SELECTIVE ALLELE LOSS IN MIXED INFECTIONS WITH T4 BACTERIOPHAGE[1]

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ABSTRACT

Evidence is presented that when *E. coli* B is mixedly infected with T4D wild type and *r*II deletion mutants, the excess DNA of the wild type allele is lost. No loss is seen in mixed infections with *r*II point mutants and wild type. In similar experiments with lysozyme addition mutants, the mutant allele is lost. We believe these results demonstrate a repair system which removes "loops" in heteroduplex DNA molecules. A number of phage and host functions have been tested for involvement in the repair of the excess DNA, and T4 genes *x* and *v* have been implicated in this process.

IT has been observed repeatedly that heteroduplex heterozygotes (Hets) for extended deletion mutations are not seen among the progeny of wild type by deletion crosses (NOMURA and BENZER 1961; BERGER 1965; SECHAUD *et al.* 1965; DRAKE 1966). The failure to observe deletion heteroduplex Hets has generally been ascribed to the large violation in base complementary which would be required to form these structures. In agreement with this view, DRAKE (1966) was able to show that some frameshift mutations containing limited base deletions or additions could be included in heteroduplex Het regions.

A number of specific explanations can be advanced to account for the failure to observe heteroduplex Hets for extended deletions:

1) The extended region of mismatched bases prevents DNA pairing (NOMURA and BENZER 1961; SECHAUD *et al.* 1965).

2) DNA pairing occurs with the formation of a single stranded DNA loop; the loop is subsequently excised, which results in loss of the excess DNA and conversion to a homozygous condition (DRAKE 1966).

3) Since the failure to observe deletion heteroduplex Hets is based on examination of viable progeny it is possible that deletion—wild type pairing occurs (with loop formation) but that these DNA molecules are selectively not packaged or, if packaged, result in non-viable phage.

These possibilities are not exclusive, and one or more could account for the failure to observe deletion Hets. Loop formation and subsequent excision is the

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simplest model to investigate, since it predicts a selective loss of the wild type allele in mixed infections with deletion and wild type phage, and a selective loss of the mutant allele in addition-wild type experiments.

In this paper we show that deletions in mixed infection with wild type and wild type in mixed infection with additions show a selective advantage most consistent with loop formation, repair, and excision of the excess DNA. Furthermore, we present genetic evidence implicating T4 genes *v* and *x* in the repair process.

MATERIALS AND METHODS

Bacteria: Escherichia coli strain B was used as host for all crosses unless otherwise indicated. The reversion rate of *r*II stocks was tested on *E. coli* CR63(λh) or *E. coli* K12(λh). Progeny of *r*II by *r*II+ infections were plated on *E. coli* S/6. *E. coli* strains JC4586 and AB2480 were obtained from Dr. John Clark; JG112 from Dr. M. Goodman; Meselson strain rec-152 from Dr. Marc Rhoades; and 1100 from our stock collection.

Phage Mutants: T4D *r*II mutants were described by Berger (1965) and Berger and Warren (1969). Extended mutations were: *r*61, *rb*17, *rdb*52, *r*J101, and *rdb*145. Point mutants were: *r*2–19, *ra*45, *r*59, *r*70, *r*2–20, *rb*41, *rb*42, *rb*45, and *rb*50. T4D *v* and *x* gene mutants were obtained from Dr. Harm and the doubles with T4D *r*II (*ra*45-*v*, *ra*45-*x*, *r*61-*v*, *r*61-*x*, *rdb*52-*v*, *r*59-*v*) constructed according to Chase and Doermann (1958) as modified by Krisch *et al.* (1972). T4B *e* gene frameshift mutants were *e*JD5, *e*JD6, *e*J28, *e*JR13, *e*JR14. T4B*ac q* was used as wild type and *am* eM41 as a base substitution mutation. All lysozyme mutants were supplied by Dr. A. Tsugita. T4D gene 43 temperature sensitive mutants *ts*G40, *ts*L56, and *ts*L42 were obtained from Dr. M. Bessman, and the *r*II doubles were constructed as described above.

