GENETIC ANALYSIS OF T4D PHAGE HETEROZYGOTES PRODUCED IN THE PRESENCE OF 5-FLUORODEOXYURIDINE[1,2]

HILLARD BERGER[3]

Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee

Received April 19, 1965

SÉCHAUD, STREISINGER, LANFORD, REINHOLD, and STAHL (unpublished work) have shown that inhibition of DNA synthesis during phage maturation has a marked effect on the frequency of heterozygous progeny (HETs) resulting from *rII* by *rII*$^+$ crosses in bacteriophage T4B. Specifically, they have performed crosses in the presence of the thymidine analog 5-fluorodeoxyuridine (FUDR) which inhibits DNA synthesis by inhibition of thymidylate synthetase (COHEN, FLAKS, BARNER, LOEB, and LICHTENSTEIN 1958). When one parent in these crosses carries an *rII* point mutant, the frequency of progeny which are heterozygous for this site is increased five- to ten-fold as compared to the HET frequency for the same marker in normal crosses. In sharp contrast, when one parent carries an *rII* deletion mutation, the HET frequency for such a marker is not increased. In this case FUDR and normal crosses yield progeny which have approximately the same frequency of HETS for the specific *rII* deletion site.

Based on these results SÉCHAUD *et al.* have visualized that there are two classes of HETs. One type is proposed to be a heteroduplex structure (LEVINTHAL 1959) in which the information on each strand of the duplex genome is genetically distinct within the heterozygous region. This type of HET is presumed to be formed by break-reunion recombination and to be lost by semiconservative replication. Since replication is presumably prevented by FUDR, SÉCHAUD *et al.* propose that this type of HET accumulates in FUDR crosses. Furthermore, it is assumed that the extensive violation of base complementarity between deletion and wild-type parental genomes prevents the formation of such heteroduplex HETs for a deletion site, thus accounting for the observation that the HET frequency for deletion markers does not increase in FUDR crosses. This latter notion is in accord with the observations by NOMURA and BENZER (1961) which showed that, in normal crosses, deletion *rII* markers exhibit approximately a ⅔ lower frequency of heterozygosity as compared to point-mutant markers. From these results NOMURA and BENZER had suggested that there are two classes of HETs, one of which is inhibited by the mismatching of bases created by the presence of a deletion in one parental genome.

The second type of HET is proposed to be a terminal-redundancy structure

[1] This work is from a thesis submitted to the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] This investigation was supported in part by Public Health Service Training Grant 5TI GM-868, and by Public Health Service research grant CA-04437 awarded to DR. A. H. DOERMANN.

[3] Present address: Department of Biological Sciences, Stanford University, Stanford, California.

(STREISINGER, EDGAR, and DENHARDT 1964) which results from the incorporation into a single duplex genome of two double-stranded segments of the homologous sequence of information. These segments are located at opposite ends of the linear genome. When this redundant information is derived from genetically distinct parents, the result is a region of heterozygosity at the marked loci corresponding to the length of the redundancy. Since this structure is presumably formed and lost by recombination, the frequency of these HETs should rapidly arrive at the same equilibrium value in FUDR crosses. Since base complementarity difficulties presumably do not interfere with the formation of terminal-redundancy HETs, it has been assumed that deletion site heterozygotes are of this type only. The proposed structure of these different HET types is shown in Figure 1.

