JOURNAL OF BACTERIOLOGY, May, 1966
Vol. 91, No. 5

Ultraviolet Mutagenesis in Bacteriophage T4

I. Irradiation of Extracellular Phage Particles

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Received for publication 18 December 1965

ABSTRACT

DRAKE, JOHN W. (University of Illinois, Urbana). Ultraviolet mutagenesis in bacteriophage T4. I. Irradiation of extracellular phage particles. J. Bacteriol. 91:1775-1780. 1966.—Ultraviolet (UV) irradiation of extracellular T4 phage particles induces about 2×10^{-4} *r* mutations per lethal hit. The mutants largely escape detection unless the irradiated phages are plated with very soft overlay agar. Multiplicity reactivation is not a prerequisite for mutagenesis. A much higher frequency of base pair substitution-type mutants is induced than is found in the spontaneous background, but sign mutants are also induced. Nearly half of the mutants map into previously identified UV hot spots. The *r*II mutants induced extracellularly are very similar to those induced intracellularly. The mutants also appear to result from direct radiation effects upon the bacteriophage deoxyribonucleic acid.

The analysis of ultraviolet (UV) mutagenesis is often greatly complicated by two factors: the putative target molecule, deoxyribonucleic acid (DNA), resides within a complex and chemically highly reactive environment; and a variety of repair processes may intercede before the UV damages are expressed as heritable lesions. Thus, doubt may arise concerning the nature of the target molecule, and specific classes of mutations may be erased in certain organisms. The first difficulty may be overcome by irradiating either free DNA molecules or extracellular virus particles. The second may be overcome by the use of mutants which are unable to carry out repair processes.

The *r* mutants of bacteriophage T4 are well suited for mutational studies because forward mutations are easily screened visually, and because fine-scale mapping and reversion analysis can be carried to a level compatible with molecular dimensions (2). A previous report has described the properties of *r*II mutants induced by UV irradiation of intracellular T4 phages (7). About half of the mutants were transitions [base pair substitutions which maintain the purine-pyrimidine orientation (13)] from guanine-cytosine (GC) to adenine-thymine (AT) base pairs. [Phage T4B contains hydroxymethylcytosine (HMC) glucosylated to various degrees, but the term cytosine will be retained for simplicity.] Most of the remainder were sign mutants, which consist of additions and deletions of small numbers of base pairs (6, 9). Similar conclusions were reached (16) in a study of mutations induced by UV in phage S13.

Previous attempts to induce mutants by UV irradiation of free phages have met with mixed success. Krieg (19) was able to demonstrate UV-induced *reversion* of *r*II mutants, but he could not consistently obtain *forward* induction of *r* mutants (*personal communication*). Folsome (11), on the other hand, reported the induction of *r*II mutants, and also characterized the mutants to a certain extent. In the temperate bacteriophage λ, mutations were detected only if the host cells were also irradiated (17, 30), whereas irradiation of the temperate phage κ alone was sufficient (18). UV mutagenesis in the semitemperate bacteriophages S13 (28), T1 (31), and T3 (27) also required the irradiation of the host cell. This report will detail methods of inducing *r* mutants in free T4 phage particles by UV irradiation, and will show that the mutants are very similar to those obtained previously by irradiating intracellular phages.

MATERIALS AND METHODS

Strains of phage and bacteria and standard methods have been described previously (1, 4, 7). *Escherichia coli* and bacteriophage T4 were used throughout. The mutant T4v_1 which was used in some experiments is about twice as UV-sensitive as the wild type (15*a*). T4*r* mutants were recognized by their plaque mor-

phology on *E. coli* strain B. Stocks of *r*II mutants were grown on strain BB, which does not discriminate between *r*II and r^+ phages. Recombinant or revertant r^+ phages among an excess of *r*II phages were selectively plated on strain OP33, a K-12 (λ) derivative which leaks *r*II phages at a lower rate than does strain KB (R. Friedman, *personal communication*).

UV irradiation was delivered from a low-pressure mercury lamp to phages suspended in buffer, at a dose rate corresponding to about 7 sec per lethal hit. All platings and incubations were performed in very dim light to prevent photoreactivation.

