

Recognition of Altered Deoxyribonucleic Acid in Recombination

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Kinetics of inactivation of transduction by phage P1bt which had been treated with ultraviolet light (UV) or nitrous acid (NA) was examined. With *Escherichia coli* B/r (radiation-resistant), low doses of UV increased transduction frequency, but the frequency was exponentially inactivated by higher doses. Little initial stimulus was observed in strain B_{s-1} (radiation-sensitive). The final rate of decay was the same as in B/r. The initial stimulus of transduction in B/r was probably a consequence of increased recombination resulting from dark repair. It was estimated that another nucleotide within 1000 nucleotide pairs had to be damaged by UV to prevent a given nucleotide from successful transduction. The NA dose response was the same for the two strains. An initial stimulus of transduction was followed by exponential decline. The UV-repair enzymes missing in B_{s-1} were not required for repair of NA-induced damage to transducing or lytic phage DNA. Low recovery of new mutations in the transductants showed that mutagen-induced damage to transducing DNA was excluded from recombinant chromosomes. The few recovered mutants may have resulted from "normal" error in recombination.

After treatment of transforming deoxyribonucleic acid (DNA) or transducing phage with a mutagen, new mutations closely linked to donor genes selected in the transformants or transductants have been recovered (1, 2, 27). The procedure appeared to be a useful one for obtaining mutations in small selected regions of the chromosome. This paper shows that in transduction of *Escherichia coli* with phage P1bt the frequency of production of such mutations is extremely low, that "damaged" regions of DNA are selectively excluded from recombinants, and suggests that the few mutants obtained resulted from "normal" error in recombination. The same general results were obtained using ultraviolet (UV) irradiation, the primary effect of which is the production of pyrimidine dimers in DNA (22), and nitrous acid (NA), which deaminates bases (31).

The results also showed that the stimulation of transduction frequency by low doses of UV irradiation requires the dark-repair enzymes missing in the UV-sensitive *E. coli* strain B_{s-1}, but they are not required for stimulation of transduction by NA, nor for repair of NA-induced damage to lytic phage DNA.

Thus, the principal questions answered here are the following. (i) Can mutagen-induced lesions in DNA of a transducing phage be successfully recombined into the chromosome of a re-

ipient cell? (ii) Are the mechanisms for repair of NA- and UV-induced lesions identical? (iii) Are dark-repair genes required for stimulation of transduction by UV or NA?

MATERIALS AND METHODS

Media. The growth media have been described (21). Glycerol (0.4%) was used as carbon source. For selection against azide-sensitive cells, the medium was supplemented with 3.4 mM sodium azide (37). Medium B consisted of 10⁻³ M MgSO₄ and 0.5 M ammonium acetate, pH 4.3. Medium T consisted of 0.02 M MgSO₄, 0.025 M CaCl₂, 4% tryptone broth (TB), 0.4% glycerol, and 0.5 M tris (hydroxymethyl) aminomethane (Sigma Chemical Co., St. Louis, Mo.; 7-9), pH 8. Medium T with cells consisted of cells grown exponentially in minimal glycerol and leucine medium, washed in 10⁻² M MgSO₄, and suspended in medium T.

Abbreviations. The abbreviations, *ara*, *leu*, and *azi*, refer to the arabinose, leucine, and azide genes. Ara⁺ and Leu⁺ refer to the ability to utilize arabinose as carbon source or to synthesize leucine. Azi^r indicates resistance to azide. Ara^r indicates ability to grow on glycerol in presence of arabinose.

Phage. Production of phage P1bt (hereafter designated P1) was as previously described (20). Titers were routinely determined by plating on a derivative of *E. coli* B/r which gives better plaques and higher efficiency of plating of P1 than other *E. coli* strains available. This property appeared in the progeny of a cell which had survived treatment with nitrosoguanidine.

Bacterial strains. A streptomycin-resistant mutant of *Shigella dysenteriae*, Sh16, came from the Bertani collection (4). *E. coli* B/r and B_{a-1} are radiation-resistant and -sensitive derivatives of *E. coli* B. Genotype of strain B/r in this respect is *lon rad*, and of B_{a-1} is probably *lex, uvrB, lon* (9, 18, 29). In every experiment, the radiation-resistance of each strain was verified by the streak-test procedure of Greenberg (17).