The primary purpose of the work to be described in this paper was to study

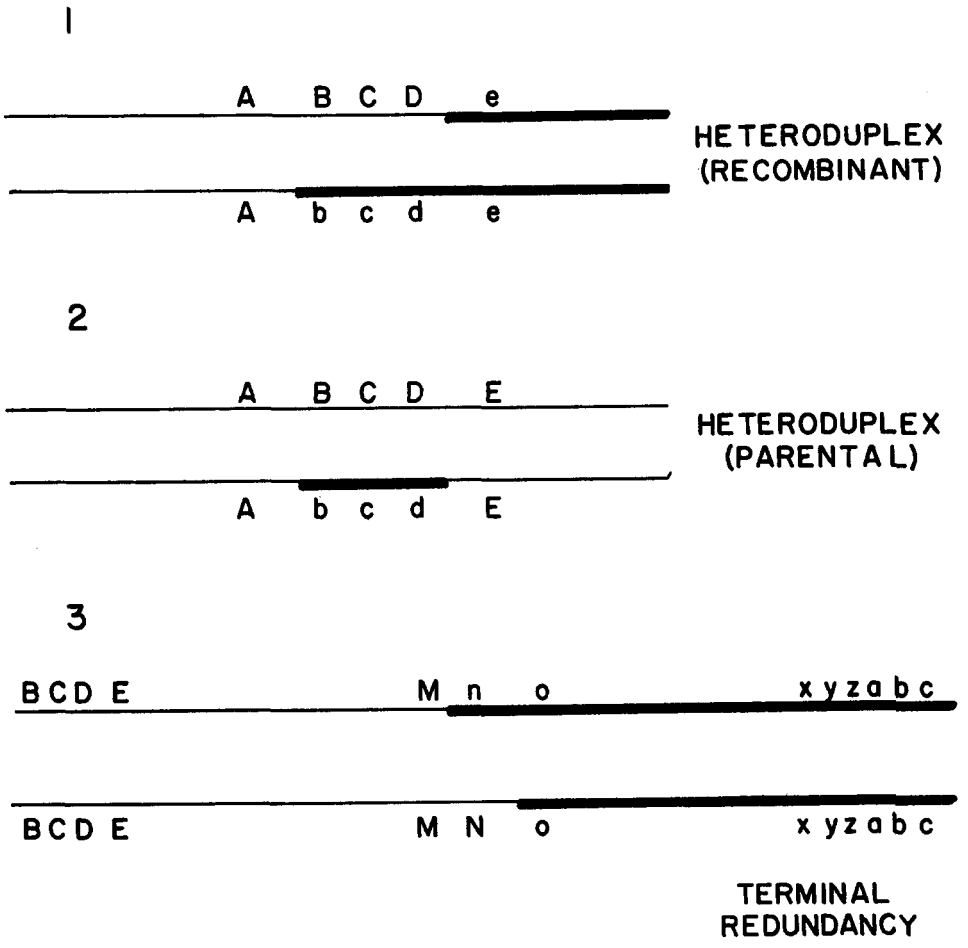

FIGURE 1.—Possible structures of HETs which are parental or recombinant for nearby outside markers. Each line represents one strand of a duplex genome. The light and heavy lines represent differently marked parental genomes.

the genetic characteristics of the HETs which are formed in FUDR crosses. Biparental mass-lysate crosses were performed in FUDR with parental stocks containing a series of *rII* point mutants as well as a group of amber point mutants (EPSTEIN *et al.*, 1963) distributed around the phage genome. The progeny were analyzed for (1) the frequency of heterozygosity for the various markers, (2) the average length of heterozygous regions, (3) the segregation pattern of HETs, and (4) whether individual HETs were recombinant or parental for markers to the immediate right and left of the heterozygous region. In addition, multifactor crosses were carried out with parents which also contained one or two *rII* deletion markers to determine the effect of deletions on the accumulation of HETs for point-mutant sites located at varying distances from the deletions.

The most striking observation resulting from these studies is that a large percentage of the HETs obtained from the crosses are parental for markers immediately adjacent to the heterozygous region. This result indicates that many FUDR HETs may have the structure shown in Figure 1-2. In addition, the frequency of heterozygosity for individual *rII* point-mutant sites (in a cross without deletions) exhibits a distinct gradient effect which is not observed in normal crosses. This result may suggest that HET formation in FUDR crosses is related to a nonrandom requirement for thymine-containing nucleotides in different regions of the genome. In the DISCUSSION a model is proposed which relates this hypothetical nonrandom thymidylate requirement both to the observed gradient effect and to the observed high frequency of HETs parental for nearby outside markers.