Media and Bacterial Growth: P-broth, H-broth, bottom and top layer agar, M-9, and M-9+ medium were as described by Krisch *et al.* (1972). The preparation of indicator bacteria and phage stocks and the standard cross procedure were also according to the methods of Krisch *et al.* (1972).

FUDR Cross Procedure: The experiments using fluorodeoxyuridine (FUDR) were performed as described by Sechaud *et al.* (1965). *E. coli* B was grown in M-9+ at 37°C to 1.5×10^8 bacteria/ml. FUDR (4×10^{-5} M), uracil (2×10^{-4} M) and tryptophane (20 μg/ml) were added to the bacteria. Parental phage mixes were assayed prior to each experiment and added at a multiplicity of about 5 for each parental type. The infections were done at 37°C. Unadsorbed phage were determined from $CHCl_3$ treated samples at 7 minutes after infection. There was greater than 95% adsorption of both parental types in all cases. Nine minutes after infection chloramphenicol (250 μg/ml) was added. At 120 minutes after infection, the cells were centrifuged and washed twice with M-9+. Aliquots from each infection were resuspended in M-9+ with FUDR, and uracil, and in M-9+ alone. Both were allowed to incubate for 1 hour at 37°C. Chloroform was then added, and the lysate titered on S/6 indicator bacteria. The plates were incubated at 34°C for 18 to 24 hours. Wild type and *r* phage were distinguished by plaque morphology. Mottled plaques were scored as contributing both a wild type and an *r* allele. The phage from experiments with lysozyme mutants were plated with B bacteria and 500 μg egg white lysozyme (Worthington Biochemical) per plate on citrate agar (Okada *et al.* 1966), and the plates were incubated for 48 hours at 34°C. Under these conditions wild type and lysozyme mutants were easily distinguished by plaque morphology.

Reversion frequency experiments: The alteration of *r* point mutant reversion frequencies in N-methyl-N′-nitro-N-nitrosoguanidine (NG) and proflavine (3,6-diamino-acridine) was tested according to Hartman *et al.* (1971). *E. coli* B was grown to 2.5×10^8 bacteria/ml in M-9+ and concentrated 4-fold in fresh M-9+ at 30°C. NG (20 μgm/ml) or proflavine (10 μgm/ml) were added; 1.5 minutes later phage were added at a multiplicity of three. After 15 minutes aeration at 30°C the proflavine cultures were diluted 50-fold into H-broth, aerated 45 minutes more, chloroformed, and assayed on *E. coli* B and K12(λh). After 25 minutes of aeration the NG

cultures were diluted 25-fold into H-broth and then treated as the proflavine cultures. In all cases simultaneous controls were performed without NG or proflavine to test the spontaneous reversion frequency.

RESULTS

We investigated the possibility of loop repair by mixedly infecting cells with wild type and *r*II deletion mutants and testing the progeny for selective loss of the wild type allele. SECHAUD *et al.* (1965) reported a great increase in the number of internal Hets in crosses of *rII* point mutants by wild type if fluorodeoxyuridine (FUDR) was used to slow DNA replication, and chloramphenicol was used to delay phage maturation. To maximize DNA-DNA interactions presumably necessary for repair we used these conditions in our experiments. Five presumed deletions in the *r*II region (BERGER 1965; BERGER and WARREN 1969) were used in separate experiments. As controls nine point mutations in the *r*II genes were also used in mixed infection with wild type. The nine point mutants have a measurable reversion frequency and their response to N-methyl-N'-nitro-N-nitrosoguanidine (NG) and proflavine is shown in Table 1. All of the mutants except *rb*42 show some increase in reversion after exposure to NG. Although the specificity of NG is not complete, it is known to induce high level reversion of base substitution mutations (BRAMMER *et al.* 1967; OESCHGER and HARTMAN 1970). Three of the mutants also respond to proflavine and thus their nucleotide change cannot be categorized.

