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Mutagenesis in *Escherichia coli*

III. Requirement for DNA synthesis in mutation by gamma rays of T4-phage complexed with *Escherichia coli*

By B. A. BRIDGES, RACHEL E. DENNIS
and R. J. MUNSON
M.R.C. Radiology Unit, Harwell, Didcot, Berkshire, U.K.

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SUMMARY

A system has been developed for the study of reversion of an amber mutation responsible for a deficiency in DNA synthesis in T4 phage E51. When complexed with bacteria able to suppress the amber mutation the induced mutation rate per phage genome per rad is

$$1{\cdot}56 \times 10^{-11} \pm 0{\cdot}13 \times 10^{-11}.$$

When complexed with bacteria unable to suppress the amber mutation (and being thus unable to synthesize phage DNA) the induced mutation rate is at least 14 times lower indicating that DNA synthesis is necessary for the production of the majority of functional reversions at the amber site. The induced mutation rate in suppressor-containing bacteria is independent of multiplicity of infection between 0·2 and 5, suggesting that recombination immediately after irradiation between phage genomes is unlikely to be a requirement for the mutation process.

1. INTRODUCTION

Recent work on base-change mutation in bacteria has revealed that the process of mutation after gamma irradiation is a metabolic process carried out by the cell in response to relatively non-specific damage to DNA and that the Exr^+ phenotype is necessary for the induction of at least 90 per cent of the mutations in some strains of *Escherichia coli* (Bridges, Law & Munson, 1968). The *exr* locus is one of the genes affecting both radiosensitivity and recombination (Witkin, 1967, 1969). It is also involved in mutation induction by ultraviolet light, and a current hypothesis suggests that it is involved in the filling (possibly by a recombinational mechanism) of gaps in newly synthesized DNA produced at the first replication of DNA containing pyrimidine dimers (Witkin, 1967, 1969; Bridges, Dennis & Munson, 1967). Its action may be described as rendering such repair more efficient while at the same time permitting a significant proportion of errors which subsequently become manifest as mutations.

For mutations induced by ionizing radiation such evidence as is available suggests that replication of damaged DNA is not necessary for mutations to be

established. For example, premutational lesions seem likely to be those single strand lesions formed directly after ionization which can be detected as single strand breaks (Munson & Bridges, 1969). In addition both daughter duplexes at the first replication after irradiation bear the newly induced mutation (Munson & Bridges, 1964), the simplest interpretation of which is that both parental strands are mutated before passing through the replication complex.

Because of the photoreversibility of premutational lesions induced by ultraviolet light in an Hcr$^-$ strain, it has been possible to show that mutations are not 'established' (i.e. do not lose photoreversibility) if DNA replication is inhibited (Bridges & Munson, 1968; Nishioka & Doudney, 1969). For ionizing radiation the most that can be achieved in bacteria is to follow the expression of mutations when DNA synthesis is inhibited (i.e. the ability of newly-induced prototrophic mutants to form colonies on unsupplemented minimal plates). Kada, Doudney & Haas (1961) found that around 50% of X-ray induced mutants of *E. coli* WP2 Trp$^-$ could be expressed in the absence of DNA synthesis. This result is, however, open to objection since one may dispute the rigour of their exclusion of DNA synthesis on two grounds. First, after 20 krads considerable breakdown of DNA is known to occur (Stuy, 1960; Emmerson & Howard-Flanders, 1965) and yet the DNA content of their population did not change for some time after irradiation. Therefore DNA synthesis must presumably have occurred to maintain the net DNA balance. Secondly, bacteria plated on minimal plates and thus deprived of the required amino acid may still complete the DNA replication cycles on which they are engaged, as they would if the same operation were carried out in liquid media (Maaløe & Hanawalt, 1961; Lark, Repko & Hoffman, 1963). This could account for further replication of some 40% of the DNA after plating. On the other hand it is possible to argue that, even if all the mutations could be functional in the absence of DNA synthesis, there might be insufficient tryptophan in the bacteria on minimal plates to permit the synthesis of the deficient enzyme in the tryptophan biosynthesis pathway.