MATERIALS AND METHODS

Bacteria: Escherichia coli strain B was used as host for all crosses. Single-factor *rII* by *rII*+ cross progeny were plated on *E. coli* strain S/6 for the determination of total progeny and the frequency of mottled plaques. *E. coli* strain CR63 was used as plating bacteria for multifactor crosses which included both *rII* and amber (*am*) mutations in the parental stocks. The genotypes of the progeny of multifactor crosses were determined by the methods of DOERMANN and BOEHNER (in preparation). *E. coli* strains B/S, CR63/S, and K-12 (λh)/S were used for this purpose. *E. coli* strain B3 which is a thymine-requiring mutant originally isolated by BRENNER, was used as host in the final experiment which was designed to determine if some of the results obtained with FUDR are also obtained when thymidylate synthesis is prevented by the use of mutant bacterial and phage strains both of which are unable to produce thymidylate synthetase.

Phage mutants: For the multifactor crosses carried out in the presence of FUDR multiple marker stocks of the following genotypes were isolated:

1. *r*2–19/*r*71/*r*59/*r*77/*r*2–20/*am*N81/*am*N82/*am*N130/*am*B251/*am*B16/*am*N131/*am*S60/*ac*41
2. *r*2–19/*r*71/*r*70/rdb52/*r*2–20/*am*N81/*am*N82/*am*N130/*am*B251/*am*B16/*am*N131/*am*S60/*ac*41
3. *rb*41/*rb*42/*rb*45/*r*65/*r*48/*am*N53/*am*B17/*am*E355/*am*N6/*am*N85/*am*N54/*am*N58/*am*N52
4. *rb*41/rdb145/*r*48/*am*N53/*am*B17/*am*E355/*am*N6/*am*N85/*am*N54/*am*N58/*am*N52

The underscored markers are deletions isolated following nitrous acid treatment of T4D*rII*+ according to the method described by TESSMAN (1962). Table 1 lists the source of the various mutants together with information on whether they are point mutants or deletions by the criteria of revertibility to wild type, accumulation of HETs in FUDR crosses, and whether they fail to yield recombinants with more than a single point-mutant site. Figure 2a shows the approximate location of the non-*rII* markers used in the multifactor crosses. Figure 2b shows the map location of the *rII* markers.

The final experiment, which was designed to determine whether the results obtained in FUDR crosses are also obtained when thymidylate synthesis is prevented genetically, used stocks

TABLE 1

Mutants used in the various experiments. . . .

Name	Source	HET accumulation in FUDR crosses*	Shown to cover more than a single reverting site
A. Mutants which exhibit a measureable reversion frequency			
*r*2–19	C. STEINBERG	yes	no
*r*71	EDGAR (1958)	yes	no
*r*70	EDGAR (1958)	yes	no
*r*59	EDGAR (1958)	yes	no
*r*77	DOERMANN	yes	no
*r*2–20	EDGAR *et al.* (1962)	yes	no
*r*b41	EDGAR *et al.* (1962)	yes	no
*r*b42	EDGAR *et al.* (1962)	yes	no
*r*b45	EDGAR *et al.* (1962)	yes	no
*r*65	EDGAR *et al.* (1962)	yes	no
*am*N81	EPSTEIN *et al.* (1963)	yes	no
*am*N82	EPSTEIN *et al.* (1963)	yes	no
*am*N130	EPSTEIN *et al.* (1963)	yes	no
*am*B251	EPSTEIN *et al.* (1963)	yes	no
*am*B16	EPSTEIN *et al.* (1963)	yes	no
*am*N53	EPSTEIN *et al.* (1963)	yes	no
*am*B17	EPSTEIN *et al.* (1963)	yes	no
*am*E355	EPSTEIN *et al.* (1963)	yes	no
*am*N131	EPSTEIN *et al.* (1963)	yes	no
*am*S60	DOERMANN	yes	no
*am*N6	EPSTEIN *et al.* (1963)	yes	no
*am*N85	EPSTEIN *et al.* (1963)	yes	no
*am*N54	EPSTEIN *et al.* (1963)	yes	no
*am*N58	EPSTEIN *et al.* (1963)	yes	no
*am*N52	EPSTEIN *et al.* (1963)	yes	no
B. Mutants which do not exhibit a measureable reversion frequency (less than 1×10^{-9})			
*r*db52		no	yes
*r*db145		no	yes
C. Mutants for which the reversion frequency has not been measured			
*ac*41	EDGAR and EPSTEIN (1961)	yes	no
*r*48	DOERMANN and HILL (1953)	yes	no
*td*8	SIMON and TESSMAN (1963)	not determined	no