Table 1 (column D) shows that irrespective of their response to mutagens these point mutants in mixed infection with wild type have a selective disadvantage. The average ratio of wild type / *r*II progeny to wild type / *r*II parental

TABLE 1

*r point mutant reversion frequencies and r-wild type mixed infections in FUDR and chloramphenicol**

r point mutation	A Spontaneous reversion frequency	B Fold increase in reversion frequency after treatment with NG†	C Proflavine	D wt/r progeny / wt/r parental in FUDR‡
*rb*41	2.6×10^{-7}	6.5	1.9	1.13 ± .05 (2)
*rb*42	2.1×10^{-7}	<1.3	2.3	1.03 ± .03 (4)
*ra*45	8.2×10^{-7}	57.5	2.9	1.20 ± .05 (12)
*rb*45	1.0×10^{-7}	3.4	1.4	1.22 ± .07 (6)
*rb*50	3.1×10^{-9}	527	1.4	1.30 ± .06 (2)
*r*59	6.0×10^{-9}	65.0	18.7	1.27 ± .06 (7)
*r*70	1.0×10^{-7}	4.5	2.6	1.18 ± .01 (2)
*r*2–19	9.3×10^{-8}	14.0	12.8	1.21 ± .04 (2)
*r*2–20	4.7×10^{-9}	38.3	12.1	1.55 ± .09 (2)
				average = 1.22 ± .03

* *r* point mutants were tested for altered reversion frequencies as described in MATERIALS AND METHODS (columns B, C). Wild type-point mutant mixed infections in FUDR and chloramphenicol (column D) were performed as in MATERIALS AND METHODS.

† NG is N-methyl-N'-nitro-N-nitrosoguanidine.

‡ Standard deviation of the mean indicated. The number in parentheses is the number of experiments performed.