We have therefore sought a system where the means of inhibiting DNA synthesis is less open to objection and have chosen a bacteriophage with a nonsense triplet in a cistron essential for DNA synthesis. We have irradiated this phage complexed with bacteria either with or without a suitable suppressor, and have assayed for back-mutants to wild-type at the site of the nonsense mutation by plating with a lawn of bacteria unable to suppress the nonsense triplet. When complexed with a suppressor-free bacterium, a potentially mutated phage could only produce progeny (and thus a mutant plaque) if the mutation could be established functionally (i.e. produce wild-type messenger RNA) in the absence of DNA synthesis. In a suppressor-containing complex this restriction does not apply and all potential mutants should give rise to plaques. A comparison of the gamma ray mutability of the phage complexed with suppressor-free and suppressor-containing bacteria thus reveals whether DNA synthesis is a requirement for the establishment of a functional mutation.

We have also used this system at different multiplicities of infection (M.O.I.) in an

attempt to estimate the importance of recombination between genomes in the mutation process.

2. METHODS

Micro-organisms

Bacteriophage T4 strain E51 (amber in gene 56) was supplied by Professor N. Symonds and Dr K. A. Stacey. It is described as 'DNA-minus' by Wiberg (1966) and is non-leaky; the probability of E51 undergoing a single cycle of lytic infection in a suppressor-free complex (M.O.I. = 5) is $\sim 1 \times 10^{-4}$ (S. Maynard-Smith, personal communication). This is an important property in so far as it establishes the maximum proportion of potentially mutant phages which would undergo a round of DNA replication in a suppressor-free host.

E51 is well suppressed by amber Su_I; without good suppression wild-type mutants would have a strong selective advantage and it would not be possible to prepare stocks with a low level of spontaneous mutants. Finally, E51 has a relatively low spontaneous mutation rate, of the order of 5×10^{-9} per replication (phage doubling). This is very considerably greater than that for a similar base change mutation at a nonsense triplet in *Escherichia coli* where it is of the order of 10^{-10} (R. J. Munson, unpublished observations). Wild-type T4 and amber mutants B17 and B22 were obtained from Dr S. Person.

The bacteria used were *E. coli* K12 CA 244 (no amber suppressors) and CA 266 Su_I^+, both strains supplied by Dr L. Barnett.

Culture conditions

A stock of E51 was obtained by growing it to a titre of 5×10^{10}/ml. on aerated CA 266 in Oxoid Nutrient Broth No. 2. Lysis was induced where necessary by addition of chloroform. The chloroform was bubbled off and the suspension filtered through a membrane filter and stored in a refrigerator.

Bacteria were used in early stationary phase. A refrigerated stationary phase culture was diluted about 20 fold into fresh Oxoid Nutrient Broth No. 2 and shaken at 37 °C until the density exceeded 10^9/ml.

Experimental procedure

Bacteria were filtered and resuspended in T4 adsorption buffer (Adams, 1959, p. 483) plus tryptophan (20 μg/ml.). Phage were added to the required level and the suspension was shaken at 37 °C usually for 10 min at which time T4 antiserum was added to K = 1 and incubation continued for a further 5 min. (Antiserum was a gift from Professor Symonds and Dr Stacey.) The suspension was then chilled and assayed for viable complexes and mutants before and after various doses of radiation.

Viable complexes were assayed by the layer plate technique using a lawn of CA 266. An assay of viable complexes and free phages together (i.e. plaque forming centres) was also performed in the same way immediately before the addition of antiserum. Mutant phages were assayed on a lawn of CA 244. A heavy

lawn inoculum ($\sim 3 \times 10^9$/plate) was necessary for the experiments at a low multiplicity of infection otherwise many of the non-mutant phages released after the first cycle in CA 266 complexes adsorbed on to uninfected CA 266 cells.