Derivative strains containing the *araD139 leuB1 azi* mutations were constructed by transduction with selection for azide-resistance, and unselected cotransfer of the other markers. The genes, *ara leu azi*, were jointly transduced at a frequency of about 7%.

NA inactivation. The reaction was carried out at 25 C essentially as described by Freese (14). A 0.5-ml amount of phage was added to 4.4 ml of medium B. A 0.1-ml amount of 5 N NaNO₂ was then added (zero time). Periodically samples were diluted 10-fold into ice-cold medium T and cells (multiplicity of infection about 0.5), and phage adsorption was allowed for 1.5 hr at room temperature (about 25 C). Samples were then plated in 5 ml of melted minimal glycerol agar medium; 4 to 8 hr later, a 15-ml top layer containing 0.4% glycerol (for Leu⁺ transductants) or 0.4% glycerol and 0.025% L-arabinose (for arabinose-resistant Leu⁺ transductants) was added. The transductants were counted after 5 days at 37 C.

UV inactivation. A thin layer of phage (constantly agitated in a glass petri dish imbedded in crushed ice) was exposed to an 8-w germicidal bulb (General Electric, model G8T5) at a distance of 50 cm. At this distance, the dose rate was 1,330 ergs/mm² per min. Periodically, samples were removed and adsorption and plating were carried out as described previously. To avoid photoreactivation, all operations were performed in dim, yellow light, and the adsorption mixtures were left in the dark.

RESULTS

Selection of arabinose-resistant mutants in transduction. An *araD139* mutant lacks a functional L-ribulose-5-phosphate-4-epimerase, and its growth is severely inhibited by arabinose, resulting from the accumulation of ribulose-5-phosphate (13). Secondary mutants resistant to the inhibition by arabinose (Ara^r), but still unable to metabolize arabinose, can be selected from an *araD139* population by plating on medium containing glycerol plus arabinose. These have been shown to contain a second mutation in the *araA*, *araB*, or *araC* gene; this mutation relieves the arabinose inhibition, presumably by preventing the accumulation of ribulose-5-phosphate (5). Thus, resistance should be genetically recessive.

To determine the time necessary for expression of the second mutation (as shown by resistance to inhibition by arabinose), a phage stock grown on such a double mutant was used to transduce an *araD139 leuB1* recipient (see Fig. 1). The infected cells were plated in minimal glycerol agar medium and periodically challenged with arabinose to

select Ara^r Leu⁺ transductants. Figure 2 shows that by 4 hr after infection, segregation was complete and a maximal number of Ara^r Leu⁺ transductants was recovered. In all subsequent experiments, at least 4 hr (usually 6 to 8 hr) was allowed for expression before selection was applied.

Inactivation by UV irradiation. As shown in

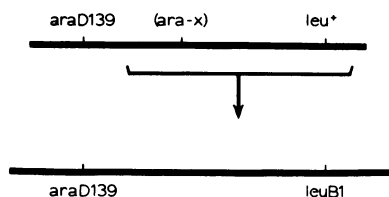


FIG. 1. The region of the transducing DNA which must be incorporated into the recipient chromosome to give an Ara^r Leu⁺ transductant. In the diagram, *ara-x* refers to any *ara* mutation which prevents the formation of a functional isomerase or kinase. In the control experiments, the phage were grown on a strain carrying such a mutation. In the other experiments, *ara-x* is any such mutation induced by treatment of the phage with UV or NA.

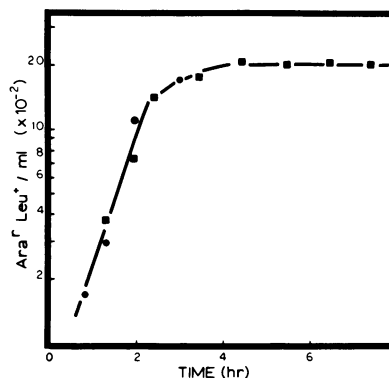


FIG. 2. Frequency of Ara^r Leu⁺ transductants as a function of time after infection at which arabinose was added for selection. Phage grown on an *araD139 ara-6032* host was allowed to adsorb to an *araD139 leuB1* recipient for 20 min at room temperature. The mixture was diluted and samples were plated in 5 ml of melted minimal salts agar and glycerol. At the times indicated, a 15-ml top layer containing glycerol and arabinose was added. At 8 hr after infection, all plates were moved to an incubator at 37 C. The maximal number of transductants was 47% of the number of Leu⁺ transductants. Symbols: ■, standard minimal medium (7); ●, H medium [twice the phosphate salts and one-fifth the ammonium sulfate as compared to standard medium (20)]. Growth of some *ara* mutants is inhibited by arabinose in standard medium, but not in H (D. Isaacson, Ph.D. Thesis, University of Pittsburgh, 1964). The similar segregation kinetics validate the use of the standard medium, since there is no obvious selection against the appearance of the Ara^r recombinants in it.