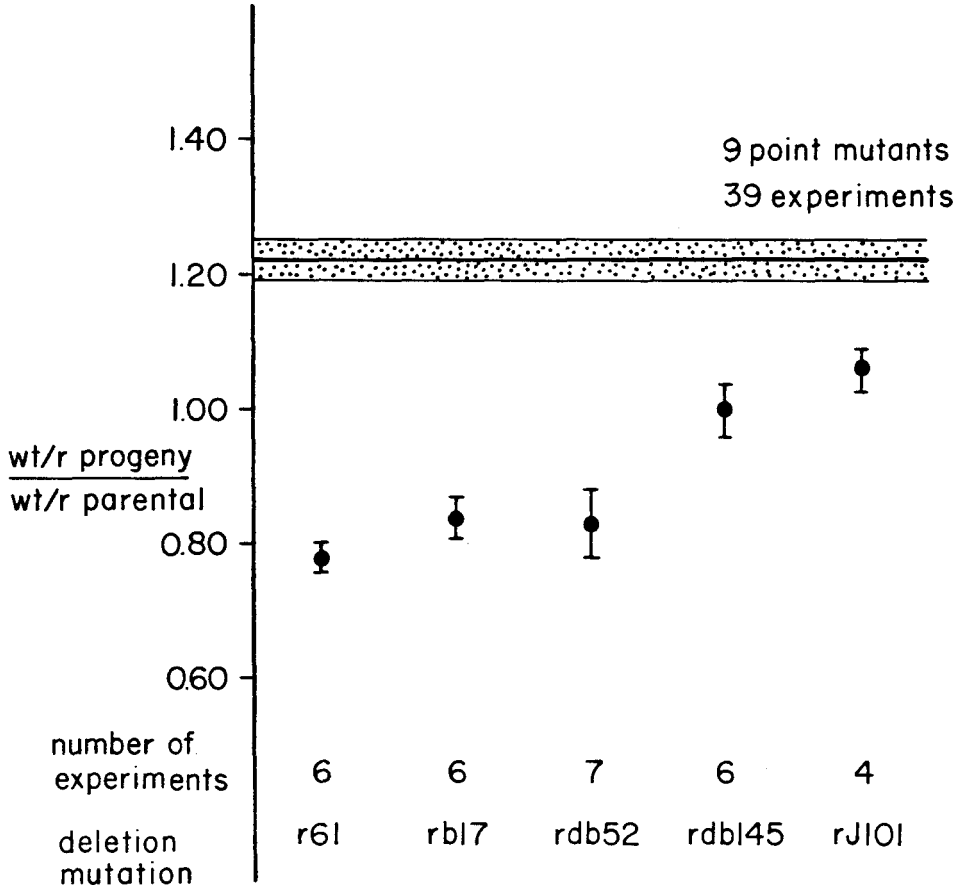

FIGURE 1.—Crosses of an *r*II deletion by wild type and control point mutants by wild type were done in the presence of FUDR and chloramphenicol as described in MATERIALS AND METHODS. The ratio of wild type/*r*II progeny is divided by wild type/*r*II parental. In the case of the point mutant crosses, all experiments were averaged and a line drawn at that value with shading indicating the standard deviation of the mean. The average and standard deviation of the mean is indicated separately for the five deletions.

is 1.22. The basis for the wild type advantage over *r*II mutants is not understood. Wild type also has an advantage in mixed infection with amber mutants (MOSIG, EHRING and DUERR 1968; SNUSTAD 1970).

In contrast, Figure 1 shows the results of mixed infections with wild type and *r*II deletions. The ratio of wild type / *r*II progeny to wild type / *r*II parental for each of the deletions is significantly less than the average ratio for the point mutant controls. The average ratio for the deletions is 0.88 ± 0.03 while that for the point mutants is 1.22 ± 0.03. Their means are different at the 99.5% significance level using the Student *t* test.

The allele ratio obtained for the deletion-wild type mixed infections is probably the result of two opposing processes: (1) the wild type advantage associated

TABLE 2

*Allele loss coefficients of rII deletions in FUDR and chloramphenicol**

Deletion mutant	Allele loss coefficient
*r*61	0.64
*rb*17	0.69
*rdb*52	0.68
*rdb*145	0.82
*r*J101	0.87

* The data from Figure 1 were used to compute allele loss coefficients. Each result from a deletion experiment was divided by the average of all the point mutant control experiments.

with mixed infections with wild type and *r*II phenotype phage and (2) a process which results in an *r*II advantage when the *r*II marker is a deletion. The actual contribution of the second process is most easily seen in Table 2 where the "allele loss coefficient" is calculated as the quotient of the wild type / *r*II progeny / wild type / *r*II parental value for a deletion and the average value of the point mutants. In all cases the deletion results in a selective wild type allele loss. This would be expected if there were repair of the excess DNA subsequent to pairing of homologous strands. With this model in mind the experiments were done in FUDR and chloramphenicol to maximize the time available for pairing. It is also possible to demonstrate the deletion-associated allele loss in the absence of FUDR and chloramphenicol. Allele loss coefficients for these experiments are shown in Table 3.

The above results demonstrate a selective loss of the wild type allele which is associated with the excess DNA. If loop repair is responsible for this observation, in the case of an addition mutation the mutant allele would be selectively lost, since it constitutes the excess DNA. To test this prediction, we have carried out a series of mixed infections using some frameshift mutations of known dimensions in the T4 *e* gene (IMADA *et al.* 1970; OCADA *et al.* 1970). A lysozyme amber

TABLE 3

*Allele loss coefficients of rII deletions without FUDR and chloramphenicol**

Deletion	wt/r progeny / wt/r parental†	Number of experiments	Allele loss coefficient
*r*61	0.99 ± .08	3	0.75
*rb*17	1.08 ± .06	3	0.82
*rdb*52	1.13 ± .05	4	0.85
*rdb*145	1.19 ± .05	3	0.90
*r*J101	1.21 ± .02	2	0.92
average of 15 point mutant control experiments is 1.32 ± .05.			

* Control experiments were done as described in MATERIALS AND METHODS for the FUDR cross procedure. However, no drugs were added, and the experiment was terminated by the addition of chloroform at 30 minutes. Allele loss coefficients were calculated as described in RESULTS and in the legend for Table 2.