Free phages were estimated immediately before the addition of antiserum by diluting an aliquot into phage buffer containing 0·1 ml. chloroform, followed by further dilution and plating on to a lawn of CA 266. In some experiments free phages were separated from complexes by membrane filtration.

Uncomplexed (viable) bacteria were estimated by plating directly on to the surface of agar plates.

Dilutions were made in phage buffer and all plates were Oxoid Nutrient Broth No. 2 solidified with 1·5% Difco Purified Agar. The semi-solid layers were 0·5% Difco Purified Agar in distilled water.

Irradiations with ^{60}Co gamma rays were carried out at a dose rate of approximately 3·6 krads per minute. The suspensions were chilled and aerated throughout the exposures.

3. RESULTS

(i) *Mutation of E51—CA 266 Su⁺ complexes at high M.O.I.*

Table 1 shows the results of a typical experiment at a M.O.I. of 2·5. Despite a decrease to about 50% in the number of viable complexes, it is clear from column 3 that there is an increase with dose in the absolute number of mutants in the

Table 1. *Gamma irradiation of E51 complexed with CA 266 at M.O.I. of* 2·5

Dose (krads)	Viable complexes per ml.	Total mutants per ml.	Induced mutants per ml.*	Induced mutants per initial phage per rad*
0	$1{\cdot}02 \times 10^8$	153	0	0
36	$8{\cdot}30 \times 10^7$	253	130	$1{\cdot}41 \times 10^{-11}$
72	$5{\cdot}75 \times 10^7$	300	213	$1{\cdot}18 \times 10^{-11}$
108	$5{\cdot}07 \times 10^7$	307	230	$8{\cdot}30 \times 10^{-12}$
144	$4{\cdot}65 \times 10^7$	350	280	$7{\cdot}85 \times 10^{-12}$

*The figure in column 4 is obtained from the figure in column 3 on the assumption that the number of spontaneous mutants declines with dose at the same rate as the number of viable complexes. The figure in column 5 is obtained by dividing that in column 4 by the number of absorbed phage per ml. and by the dose.

suspension. These are shown as the number of induced mutants per rad per complexed phage making no allowance for inactivation (column 5). This calculation yields a value which decreases with dose at a rate determined by the survival of the mutated gene in a viable genome (Fig. 1, lower curve, cf. Munson & Bridges, 1964, 1966). This is seen to be parallel to that for the number of viable complexes (upper curve), a fact which may reflect a certain amount of multiplicity reactivation within the multiply infected complexes. Extrapolation of the mutation

curve to zero dose yields a value for the mutation rate per phage genome per rad in the absence of inactivation. In this experiment the value was $1{\cdot}7 \times 10^{-11}$. The mean value from a total of four such experiments is $1{\cdot}56 \times 10^{-11} \pm 0{\cdot}13 \times 10^{-11}$.

In order to estimate the number of induced mutants at any point it is necessary to subtract the number of spontaneous mutants. The latter may be divided into two classes, those pre-existing in the population before irradiation and those occurring during the first cycle of infection in the complexes. The former may be presumed to be inactivated at the same rate as the non-mutant population. The latter are presumably produced in proportion to the number of infectious centres and may again be assumed to decrease in proportion to the total population

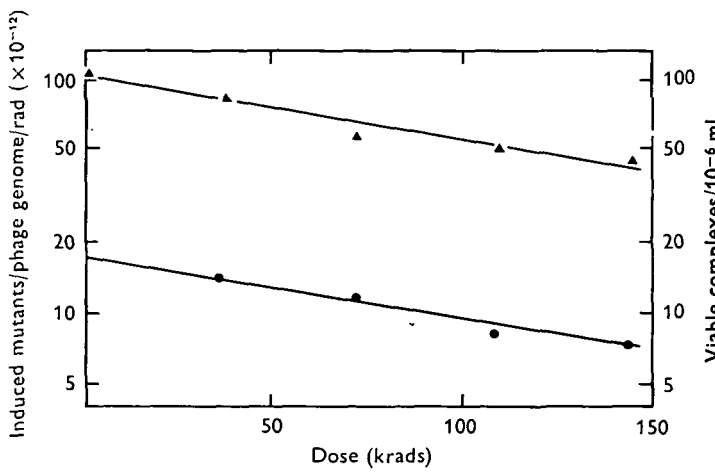

Fig. 1. Gamma radiation mutagenesis of T4 strain E51 complexed with *E. coli* CA 266 at a multiplicity of infection of 2·5. ▲, viable complexes; ●, induced mutations per phage genome per rad.