Fig. 3, the lytic phage was inactivated by UV much more rapidly when titered on the radiation-sensitive B_{s-1} than on B/r (10). *Shigella* appears to have a mechanism for repairing UV damage to P1 which is about as efficient as the system in strain B/r.

The phage population, which was grown on an *araD139 leu⁺* host, was used to transduce an *araD139 leuB1* recipient to *Leu⁺* (see Fig. 1). The kinetics of the inactivation of transduction by UV when strain B/r was recipient is shown in Fig. 4. An initial rise in transducing titer was followed by exponential decline. Such an initial rise was observed first by Garen and Zinder (15) and

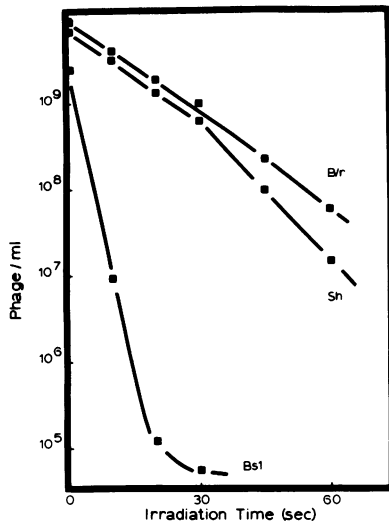


FIG. 3. UV inactivation of phage P1 as titered on *Shigella* or on *E. coli* B_{s-1} or B/r.

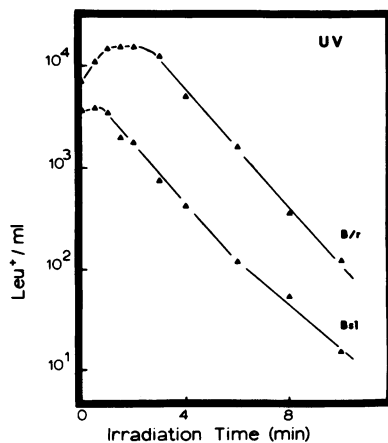


FIG. 4. Number of *Leu⁺* transductants of strain B/r or B_{s-1} as a function of the amount of irradiation of the phage.

subsequently explained as the result of conversion of abortive to complete transductants by increased recombination (3). A similar phenomenon caused by NA was explained in the same way (1).

The same phage population was used to transduce an *araD139 leuB1 azi* derivative of strain B_{s-1} to *Leu⁺* (Fig. 4). In this recipient, little initial increase in transducing titer was seen. Instead, the decay in numbers of *Leu⁺* transductants was continuous, almost from zero dose. Consistently, the B_{s-1} recipient showed a two- to threefold lower transducing efficiency than B/r at zero dose. The rate of decay did not differ from the final exponential decay rate in strain B/r (Fig. 4); the rate was about 0.14 of the rate of decay of lytic titer as measured on B/r, or 0.02 as measured on B_{s-1} . The similarity of final inactivation rates for transduction probably reflects saturation of repair enzymes in B/r; however, if damaged DNA is repaired, it may still not be capable of undergoing recombination. This appears to be true of irradiated DNA transferred in conjugation (35).