† Standard deviation of the mean indicated.

TABLE 4

*Allele loss coefficients of lysozyme frameshift mutants in FUDR and chloramphenicol**

Frameshift mutant	Number of bases altered†	Number of experiments	wt/e progeny / wt/e parental‡	Allele loss coefficient
*e*JR13	+5	7	1.54 ± .13	1.29
*e*J28	+4	6	1.35 ± .06	1.13
*e*JR14	+2	8	1.43 ± .12	1.20
*e*JD6	+1	6	1.41 ± .12	1.18
*e*JD5	−2	6	1.00 ± .07	0.84
average of 33 lysozyme *amber* control experiments is 1.19 ± .04.				

* Lysozyme frameshift-wild type and control *amber*-wild type mixed infections in FUDR and chloramphenicol were performed as described in MATERIALS AND METHODS, and the allele loss coefficient computed as described for Table 2.

† IMADA *et al.* 1970, OCADA *et al.* 1970.

‡ Standard deviation of the mean indicated.

mutant was used in control infections. Table 4 shows the allele loss coefficient for each of 5 frameshift mutants. There is loss of the mutant allele with the four addition mutations and loss of the wild type allele in the case of the deletion mutation. There is a suggestion that the efficiency of allele loss is related to the size of the DNA excess, since maximum loss is observed with the 5 base addition.

We have investigated possible association of several phage functions with the allele loss demonstrated above. Two likely candidates, which have been previously implicated in DNA repair in phage infected cells (the repair of ultraviolet irradiated DNA), are the *v* and *x* gene products (HARM 1963). We also examined some phage polymerase (gene 43) temperature sensitive mutants which at low temperatures have mutator, antimutator, or neutral properties (DRAKE *et al.* 1969). Recently, it has been shown that these mutants have altered ratios of gene 43 associated polymerase to nuclease activity (BESSMAN, personal communication). The mutator mutants have a low nuclease to polymerase ratio and the antimutators have a high ratio.

Double mutants containing *r*II deletion or point mutation markers, and *v*, *x* or one of the three gene 43 temperature sensitive mutations, were constructed, and experiments identical to those described in Figure 1 were performed. As shown in Table 5, the *x* and the *v* gene mutations both prevented specific allele loss in mixed infections with deletions. The allele loss occurred with the altered polymerases. Bacterial host functions were also examined for possible effect on the allele loss phenomenon. Bacterial strains mutant in DNA polymerase (polA), nuclease (EndoI), recombination functions (recA, recB) and in uv repair (uvrA6) were used as hosts in experiments otherwise identical to those in Figure 1. Table 6 shows that alleles associated with the excess DNA were lost in the absence of any of these host products.

Since mixed infections in the absence of *v* or *x* gene products show no evidence of repair, it was possible that deletion Hets could now be found among the viable progeny of mixed infection with deletions and wild type in a *v* gene mutant background. However, as shown in Table 7, the frequency of deletion Hets remained

TABLE 5

*Allele loss coefficients of rII deletions in the absence of various phage gene products**

Deletion	Control point mutant	Gene function tested for repair	Number of experiments	Allele loss coefficient†
r61	*ra45*	control	6	0.65 ± .02
r61	*ra45*	*x*	4	0.96 ± .07
r61	*ra45*	*v*	6	0.98 ± .06
rdb52	*r59*	control	7	0.68 ± .06
rdb52	*r59*	*v*	4	0.99 ± .02
rdb52	*r59*	gene 43-neutral (tsG40)	4	0.61 ± .02
rdb52	*r59*	gene 43-mutator (tsL56)	4	0.61 ± .01
rdb52	*r59*	gene 43-antimutator (tsL42)	4	0.75 ± .07

* *r*II-*v* by *v* and *r*II-*x* by *x* crosses were performed in FUDR and chloramphenicol as described in MATERIALS AND METHODS. Mixed infection with phage containing gene 43 temperature sensitive mutants were performed at 32°C, chloramphenicol was added at 11 minutes after infection, and the infected bacteria were exposed to FUDR and chloramphenicol for 3 hours. The experiment was terminated with chloroform at 80 min after removal of the chloramphenicol. The progeny were plated on S/6 indicator bacteria and incubated at 30°C for 18 to 24 hours.