provided irradiation does not alter the burst size of the infected complex. If, as is possible, the burst size is reduced, then the number of spontaneous mutants at the higher doses may have been slightly overestimated and thus the number of induced mutants slightly underestimated. The mutation rates have, however, been obtained by extrapolating the curve back to zero dose and the extrapolate would not be significantly affected by a slight overestimate of the slope of the line.

It is noteworthy that the induced mutation rate per phage per rad is very close to that for similar base-change mutations in bacteria. In *E. coli* WP2, for example, AT–GC transitions in an ochre nonsense triplet are induced at a rate of about 10^{-11} per genome per rad. In contrast the spontaneous mutation rates, as described above, differ by a factor of the order of fifty.

(ii) *Attempted mutation of E51—CA 244 Su⁻ complexes at high M.O.I.*

Calculation of the frequency of induced mutants arising in E51–CA 244 Su⁻ complexes is a less straightforward operation because of the absence of the amber

suppressor. Although the latter simplifies the allowance for spontaneous mutations, since no significant amount of replication occurs and no mutants arise on the plates, it also prevents a direct determination of the number of viable complexes. We have followed the adsorption of E51 to CA 244 by estimating the decline in titre of unadsorbed phage in the complexing mixture, and the formation of complexes by estimating the loss of viability of the bacteria. Neither of these quantities differed significantly from those observed with CA 266 indicating that the same number of complexes were probably formed. We also tried to demonstrate the injection of E51 DNA into CA 244 by simultaneous infection with another amber strain (either B22 or B17). In theory the phages should complement each other and

Table 2. *Gamma irradiation of E51 complexed with CA 244 Su⁻ at high M.O.I.*

	Total mutants			Total mutants
Dose (Krads)	Per ml. (Expt 1)	Per ml. (Expt 2)	Dose (Krads)	per ml. (Expt 3)
0	83	74	0	52
			36	56
47	74	69	72	48
94	57	50	108	58
141	58	33	144	54
M.O.I.	5	5	—	2·5
Infected bacteria per ml.*	$1{\cdot}90 \times 10^8$	$1{\cdot}55 \times 10^8$		$1{\cdot}50 \times 10^8$

*Total number of bacteria per ml. less those able to form colonies on nutrient agar plates.

produce a plaque when plated on a lawn of CA 266. In practice the number of plaques was about half of those expected (the expected number being about half the number of non-viable bacteria, the other half being infected abortively, as is common with adsorption in buffer). There would thus seem to be evidence either of a failure to complement in all cases or of a higher rate of abortive infection when CA 244 is mixedly infected. Notwithstanding, it can be said that at least half of the expected E51–CA 244 complexes do exist and can be rescued by complementation. In our calculations we have assumed that the rate of complexing is the same as in CA 266 and that the frequency of abortive infections in CA 244 is 50 %. We also assume that any mutants induced in CA 244 survived irradiation to the same extent as if they were in a CA 266 host, since the dominant nature of the mutation should enable all the phages in the complex to replicate and recombine, whether mutant or not, and one would thus expect the normal amount of multiplicity reactivation. We have found no significant difference in the survival of wild-type T4 whether complexed with CA 266 or CA 244.

Of three experiments carried out (Table 2) two showed that the number of mutants per plate fell with increasing dose at a rate which did not differ significantly from the expected theoretical inactivation rate for complexes (cf. Fig. 1 upper curve). In these experiments there was thus no indication that any mutants

whatsoever were induced. In the third experiment, the number of mutants per plate did not change significantly up to 144 krads.