Spot tests were employed both for mapping (3) and for base analogue reversion tests (14). Low-titer stocks were first grown by picking an isolated *r*II plaque into 2 ml of broth, adding a drop of stationary-phase BB cells, incubating for 4 hr at 37 C, and adding a few drops of chloroform. These stocks contained from 10^9 to 10^{10} phages per milliliter. They were diluted 50-fold, and 0.1 ml was plated with 0.05 ml of log-phase OP33 cells concentrated to 3×10^9 per milliliter, plus 0.05 ml of log-phase B cells at 10^8 per milliliter. After 15 min, the plates were spotted with small drops of 2-aminopurine (2AP) at 10 mg/ml and 5-bromouracil (5BU) at 2.5 mg/ml. After another 90 min, the plates were placed upright in an incubator at 37 C. The mutants multiply to a limited extent in the B cells, but only revertants grow into full plaques. Unspotted regions of the plate provide a background control. In a responding mutant, the area around the spot shows a strikingly increased density of revertant plaques. The test was calibrated with about 100 well-studied (7) *r*II mutants. All of the mutants known to respond to base analogues responded to the spot tests, while all of the sign mutants were negative. A reliable spot test for proflavine mutagenesis could not be developed.

RESULTS

Induction of mutations by irradiation of extracellular phages. Preliminary attempts to detect *r* plaques among the survivors of UV irradiation were at best irregularly successful. During the course of an unrelated experiment, however, UV-irradiated phages were plated with very soft plating agar, and large numbers of *r* plaques were observed. Further manipulation of plating conditions revealed that the relative frequency of *r* plaques was at most a weak function of (i) number of phage particles plated; (ii) quantity of plating cells; (iii) interval between UV irradiation and plating, up to several weeks; and (iv) trauma to the irradiated phage stock, such as heating for 60 min at 46.5 C. The frequency of *r* plaques was a strong function of the agar concentration in the plating agar. The maximal recovery was achieved with about 0.55% agar, which could be obtained by diluting standard soft agar either with L broth or with water. Lower agar concentrations produced an unstable overlay without improving the detection of mutants. Higher concentrations (up to 0.7%) made mutant detection less efficient and much more difficult and variable. Older plates produced an effect similar to increasing the overlay agar concentration.

Kinetics of mutation induction. The production of *r* mutants as a function of UV dose is shown in Fig. 1. The kinetics appear to be linear, the slope of the curve being 2.7×10^{-5} mutations per second. These data were obtained simultaneously with a careful determination of the inactivation rate, which followed 1.4-hit kinetics with a rate of 0.14 lethal hits per second. The mutation rate is therefore 1.9×10^{-4} mutational hits per lethal hit. (The two points represented by squares were obtained with T4*v*, and the corrected UV dose was calculated from the ratio of the UV sensitivities of T4*v* and T4v^+. The two sets of mutants were indistinguishable in further tests, and have therefore been grouped.)

Phenotypic characterization of the mutants. Several dozen spontaneous mutants, and several hundred UV-induced mutants, were streaked on B, BB, and OP33 cells. The phenotypic classification of these mutants is summarized in Table 1. All of the UV-induced mutants were obtained from the populations represented by the 54- and 60-sec points of Fig. 1, and therefore contain 20 to 25% of spontaneous mutants. Frequencies of *r* types among the UV mutants have therefore been corrected for the spontaneous background. The following phenotypes were observed.

(*i*) *The rI phenotype.* Isolates which exhibit the *r* plaque morphology on all three cell types are *r*I, and map in a region far from the *r*II cistrons (2). They are strongly induced by conditions leading to base pair substitutions, but are not induced by conditions generating sign mutations (Drake, *unpublished data*; Brenner, *personal communication*). A relatively small fraction of the spon-

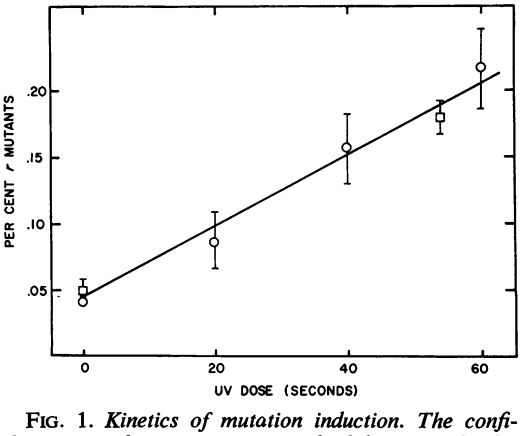

FIG. 1. *Kinetics of mutation induction. The confidence intervals represent one standard deviation. Circles and squares are different experiments.*