Inactivation by NA. The inactivation rate of the lytic phage was the same whether titered on B/r or B_{s-1} (Fig. 5). (A lower efficiency of plating on B_{s-1} is shown in both Fig. 3 and 5.) Howard-Flanders has reported that a strain radiation-sensitive because of a *uvrA*, *uvrB*, or *uvrC* mutation is also more sensitive to killing by NA (23, 24). Thus, the *uvrB* product (which is probably defective or missing in B_{s-1}) is involved in repair of NA-induced damage to the host chromosome, but not of damage to DNA which is external to

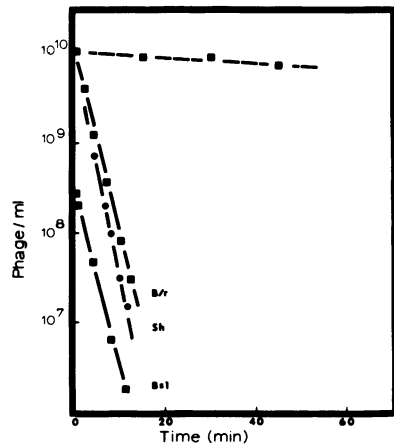


FIG. 5. NA inactivation of phage P1 as titered on *Shigella*, or on *E. coli* B/r or B_{s-1} . Titer on B_{s-1} should be multiplied by 10 to obtain the true titer. Control without NA (dotted line) is shown for B/r only because curves were similar for all three indicator strains. Other experiments showed NA-inactivation to be exponential for at least five logs.

the chromosome. This is consistent with results indicating that NA-treated phage T1 shows the same inactivation rate whether plated on normal or UV-irradiated hosts (30).

The kinetics of inactivation of Leu^+ -transducing capacity appear similar on B/r and B_{s-1} (Fig. 6). The same initial rise is seen, and the difference in final inactivation rates is probably not significant. Obviously, the dark-repair processes which are defective in B_{s-1} play no essential role in repair of NA-induced damage to the phage genome, or in the formation of complete transductants. The final inactivation rate may reflect damage which prevents DNA injection into the host. The extent of such damage is probably not large, because the lytic phase is inactivated much more rapidly than the transducing phage.

Ara^r Leu^+ transductants. At the same times as Leu^+ transductants were selected, selection was also made for Ara^r Leu^+ transductants. Such transductants might result from a mutation induced in the phage by the mutagenic treatment and cotransduced with leu^+ , or from spontaneous mutation during the processes involved in transduction. The probability of transducing to Leu^+ a recipient which had spontaneously mutated to Ara^r prior to infection was extremely low. In these experiments, the frequency of Ara^r cells within the recipient population was about 7×10^{-7} in both strains B/r and B_{s-1} (Table 1).

Table 1 compares the frequencies of appearance of Ara^r Leu^+ transductants after various treatments of the phage. The frequency is extremely low in both B/r and B_{s-1} , indicating (i) mutagen-induced damage is excluded from recombination, (ii) it is repaired with complete efficiency, or (iii) if damaged regions are involved in recombination, lethality occurs. Complete repair can be ruled out because of the obvious inactivation of both lytic and transducing titer, and, also, because final inactivation rates of Leu^+ transducing ability are the same in B/r (which can repair UV-induced damage) and in B_{s-1} (which is defective in such repair). Thus, damaged regions can not be incorporated into the recipient genome or such an event is lethal to the cell, and the extent of incorporation of repaired DNA is small.

The frequency of Leu^+ transductants which were Ara^r appeared to be independent of the dose of mutagen applied. Since the frequency in B/r, although low, is 10- to 100-fold higher than the frequency of Ara^r cells in the recipient population (Table 1), these mutants may have resulted from "normal" error in the recombination events which gave rise to transductants. Ara^r Leu^+ transductants were recovered from the radiation-sensitive recipient B_{s-1} at about the same frequency (Table 1). Treatment of the infected cells and the

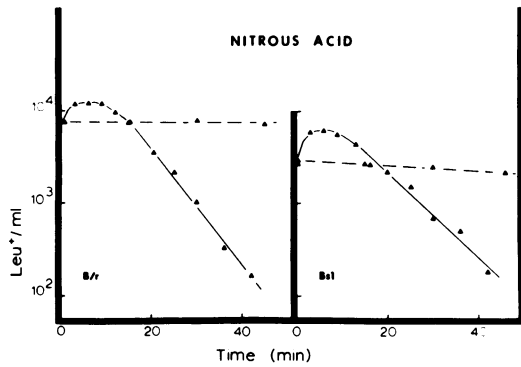


FIG. 6. Number of Leu^+ transductants of strain B/r or B_{s-1} as a function of time of treatment with NA. Transductants from control phage not treated with NA are indicated by the dotted line.