One possible explanation for this third experiment is that it reflected an unusually shallow survival curve for the whole population such as has occasionally been observed in complexes with CA 266. On the other hand it might be the result of a low rate of mutation induction which approximately balanced the loss of spontaneous mutants by inactivation. For example, if as many as half the spontaneous mutants were inactivated by 144 krads (as might be expected from Table 1), an equal number of induced mutants would represent an induced mutation rate of $1{\cdot}04 \pm 10^{-12}$ per phage per rad, which is about 7 % of the rate in CA 266 complexes. We may therefore conclude that in the absence of an amber suppressor (and thus in the absence of DNA synthesis), E 51 is mutated by gamma radiation at a rate which may be zero but which could be as much as 7 % of that in the presence of a suppressor.

(iii) *Mutation of E 51—CA 266 Su⁺ complexes at low M.O.I.*

The object of this experiment was to determine whether the presence of at least two phage genomes within a cell is a prerequisite for gamma-ray induced mutation. Accordingly complexes were made with average multiplicities of infection between 0·2 and 0·5. Lower multiplicities of infection proved impracticable since uninfected suppressor-containing bacteria which were carried over on to the plates supported a second cycle of growth of many of those phages released by lysis of the original complexes. Such plates were unscoreable.

The experimental procedure was similar to that described for multiply infected complexes and results of one experiment are given in Table 3. The M.O.I. in this case was 0·5, at which level the proportion of complexes that are multiply-infected should be 0·23. In this experiment the complexes were more sensitive to inactivation by gamma radiation presumably since the opportunity for multiplicity reactivation occurs rarely. Therefore even if the mutation rate was the same as with multiply infected complexes, with increasing dose one would expect to find an absolute decline in the total number of mutants per ml. The rate of mutation induction could only be determined from measurement of the mutant *frequency* after various doses of radiation on the assumption that the rate of decline of spontaneous mutants was equal to the rate of decline in the viability of the total population. If it were actually greater, the induced mutation rate so found would be an underestimate and vice versa. While there is no theoretical reason to suggest that the rate of decline of spontaneous mutants should be different from that of the total population there seems to be no way at present in which this could be experimentally established.

The validity of this experiment also depends upon the assumption that the phage stock does not contain a significant proportion of inviable phages which, though unable to give rise to plaques on CA 266 can nevertheless adsorb and inject their DNA.

From Fig. 2 it can be seen that phage genomes bearing mutations are inacti-

vated at the same rate (lower curve) as the total population of complexes (upper curve). The mutation rate per phage genome per rad, found by extrapolating the mutation curve to zero dose, was $1{\cdot}2 \times 10^{-11}$. The mean value of four such experiments was $1{\cdot}63 \times 10^{-11} \pm 0{\cdot}21 \times 10^{-11}$. In the other three experiments the sensitivity of complexes to inactivation was smaller than this and in two of them there was an absolute increase in the number of mutants per plate with increasing dose.

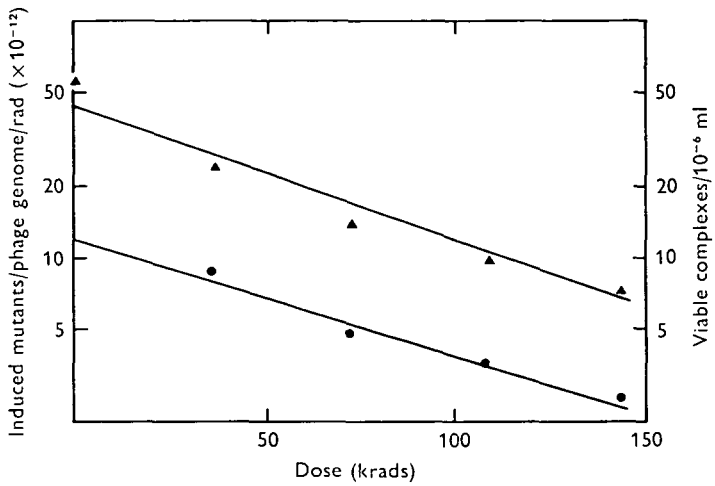

Fig. 2. Gamma radiation mutagenesis of T4 strain E51 complexed with *E. coli* CA 266 at a multiplicity of infection of 0·5. ▲, viable complexes; ●, induced mutations per phage per rad.