TABLE 1. *Classification of spontaneous and UV-induced r mutants*

UV*	Total r mutants	rI	Ordinary rII	Anomalous rII (+ rIII)
0	82	24 (29%)	49 (60%)	9 (11%)
+	457	263 (58%)	156 (34%)	38 (8%)
+ (corrected)		(66%)	(27%)	(7%)

* The UV-induced mutants were obtained from the populations represented by the 54- and 60-sec points of Fig. 1, and therefore contain an average of 23% of spontaneous mutants. Subtracting the contribution of the spontaneous mutants yields the corrected values.

taneous mutants (29%), but a majority of the induced mutants (58%), were *r*I.

(*ii*) *Ordinary rII mutants.* Isolates which exhibit the *r* plaque morphology on B cells, the r^+ plaque morphology on BB cells, and which fail to grow (except for infrequent revertants) on OP33 cells, are *r*II. Since the *r*II function is dispensable for growth in B cells, any type of mutational lesion may be recovered. The majority (60%) of spontaneous mutants, but only 34% of the UV-induced mutants, were *r*II.

(*iii*) *Anomalous rII mutants.* This is a heterogeneous group, forming plaques on OP33 and other λ-lysogens at relatively high frequencies (0.1 to 100%), and exhibiting the r^+ plaque morphology on BB cells. Approximately 10% of the mutants, both spontaneous and UV-induced, fall into this class. Many revert to wild or pseudowild types at high rates, so that up to several per cent of the plaques on B cells are r^+. They are therefore only very approximately mappable within the *r*II region. The molecular structure of highly reverting lesions is largely unexplored; recombination may be involved, rather than a simple chemical instability. Others tend to form minute plaques on OP33, and a variety of plaque morphologies on B cells ranging from fully *r* to nearly r^+. These may be shown under appropriate conditions to revert at relatively *low* frequencies. They are leaky *r*II mutants, and probably include most of the mutants previously designated *r*III (2, 10). Most are also temperature-sensitive, plating even more poorly on OP33 cells at 42 C than at 37 C. Pseudowild revertants from highly reverting *r*II mutants are also often temperature-sensitive.

Genotypic characterization of the mutants. Techniques are only available for the detailed study of *r*II mutants. Such mutants are well described by two parameters: nature of the mutational lesion, and map location. The nature of the mutational lesion in an *r*II mutant is fairly well specified by determining the reversion response of the mutant to specific mutagens (7, 9, 13, 14). Mutants revertible by base analogues contain base pair substitutions, usually transitions. Mutants revertible by proflavine contain sign mutations. Mutants revertible by neither may contain transversions (base pair substitutions in which the purine-pyrimidine orientation is reversed). Simple and reliable spot tests (see Materials and Methods) rapidly characterize the responses of *r*II mutants to base analogues. These tests were applied to the present collection of *r*II mutants, and the results are summarized in Table 2. Few of the spontaneous mutants (22%) contained base pair substitutions, the majority probably containing sign mutations. About half of the UV-induced *r*II mutants contained base pair substitutions, and the remainder also probably contained sign mutations (7).

It was anticipated that mutants induced by UV irradiation of extracellular phages would resemble those previously induced intracellularly (7). Most (192 of 328, or 59%) mutants in the former collection mapped into a few hot spots (sites where identical mutations frequently recur). The present collection was therefore compared in recombination spot tests with known UV hot spots, composed of sites with five or more recurrences (except for UV199, with only four recurrences). Many (69 of 150, or 46%) of the extracellularly induced mutants did indeed fall into previously identified UV hot spots (Fig. 2). (The sizes of the original hot spots were normalized to the present sample size in Fig. 2.) Only two UV hot spots, UV46 and UV202, went unrepresented. The size of the mapped sample (150) was probably sufficiently small to allow one or two sites to escape detection. UV2 and UV6 are also large spontaneous hot spots, and UV16 and UV24 are smaller spontaneous hot spots. The results of the base analogue spot tests are also represented

TABLE 2. *Responses of spontaneous and UV-induced rII mutants to base analogues*

UV*	Total rII mutants	Responding to 2AP and/or 5BU
0	49	11 (22%)
+	156	69 (44%)
+ (corrected)		(59%)

* The UV-induced mutants were obtained from the populations represented by the 54- and 60-sec points of Fig. 1, and therefore (see Table 1) contain an average of 41% of spontaneous *r*II mutants. Subtracting the contribution of the spontaneous mutants yields the corrected values.