* See text for experimental techniques.

of *r*71, *r*db52, and *rII*+ which all, in addition, contained the *td*8 mutation described by SIMON and TESSMAN (1963). This mutant is incapable of initiating the synthesis of phage directed thymidylate synthetase.

Media: Crosses were carried out using M-9 medium prepared according to ADAMS (1959) and supplemented with 0.5% Difco Casamino acids-technical (M-9+). Stocks were prepared in this medium to which 250 μg of tryptophan per ml had been added to facilitate phage adsorption. Plate and soft agar were prepared as described by CHASE and DOERMANN (1958).

Cross procedure in FUDR: A host culture of *E. coli* B was prepared by adding 0.1 ml of an overnight culture grown with aeration in M-9+ medium to 100 ml of fresh medium and incubating with aeration for 205 min at 37°, yielding a culture containing approximately 3×10^8 viable cells per ml. Three minutes prior to phage infection 0.9 ml of a solution containing FUDR, uracil, and DL tryptophan was added to 2.1 ml of the bacterial culture giving respective

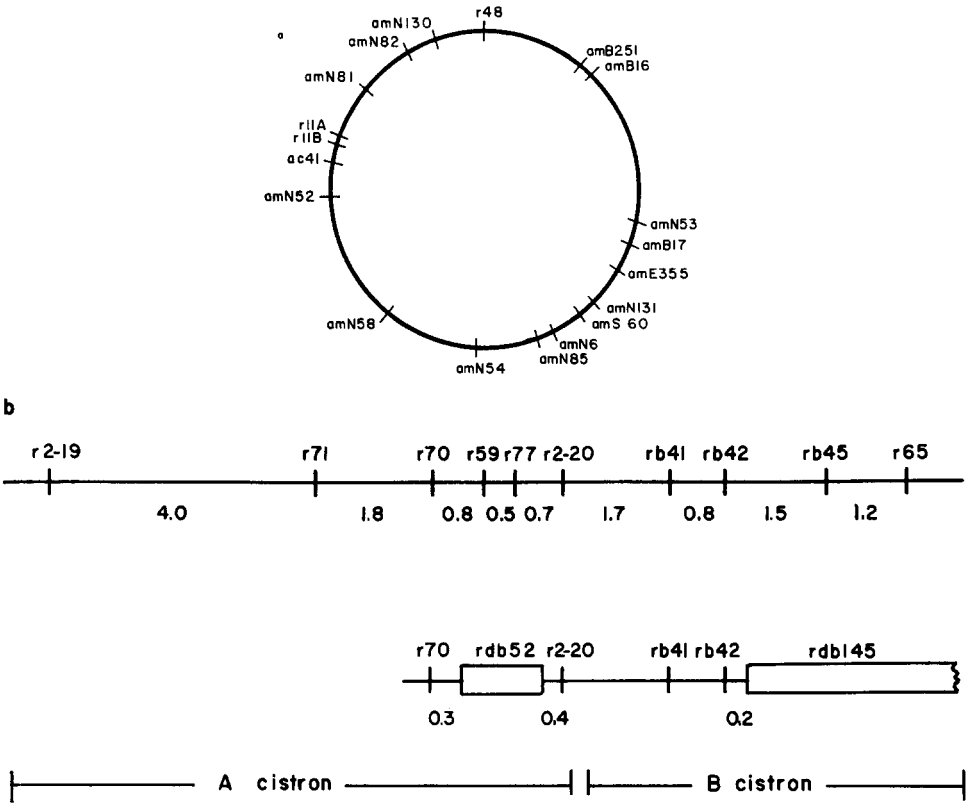

FIGURE 2.—a. Approximate map location of markers. b. Map location of markers within the *rII* region. The lower map indicates the approximate location of the deletion mutants *r*db52 and *r*db145.

concentrations of the three reagents of 8×10^{-5}M, 4×10^{-4}M, and 4.5×10^{-4}M. At time "0", 3 ml of phage suspension, containing 1×10^9 phage per ml of each parent was added to the 3 ml of bacterial culture, giving a multiplicity of approximately 5 of each parental phage. Nine minutes after addition of the phage, chloramphenicol was added to a concentration of 250 μg per ml. The culture was then incubated at 37° with aeration for 120 min. At this time the infected cells were centrifuged in the cold, washed three times with fresh chilled M-9+ medium containing FUDR, but no chloramphenicol, and finally resuspended in 6 ml of this solution. The cells were then incubated at 37° with aeration for an additional 60 min to permit maturation. At this time $CHCl_3$ was added to lyse the cells; DNAase was added to the lysate which was then incubated for an additional 30 min at 