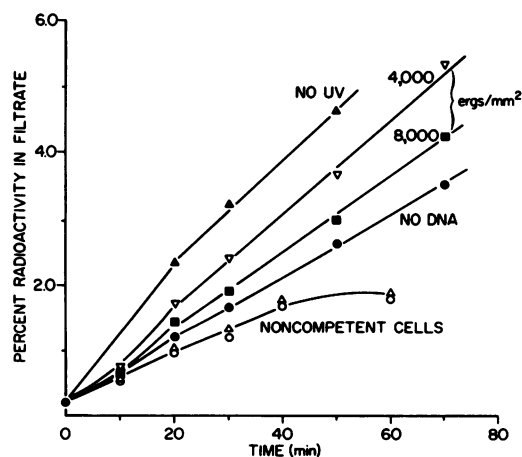


FIG. 3. Kinetics of release of radioactivity into the medium from *H. influenzae* strain DB116. Ultraviolet irradiation of the DNA was at 254 nm. The total radioactivity in the solution was 13,100 counts/min, and the percentage transformation from the unirradiated DNA was 0.7. The exponentially growing (noncompetent) cells were either exposed (Δ) or not exposed (\circ) to transforming DNA.

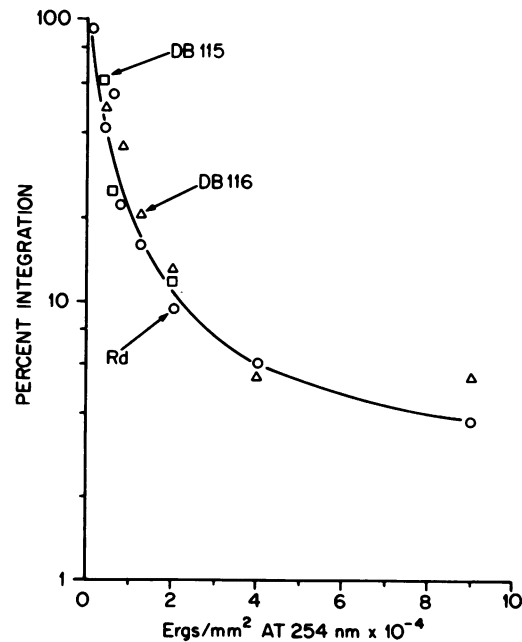


FIG. 4. Dose effect curve for loss of integration in three strains of *H. influenzae*. Calculations made from data such as those of Fig. 1 and 3 as described in the text.

No difference was observable in the effect of ultraviolet irradiation on DNA integration in the three strains of *H. influenzae* (Fig. 4). Thus, the cell's ability to excise ultraviolet-induced pyrimidine dimers, which is important in the survival of ultraviolet-irradiated transforming DNA (11), plays no part in the resistance of DNA to loss of integration by irradiation. Furthermore, the defect in DB115, which consists in slow rejoining of single-strand gaps in its DNA made by excision of pyrimidine dimers (11), also seems to have no influence on the sensitivity to integration loss. These results are in accord with previous work indicating that all repair of transforming DNA takes place after integration (Setlow and Beattie, unpublished).

From the data of Fig. 4, it is possible to assess the contribution by loss of integration to the biological inactivation of transforming DNA by ultraviolet irradiation. A dose of 5,000 ergs/mm² at 254 nm to the DNA reduced the integration to about 50%, whereas the survival of the very resistant cathomycin marker on transforming DNA irradiated with such a dose and assayed on DB116 is about 0.7% (10). Obviously, the contribution of the loss of integration to inactivation of the DNA is a minor one. Even with the wild-type recipient, the transformation to cathomycin resistance is inactivated down to about 6% after such a dose (10). Thus, much of the ultraviolet inactivation of transforming DNA must result from processes that occur, or do not occur, after integration. Except after enormous ultraviolet doses, more than 90% of the biological damage assayed in excising and nonexcising strains is the result of pyrimidine dimers (10). Because there is evidence that pyrimidine dimers in transforming DNA are incorporated into the recipient genome (1, 11), we conclude that either some of these are not repaired in the wild-type cell, or a certain number are not repaired correctly.