TABLE 1. Frequency of Ara^r Leu^+ transductants after treatment of the phage with NA or UV

Treatment	Ara ^r Leu^+ / Leu^+ $\times 10^6$	
	B/r recipient	B_{s-1} recipient
Ultraviolet irradiation	1.3 (2/159,000) ^a	— (0/25,900) ^a
Nitrous acid	7.3 (8/110,000)	— (0/18,400)
None ^b	7.6 (5/65,500)	5.4 (4/73,500)
Total	4.5 (15/334,500)	3.4 (4/117,800)

^a Actual numbers of Ara^r Leu^+ transductants per total Leu^+ transductants plated are indicated in parentheses. The frequency of Ara^r mutants in the uninfected populations ranged from 4×10^{-7} to 9×10^{-7} in these experiments. Samples containing equal numbers of infected cells were plated selecting for Ara^r Leu^+ transductants at each of the times at which Leu^+ transductants were selected in the experiments of Fig. 4 and 6. Numbers from treated phage are cumulative over the dose range examined, and are combined from several experiments. Following are the actual doses at which mutants were obtained and the number of mutants isolated: UV, 2 min (1), 4 min (1); NA, 6 min (2), 9 min (1), 12 min (2), 15 min (1), 20 min (1), 42 min (1).

^b Zero dose controls from the above experiments.

uninfected controls was essentially identical, except for the presence or absence of the phage and the addition of leucine to the control. If the higher frequency of mutations among the transductants resulted from spontaneous mutations occurring during growth on the plate prior to the addition of the selective agent, then the transductants would have to undergo a minimum of three more generations of such growth than the control population. This possibility seems unlikely, since it implies that growth of the control population is almost completely stopped on plating, whereas growth of the treated population is unaffected. It appears unlikely that the excess mutants after transduction result from some defect in the method used to demonstrate their presence.

To confirm the conclusion that altered DNA is excluded from recombinant chromosomes or is lethal, selection was also made for Azi^r Leu⁺ transductants in one experiment. The *azi* and *ara* genes are linked at about the same distance from *leu* (on opposite sides). No Azi^r Leu⁺ were found among approximately 7,200 (B/r recipient) or 3,600 (B_{s-1} recipient) Leu⁺ transductants from untreated phage, nor among 75,000 (B/r recipient) or 13,000 (B_{s-1} recipient) Leu⁺ transductants from phage treated with UV (doses ranging from 30 sec to 10 min). Such transductants were easily obtained when the phage had been grown on an *azi* host.

It was not possible to demonstrate directly the production of new Ara^r or Azi^r mutations in the recipient after infection with treated phage because the background frequency of such mutants in the recipient population was too high.

DISCUSSION

Extracellular treatment of both phage and transforming DNA by UV or NA has been shown to lead to mutation in progeny phage and in transformants (11, 28, 32). Failure to observe new *ara* mutations in these experiments probably is a consequence not only of the low frequency of incorporation of altered DNA, but also of the large distance between the *ara* and *leu* genes and the low probability for joint transduction of genes this far apart after UV- or NA-induced damage. Thus, the procedure is of doubtful use as a general means for obtaining new mutations at specific loci, except possibly where the mutation induced is very close to the selected locus. Such a procedure has been used for mutagenizing transforming DNA and transducing phage, and later recovering new mutations closely linked to the donor locus selected in transformation or transduction (1, 2, 27). However, the frequency of recovery of such mutants was low (see 6) and many of the mutants obtained were leaky (1, 32). Leaky mutants would not be obtained through the selection procedure used here, although several leaky *ara* mutants were obtained in other experiments by directly selecting Leu⁺ transductants and then examining their Ara phenotypes.

It is plausible that the mutants which have been recovered resulted not from the mutagenic treatment, but rather from mutations produced by the transduction or transformation process itself (Table 1). Such mutation has been reported to occur at high frequency in transformation of *Bacillus subtilis* (36); also, Demerec found that when many auxotrophs were transduced with phage grown on the same strain, significantly larger numbers of wild-type variants than occurred by spontaneous reversion in the uninfected

control bacteria were produced (8). He considered it likely that these "selfers" resulted from increased mutability of a selfer gene in the presence of a transducing fragment. The mutations observed here may result from the same general process, although such mutation seems to me more likely to result from rare transduction by another region of the genome which shows partial, but not complete, homology with the recipient genes replaced.