37°. Following DNAase treatment the lysate was centrifuged to remove bacterial debris. The lysates were then diluted 1:100 into H-broth for storage. Control non-FUDR crosses were performed in a similar manner but using medium which did not contain FUDR or chloramphenicol. The latter were lysed 80 min after phage infection and treated in the manner described above.

Cross procedure-using td*8 phage mutants and B3 bacteria:* These crosses differed from the FUDR crosses in the use of M-9+ medium to which the only preinfection supplement was tryptophan. The B3 host bacteria which were grown in thymidine supplemented media were washed twice with fresh M-9+ medium before use. Control crosses in which DNA synthesis was not inhibited were performed by adding 10 μg of thymidine to the M-9+ medium.

Determination of progeny genotypes: The cross lysates were plated to yield no more than 20

plaques per plate. Representative individual progeny plaques were picked with a glass rod and resuspended in 2 ml of H-broth containing approximately 0.2 ml of $CHCl_3$. All plaques which were clearly nonoverlapping were sampled without regard to plaque morphology. After overnight storage in the cold, the plaque samples were plated to give no more than 50 plaques per plate and ten of these plaques, representing the segregants from a single progeny phage, were tested to score for each of the markers present in the original parental stocks. In all cases where both alleles for a given marker were found among the ten segregants, the original progeny phage was classified as heterozygous for that marker. Heterozygosity for the *r*48 marker was determined visually by observing if the plating of the resuspended plaque yielded progeny plaques of both the *rI* and the *rI*+ plaque morphology on strain CR63.

EXPERIMENTAL RESULTS

Figure 3 shows the parental *rII* markers in each of the four multifactor crosses that were carried out in the presence of FUDR. Cross 1 which contains only point-mutant markers was also done without FUDR and the progeny were analyzed in the same manner. The average burst sizes of the FUDR crosses were quite similar, ranging from 0.2 to 0.5 phage per infected bacteria.