Notani and Goodgal (6) found that only about 25% of the integration of radioactively labeled transforming DNA remained after a dose similar to the one that yielded 50% survival of integration in our experiments. The lower figure in their experiment may reflect the loss of labeled material by excision of dimers from integrated transforming DNA. Our failure to find a strain difference in the loss of integration suggests that little or none of the released host radioactivity is re-incorporated in subsequent synthesis. The lack of strain difference also suggests that there is little breakdown of recipient DNA resulting from excision of dimers in integrated donor DNA.

Notani and Goodgal suggested that the photochemical event responsible for the decrease in

integration is a cross-link between DNA strands. Since it is now believed that single-stranded pieces of transforming DNA become integrated (7), cross-links would be expected to interfere with the strand separation that must occur in integration. At doses such as 5,000 ergs/mm², however, there are few cross-links in DNA (8). Data presented in Fig. 5 show that, after moderate doses (such as 10⁴ ergs/mm²), much of the integration loss is photoreactivable. Therefore, at such a dose about two-thirds of the photochemical lesions produced, which cause DNA to lose its ability to be integrated, must be pyrimidine dimers. Above this dose, the rate of formation of pyrimidine dimers begins to drop off, and the number of dimers finally reaches a saturating level (17). As a result, the relative number of other photoproducts increases, and the photoreactivability would be expected to decrease (Fig. 5). It must be pointed out, however, that there is considerable uncertainty in the measurement of integration after high doses. Since ultraviolet-induced cross-links in DNA are not photoreactivable (R. W. Barton and J. K. Setlow, unpublished), they become an obvious candidate for the lesion causing the decrease in integration at the higher doses.

The 1/e dose for inhibition of integration (Fig.

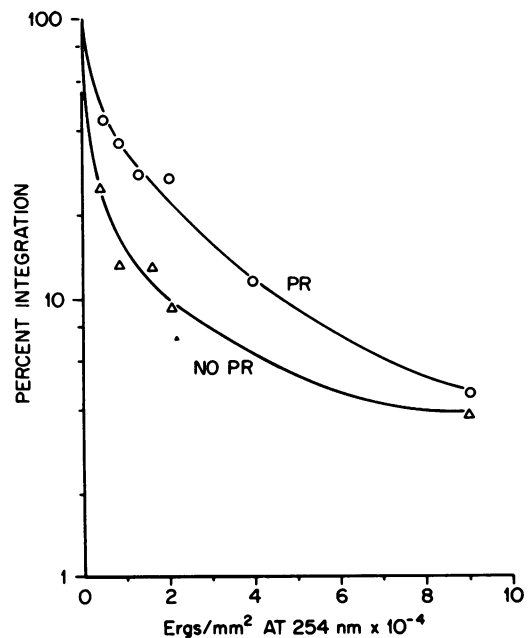


FIG. 5. Dose effect curve for loss of integration in wild-type *H. influenzae*, with and without prior photoreactivation of the DNA with yeast photoreactivating enzyme. The curve labeled no PR represents samples incubated in the dark with the enzyme.

4) is about 5,000 ergs/mm². Such a dose produces pyrimidine dimers that are, on the average, separated by about 400 nucleotides on a single strand (12). It is an interesting coincidence that this is the same number given for "excluded length" in *H. influenzae* transformation (4). The "excluded length" is a number derived from an investigation of DNA length and transformation efficiency (4), and represents the maximal length that is too short for integration.

We conclude that pyrimidine dimers in transforming DNA inhibit integration, but only partially, and that the inhibition is independent of repair mechanisms. Some dimers become integrated, and some of these integrated dimers inhibit the transformation process. The extent of this type of inhibition depends to a large extent on the repair capacity of the recipient cell. Some dimers may be more important than others for integration inhibition. These could be dimers close to or in a region important for pairing of transforming DNA with the recipient genome, a process that presumably must precede physical integration.