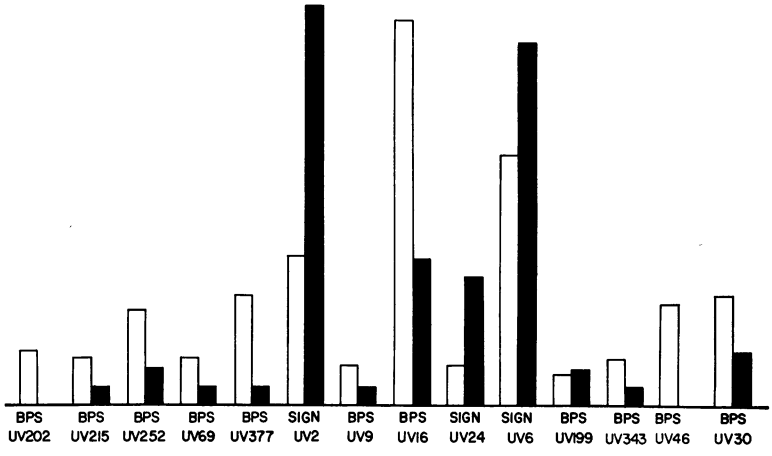

FIG. 2. *UV hot spots and mutational lesion types. BPS = all base pair substitution mutants. SIGN = all sign mutants. Solid columns, extracellularly induced mutants. Open columns, intracellularly induced mutants (7).*

in Fig. 2. The mutations at each site were either all base pair substitutions (BPS), or all sign mutations (SIGN). The original BPS mutants were all transitions containing adenine-thymine base pairs (7).

A small fraction—about 4%—of both the spontaneous and the UV-induced *r*II mutants were deletions, as determined by their failure to revert and their failure to recombine with two or more point mutants. The numbers involved were too small to estimate whether or not deletions were actually induced by irradiation.

DISCUSSION

Method. Several factors affect the plaque morphology of *r* mutants on the screening plates. The survivors of UV irradiation form small and irregular plaques. Most of this variation is phenotypic, the phages in the plaques being genotypically wild type. The sharp reduction in plaque size and the concomitant increase in plaque size range made identification of *r* plaques increasingly difficult as a function of UV dose. Most of the induced mutants also appeared in mottled (heterozygous) plaques. Since r^+ phages may be selected during plaque growth on B cells, only those plaques which experience break-away growth of *r* sectors tend to be scored as mutants. The very soft agar overlay increased plaque sizes generally, and probably preferentially increased the sizes of *r* regions.

The mutant frequency did not depend upon cell or phage densities on the screening plates. Anomalously large mutant frequencies (up to 0.6% at 60 sec) were occasionally noted, but were found to be artifacts due to multiplicity reactivation (MR; 21), appearing selectively on the mutant screening plates, but not on the plates used for counting r^+ plaques (with about four-fold fewer phages). In fact, both mutant and wild-type plaque frequencies were approximately equally increased by MR on the plate. MR apparently is not a prerequisite for recovery of induced mutants (11, 12). Attempts to detect induced mutants either by MR or by cross-reactivation (multiple infection between irradiated and unirradiated phages leading to marker rescue from the irradiated phages) consistently led to *reduced* mutant frequencies in the resulting lysates (Drake, *unpublished data*). This result suggests that the proportion of *r* mutants to r^+ in any given burst is quite small, and is reminiscent of the delayed origin of mutants induced by ethyl methanesulfonate (15).

The UV-induced mutants differed strikingly from the background of spontaneous mutants (Tables 1 and 2), particularly in exhibiting a larger fraction of *r*I among the *r* mutations and a higher frequency of transitions among the *r*II mutations. Specific UV hot spots were also induced (Fig. 2). The origin of the mutants by selection of pre-existing spontaneous mutants is therefore excluded. Reconstruction controls had also previously established that the UV hot spots were not selection artifacts (7).