Table 3. *Gamma irradiation of E51 complexed with CA 266 at a M.O.I. of* 0·5

Dose (krads)	Viable complexes per ml.	Total mutants per ml.	Induced mutants per ml.	Induced mutants per phage per rad.*
0	$5{\cdot}6 \times 10^{7}$	110		
36	$2{\cdot}4 \times 10^{7}$	69·5	22·3	$8{\cdot}75 \times 10^{-12}$
72	$1{\cdot}4 \times 10^{7}$	52	24·5	$4{\cdot}78 \times 10^{-12}$
108	$1{\cdot}0 \times 10^{7}$	47·5	27·9	$3{\cdot}63 \times 10^{-12}$
144	$7{\cdot}5 \times 10^{6}$	41·5	26·8	$2{\cdot}62 \times 10^{-12}$

*Assuming total number of complexed phages is 0·5/0·394 × number of complexes.

On the basis of the assumptions stated above, we may therefore conclude that the induced mutation rate per phage genome per rad in singly and multiply infected complexes does not differ significantly.

4. DISCUSSION

Our results indicate clearly that DNA synthesis is essential for the functional expression of at least 93% of gamma-ray induced mutations of the amber bacteriophage T4 E51. This would seem to rule out any direct conversion of one base or

base pair to another *in situ* (cf. Ponnamperuma, Lemmon & Calvin, 1962). It has been postulated that a single-strand gap in DNA is the premutational lesion induced by ionizing radiation and that the mutational error is produced during the repair of this gap (Bridges, Law & Munson, 1968). A requirement for DNA synthesis would seem to be entirely consistent with this hypothesis.

It is, however, likely that some DNA synthesis is necessary for genetic recombination to occur and it could be that recombination is a necessary part of the mutagenic process (Grigg, 1964; Magni & Von Borstel, 1962; Witkin, 1969; Bridges, Dennis & Munson, 1967; see discussion in Bridges, 1969) by virtue of its involvement in the repair of single-strand gaps formed at the time of irradiation. The evidence that singly infecting phage genomes are as mutable by ionizing radiation as multiply infecting genomes, however, argues against a genuine recombination process being involved. Nevertheless, it does not exclude the possibility that the enzymes involved in mutagenesis also have a function in the process of recombination.

An alternative hypothesis consistent with the present data is that gamma radiation produces chemical alterations in the DNA which are not removed by excision and which may give rise to errors (mutations) during subsequent passages through the DNA replication complex possibly with the involvement of daughter strand recombination. This would be formally analogous to the persistence of UV-induced pyrimidine dimers in strains of bacteria deficient in excision-repair (Bridges & Munson, 1968) and although there is evidence against such a model for mutagenesis in *E. coli* by ionizing radiations it cannot be ruled out for T4 on present data.

It is noteworthy that although the induced mutation rate for mutation at the amber triplet in T4 E51 is almost the same as that at the ochre triplet in *E. coli* WP2, the spontaneous mutation rate is about 50 times greater. The most characteristic feature of T4 replication is the high frequency of recombination. The relatively high spontaneous mutation rate thus lends support to the notion that recombination is involved in spontaneous mutation.

In conclusion, we have shown that DNA synthesis is generally necessary for the production of a functional mutation following gamma irradiation of T4 genomes in *E. coli* bacteria. A single genome per complex appears to be mutable. The data are consistent with the hypothesis that mutations arise as errors during the repair of single-strand gaps in DNA. There is no evidence that a recombinational process is involved but one cannot exclude the possibility that enzymes involved in the production of mutations also have a function in recombination.

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