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LITERATURE CITED

1. Beattie, K. L., and J. K. Setlow. 1969. Killing of *Haemophilus influenzae* cells by integrated ultraviolet-induced lesions from transforming deoxyribonucleic acid. *J. Bacteriol.* **100**:1284-1288.
2. Boling, M. E., and J. K. Setlow. 1969. Dependence of vegetative recombination among *Haemophilus influenzae* bacteriophage on the host cell. *J. Virol.* **4**:240-243.
3. Goodgal, S. H., and R. M. Herriott. 1961. Studies on transformations of *Haemophilus influenzae*. I. Competence. *J. Gen. Physiol.* **44**:1201-1207.
4. Guild, W. R., A. Cato, Jr., and S. Lacks. 1968. Transformation and DNA size: two controlling parameters and the efficiency of the single strand intermediate. Cold Spring Harbor Symp. Quant. Biol. **33**:643-645.
5. Muhammed, A. 1966. Studies on the yeast photoreactivating enzyme. I. A method for the large scale purification and some properties of the enzyme. *J. Biol. Chem.* **241**:516-523.
6. Notani, N. K., and S. H. Goodgal. 1965. Decrease in integration of transforming DNA of *Haemophilus influenzae* following ultraviolet irradiation. *J. Mol. Biol.* **13**:611-613.
7. Notani, N., and S. H. Goodgal. 1966. On the nature of recombinants formed during transformation in *Haemophilus influenzae*. *J. Gen. Physiol.* **49**(2):197-208.
8. Setlow, J. K. 1967. The effects of ultraviolet radiation and photoreactivation, p. 157-209. In M. Florkin and E. H. Stotz (ed.), *Comprehensive biochemistry*, vol. 27. Elsevier Publishing Co., Amsterdam.
9. Setlow, J. K., M. E. Boling, and F. J. Bollum. 1965. The chemical nature of photoreactivable lesions in DNA. *Proc. Nat. Acad. Sci. U.S.A.* **53**:1430-1436.
10. Setlow, J. K., D. C. Brown, M. E. Boling, A. Mattingly, and M. P. Gordon. 1968. Repair of deoxyribonucleic acid in *Haemophilus influenzae*. I. X-ray sensitivity of ultraviolet-sensitive mutants and their behavior as hosts to ultraviolet-irradiated bacteriophage and transforming deoxyribonucleic acid. *J. Bacteriol.* **95**:546-558.
11. Setlow, J. K., M. L. Randolph, M. E. Boling, A. Mattingly, G. Price, and M. P. Gordon. 1968. Repair of DNA in *Haemophilus influenzae*. II. Excision, repair of single-strand breaks, defects in transformation and host cell modification in UV-sensitive mutants. Cold Spring Harbor Symp. Quant. Biol. **33**:209-218.
12. Setlow, R. B., and W. L. Carrier. 1966. Pyrimidine dimers in ultraviolet-irradiated DNA's. *J. Mol. Biol.* **17**:237-254.
13. Setlow, R. B., and J. K. Setlow. 1962. Evidence that ultraviolet-induced thymine dimers in DNA cause biological damage. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1250-1257.
14. Spencer, H. T., and R. M. Herriott. 1965. Development of competence of *Haemophilus influenzae*. *J. Bacteriol.* **90**:911-920.
15. Steinhart, W. L., and R. M. Herriott. 1968. Fate of recipient deoxyribonucleic acid during transformation in *Haemophilus influenzae*. *J. Bacteriol.* **96**:1718-1724.
16. Steinhart, W. L., and R. M. Herriott. 1968. Genetic integration in the heterospecific transformation of *Haemophilus influenzae* cells by *Haemophilus parainfluenzae* deoxyribonucleic acid. *J. Bacteriol.* **96**:1725-1731.
17. Wulff, D. 1963. Kinetics of thymine photodimerization in DNA. *Biophys. J.* **3**:355-362.