† Standard deviation of the mean indicated.

TABLE 6

*Allele loss coefficients of rII deletions in the absence of various host functions**

E.coli strain	Host functions altered	Number of experiments	Allele loss coefficient†
Meselson 152rec⁻	RecA⁻	4	0.70 ± .04
AB2480	RecA⁻, uvrA6⁻	4	0.57 ± .02
JC4586	RecB⁻, Endo 1⁻	2	0.70 ± .09
JG112	PolA⁻	4	0.71 ± .05
1100	Endo 1⁻	4	0.69 ± .03
B	control	6	0.65 ± .02

* *r61* by wild type and *ra45* by wild type mixed infections were performed in FUDR and chloramphenicol as described in MATERIALS AND METHODS, using the hosts listed. 20 μgm/ml each of thiamine and thymine were added to the M-9+ medium.

† Standard deviation of the mean indicated.

TABLE 7

Deletion Het formation in v *gene mutants*

Cross	Number of mottled plaques	Total plaques counted	Percent heterozygotes
ra45 by wt	101	1595	6.3%
ra45-v by *v*	140	1905	7.3%
r61 by wt	70	8318	0.84%
r61-v by *v*	39	4088	0.95%

Crosses were performed in FUDR and chloramphenicol as described in MATERIALS AND METHODS. After the chloramphenicol was removed the crosses were left in FUDR for the last hour, plated on S/6 indicator bacteria, incubated 14 to 18 hrs at 34°C, and the progeny examined for mottled plaques.

at a low level irrespective of the presence of the v gene mutation. Thus it appears that the failure to observe deletion Hets cannot be explained by a loop repair system alone. Deletion Hets (containing loops), even if unrepaired, may result in inviable progeny. It is important to note that the high level of Het formation in the point mutant v infections rules out the possibility that poor pairing in the absence of v gene product is responsible for lack of deletion Het formation.

DISCUSSION

We have presented genetic evidence for a novel T4 repair system. When one member of a pair of interacting genomes contains an extended addition or deletion mutation we have found a selective loss of the genetic markers associated with the excess DNA. The best explanation for the results is that heterologous pairing occurs, and the excess DNA in the resultant loops is removed, by a repair process. This model is shown for deletion by wild type mixed infection in Figure 2.

While the overall efficiency of repair is high, it is impossible to evaluate the relative contributions of step I (pairing) or steps II and III (excision and repair)