Kinetics of mutation induction appear to be linear (Fig. 1), so long as MR artifacts are avoided by determining *r* and r^+ frequencies on strictly equivalent plates. Thus, multiple-hit mechanisms of UV mutagenesis—such as dimer formation followed by dimer dissolution—are made improbable. The induction rate was about 2×10^{-4} *r* mutations per lethal hit (where one lethal hit reduces survival by e^{-1}). This is a frequently recurring value for a variety of mutagens, such as heat and low *p*H, 3.5×10^{-4} (13), and radio-

mimetic polymines, 3×10^{-4} (8). A value of about 2.5×10^{-4} may be calculated for UV from the data of Folsome (11), but other aspects of those data are in serious disagreement with the present results. The value for nitrous acid is about 2×10^{-3} (29). Other mutagens, however, are much less lethal, such as hydroxylamine, 2.1×10^{-2} (23), and ethyl methanesulfonate, about 10^{-2} (15). The reasons for this variation among mutagens are not presently clear, but probably reflect the different modes of chemical attack on different bases (29).

Other systems. UV mutagenesis has been studied in two distinctly different types of bacteriophage systems. In the first, typified by the T-even phages, the phage DNA lacks homology altogether with the host cell DNA (22), and is, in fact, chemically distinct in its content of glycosylated HMC. Here, irradiation of extracellular phages is sufficient to produce mutations (11, 12, 19). [An earlier failure to detect T2*h* mutants after extracellular irradiation (20) probably resulted from phenotypic mixing.] The only previous report of the induction of T4*r* mutants (11) presented results quite different from those obtained here. Only 16% of the spontaneous and 21% of the UV mutants were *r*II. compared with about 70% and about 34%, respectively, in the present collection. Many more deletions appeared among both the spontaneous and the UV mutants than were seen here. Base pair substitution-type mutants constituted only 11% of the *r*II mutants, versus 59% in the present collection. As far as can be estimated from the data, only a few of the 14 largest UV hot spots could have been represented in the Folsome collection. On the whole, fewer differences were seen between spontaneous and induced mutants in the Folsome collection than in the present collection.

Temperate and semitemperate phages (mostly of *E. coli*) constitute the second type of system. Here, the phage DNA is usually chemically indistinguishable from, and may in fact contain regions of homology with, the host DNA (5). Mutagenesis requires irradiation of both the phage and the host cell [λ (30), T1 (27), T3 (31), S13 (28)], or else of the host cell only [λ (17)]. The induction of *c* mutations by irradiation of phage κ alone (18, 25) may represent a special case: other κ mutations are not induced by extracellular irradiation, and *c* mutations are only detected by irradiating phages grown on certain host cells, and by plating them on specific host cells. It is possible in all of these systems that only a few mutations (about 10^{-4} per lethal hit) are produced directly, but that the great majority of mutations (up to several per cent of the survivors) result from UV-induced recombination with the host cell (26). It is also possible that many mutations are normally erased by cellular repair systems, and that irradiation of the host inhibits this repair.

The primary mutagenic target is strongly suggested by the present results to be DNA, and by previous results (7, 16) to be cytosine, at least for the induction of transitions. On the other hand, the UV sensitivity of a certain strain of tobacco mosaic virus depends upon the association of the viral nucleic acid with its coat protein (24), so that modifying effects of the phage coat protein cannot be ruled out even in the present case. Experiments to be presented in a succeeding paper of this series do, however, indicate that neither irradiation of the host cells alone, nor of the suspending buffer, is mutagenic.

The present system makes possible new experiments on the molecular mechanism of UV mutagenesis. Photoreactivation, which is technically impossible after intracellular irradiation but easy after extracellular irradiation, may be used as a test of the role of pyrimidine dimers in UV mutagenesis. Chemical modifications of irradiated but unplated phages are also feasible.

ACKNOWLEDGMENTS

This investigation was initiated by a fortunate accident at the Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, England, while the author was a Guggenheim Fellow. I am very grateful to Sydney Brenner and Francis Crick for providing a constantly stimulating environment during my visit to their laboratory in 1964–1965. I am also grateful to Mrs. Leslie Barnett for so often permitting me to keep the lights turned off in her laboratory, in order to avoid photoreactivation.

This investigation was supported by Public Health Service research grant AI-04886 from the National Institute of Allergy and Infectious Diseases and by grant E59 from the American Cancer Society.

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