The final rate of UV inactivation of Leu⁺-transducing titer was 0.02 of the rate of inactivation of lytic phage as measured on B_{s-1} (Fig. 3 and 4). Because it was shown that neither damaged nor repaired regions are recombined into the recipient chromosome to any appreciable extent, and because the inactivation curve of the lytic phage does not reflect mutations which were repaired or which did not cause lethality, I estimate that, to prevent a given nucleotide from successfully recombining into the host chromosome, another nucleotide within no more than 1% of the length of the transducing DNA fragment must be damaged by UV. This corresponds to about 1,000 nucleotide pairs, since the transducing phage contains about 10⁶ nucleotide pairs (25).

Assuming a yield of 2.6×10^{-6} pyrimidine dimers/thymine per erg/mm² (22), about 170 dimers were formed per phage particle per min of irradiation. Since there were approximately 33 lethal hits per min to lytic phage titered on B_{s-1}, this suggests that not all dimers were lethal. This evidence that some UV repair still occurs in PI-infected B_{s-1} is consistent with the small shoulder on the inactivation curve of the transducing phage on B_{s-1} (Fig. 4).

The number of lethal hits/min to the transducing phage was 0.68. Thus, only about one dimer in 250 (or more) inactivates the ability to transduce *leu*⁺; or, less than 1% of the dimers caused by UV prevent the formation of a transductant.

The questions posed in the introduction can be answered as follows. (i) Mutagen-induced lesions in DNA of P1 transducing phage can not be successfully recombined into the chromosome of a recipient cell. After 6 min of irradiation, every transducing particle contained about 1,000 dimers, an average of one dimer per every 100 nucleotide pairs. The joint transduction frequency of *leuB1* and *araD139* (the farthest *ara* mutation from *leuB1*) is about 35 to 40% with unirradiated phage. This distance is certainly in excess of 5,000 nucleotide pairs (based on the number of genes known within this span); thus, after 6 min of irradiation, the average transducing particle contained more than 50 dimers (probably over 100) in this region. Nevertheless, among several thou-

sand Leu⁺ transductants from phage irradiated for 6 min or longer, no Ara^r mutants were recovered. This, and the slow inactivation of Leu⁺-transducing ability, must be explained by recombination; this process rescued the Leu⁺ marker, while excluding dimers and other unnatural nucleotide derivatives which could lead to mutation. Results can not be explained by the lethality of transductants incorporating dimers into their chromosomes without increased recombination, because the rate of inactivation of Leu⁺-transducing ability is far lower than the rate of dimer formation in this region. Increased recombination after UV irradiation was first shown by Jacob and Wollman (26), and, for this type of system, by Benzinger and Hartman (3).

(ii) The repair mechanisms for UV- and NA-induced damage are not identical, for they do not require the same genes. Because NA-induced stimulation of Leu⁺ transduction was still observed in B_{s-1}, the enzymes missing in B_{s-1} which are involved in repair of UV-induced damage have no essential role in the repair of deaminated bases in DNA which is external to the host chromosome. The frequency of Leu⁺ transduction was lower in B_{s-1} than in B/r, even with untreated phage; whether this is the result of the *uvrB*, *lex*, or some other mutation is unknown. Furthermore, repair of NA-induced damage to the lytic phage required no genes missing in B_{s-1} (Fig. 5), but repair of UV-induced damage to lytic phage did require at least one gene missing in B_{s-1} (Fig. 3).

(iii) Dark-repair genes are required for stimulation of transduction by UV and probably by NA. The UV-induced stimulation of Leu⁺ transduction was largely eliminated in the dark-repair defective recipient B_{s-1}, showing that the dark-repair enzymes are required for the increased recombination leading to complete transductants. Both ³²P-decay and X irradiation, which result in single- and double-strand breaks in DNA, exponentially inactivate transduction with no initial stimulus (19, 33, 34). Thus, production of strand breaks in DNA is insufficient to increase recombination. Increased recombination probably results from the appearance of undamaged single-stranded regions of donor DNA produced by the excision enzymes. Such regions (or newly synthesized copies) may pair with a replicating strand of the host chromosome, and covalently link at each end to complete recombination. Current models of DNA synthesis nicely accommodate such a procedure (12, 16).

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ADDENDUM IN PROOF

Using P22 and *Salmonella*, H. Takebe (Biochem. Biophys. Res. Commun. 31:938-944, 1968) also found that enhancement of transduction by UV depended on host-cell reactivation.

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