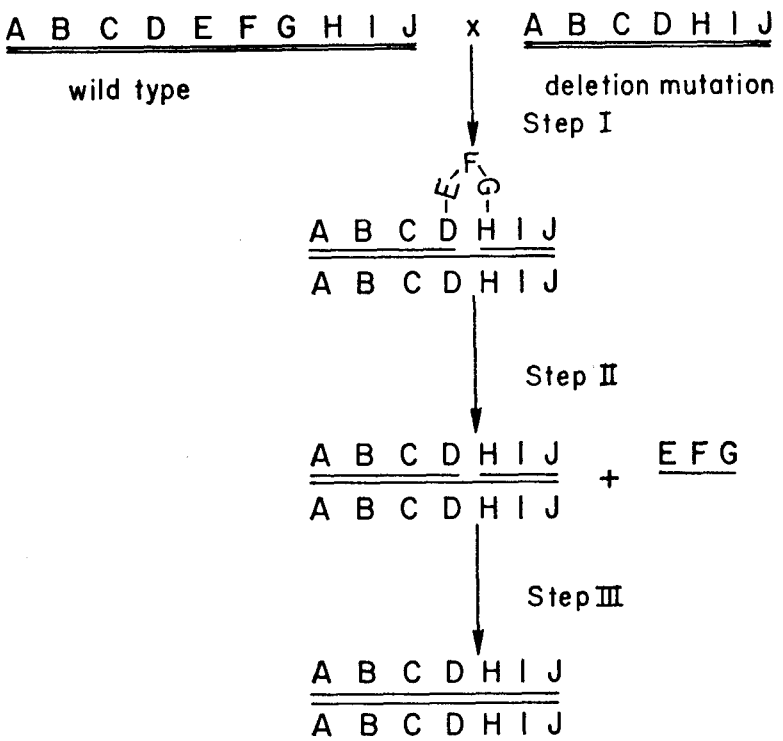

FIGURE 2.—This is a schematic representation of a cross between wild type and a deletion mutation. Step I represents pairing between the wild type and the deletion DNA. Step II is excision of the loop by a phage or host repair mechanism. Step III is repair of the nick or gap caused by the loop excision.

to the allele loss coefficients observed with different mutations. Perhaps the size of the mismatch is important. For example, the data in Table 4 suggest that loops of less than five bases are not as efficiently repaired as larger mismatches. Small mismatches may readily pair but be poorly repaired. The *rdb*145 deletion (Figure 1 and Tables 2 and 3) shows less repair than some *r*II deletions. This is the largest deletion, including greater than one-half of the *r*IIB cistron (BERGER 1965). In this case poor pairing may limit the amount of heteroduplex DNA available for repair. The *v* and *x* gene functions are required for repair. Presumably these functions are involved in steps II and III in the postulated repair process. The possibility that the absence of their functions limits pairing (step I) is made unlikely by the data in Table 7 showing that heterozygotes for a point mutant are not reduced by the absence of these functions. The *v* gene product has been isolated (YASUDA and SEKIGUCHI 1970a,b; FRIEDBERG and KING 1971) and is a nuclease with a high specificity for UV irradiated DNA. It is possible that loops in heteroduplex DNA are also substrate for this enzyme.

To obtain more detailed evidence about the mechanism, repair of looped DNA will be examined *in vitro*. Loop-containing heteroduplexes can be constructed (WESTMORELAND, SZYBALSKI and RIS 1969), and we intend to examine the *v* gene product as well as other phage and bacterial enzymes for involvement in *in vitro* repair of this substrate.

It is clear from the data in Table 7 that loop repair does not completely account for the reduced frequency of heterozygotes for deletion markers observed in normal and FUDR crosses (NOMURA and BENZER 1961; BERGER 1965; SECHAUD *et al.* 1965; DRAKE 1966). The observation that *v* gene mutation eliminates repair but does not restore a high frequency of deletion Hets suggests that loop-containing heteroduplex DNA, even when unrepaired, is not converted into mature whole phage. The molecules may not be normally packaged or, if matured, may not be properly ejected from the phage head upon subsequent infection.

The loop repair suggested by our observations on specific allele loss makes some predictions concerning the outcome of genetic crosses involving extended deletion markers. In two-factor crosses involving non-overlapping deletion and point mutants, repair could yield gene conversion resulting in a loss of wild type recombinants and a possible increase in double-mutant recombinants. In FUDR crosses involving *rdb*52 and the nearby point mutant site *r*70, we find that the frequency of unselected double-mutant recombinants, as determined by the methods of DOERMANN and BOEHNER (1970), is 4.8%. The frequency of unselected double-mutant recombinants in parallel crosses using the point mutant *r*70 and replacing the deletion by point mutant *r*59 is 3.1%. This is in contrast to the recombinational distances between *rdb*52 or *r*59 and *r*70 measured by the frequency of wild type recombinants in conventional crosses. It is 0.3 map units for *rdb*52-*r*70, and 0.8 map units for *r*59-*r*70. These inconsistencies in linkage relationships may be due to gene conversion resulting from repair of heteroduplex DNA. It is also possible that the reduction in high negative interference, observed when the central marker in three-factor crosses is a deletion (BERGER

and WARREN 1969), results from the suggested repair process. Additional experiments reinvestigating these phenomena in the presence of v and x gene mutations are underway.

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