The genetic analysis to be presented in detail in the succeeding paragraphs revealed the following characteristics of the HETs which accumulate in FUDR crosses: (1) The number of HETs recombinant for markers on opposite sides of the heterozygous region is approximately equal to the number of parentals. When all HETs are considered there is an excess of parental types, but the overall difference from a 1:1 ratio is not appreciable. (2) They have the same average

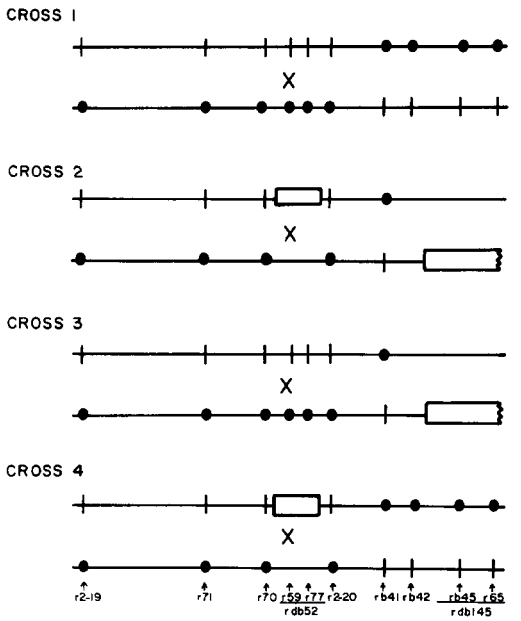

FIGURE 3.—Parental genotypes in multifactor crosses. The solid circles indicate wild-type alleles. The vertical lines indicate mutant alleles. Deletion sites are indicated by the rectangular blocks.

length as HETs formed under normal conditions of DNA synthesis. (3) They usually exhibit nonpolarized segregation. In addition, it was found that HETs for a deletion marker do not accumulate, in accord with the results of SÉCHAUD *et al.* A deletion, however, only inhibits HET accumulation for point mutant sites which are located quite close to the deletion; *i.e.*, point mutants greater than 1 to 2 recombination units from a deletion show an accumulation of HETs which is independent of the presence of the nearby deletion. This is seen for both *rII* point mutants and for the various non-*rII* markers distributed around the genome.

The analysis also revealed a surprising result concerning the frequency of heterozygosity for the *rII* point-mutant sites among the progeny of the cross containing no deletion markers. The frequency of heterozygosity accumulated in FUDR for the individual markers exhibits a distinct gradient pattern which is not seen under normal cross conditions. The *rII* markers closest to the divide between the *A* and *B* cistrons show a higher accumulated-HET frequency than markers located to either side of this central region.

Figure 4 shows the frequency of heterozygosity for each *rII* marker in the FUDR crosses plotted as a function of map position. The results of Crosses 2, 3, and 4 will be considered first. As expected, HETs for a deletion marker remain at a low frequency. Only point-mutant markers located very close to a deletion show a similar nonelevated HET frequency. In order to assess more fully the effect of a deletion on nearby point mutants, the ratio of the HET frequency for a given *rII* point mutant in a cross containing a deletion to the HET frequency for the same marker in Cross 1 (without deletions) is plotted as a function of map position in Figure 5. This figure clearly shows that only markers located quite close to a deletion show a nonelevated HET frequency. Table 2 shows the number of HETs which were found for non-*rII* markers in the FUDR crosses. The similarity of the average percentage heterozygosity per marker in the four crosses also supports the conclusion that an *rII* deletion does not affect appreciably the

TABLE 2

*Number of HETs for non-*rII *markers in FUDR multifactor crosses*

Cross*	*am*N81	*am*N82	*am*N130	*r*48	*am*B251	*am*B16	*am*N53	*am*B17	*am*E355
1	11	16	7	11	8	16	14	13	16
2	7	11	7	7	12	16	11	19	12
3	13	16	7	11	14	13	21	18	9
4	12	12	10	10	12	13	22	21	15

Cross*	*am*N131	*am*S60	*am*N6	*am*N85	*am*N54	*am*N58	*am*N52	Average percent heterozygosity per marker†
1	8	8	16	15	14	22	13	7.2
2	10	11	13	17	13	28	16	7.3
3	7	9	10	22	22	23	21	8.2
4	8	13	13	17	18	38	17	8.6

* The *rII* region parental genotypes are shown in Figure 3.
† From each cross 180 progeny were tested for heterozygosity at each of the marked sites.

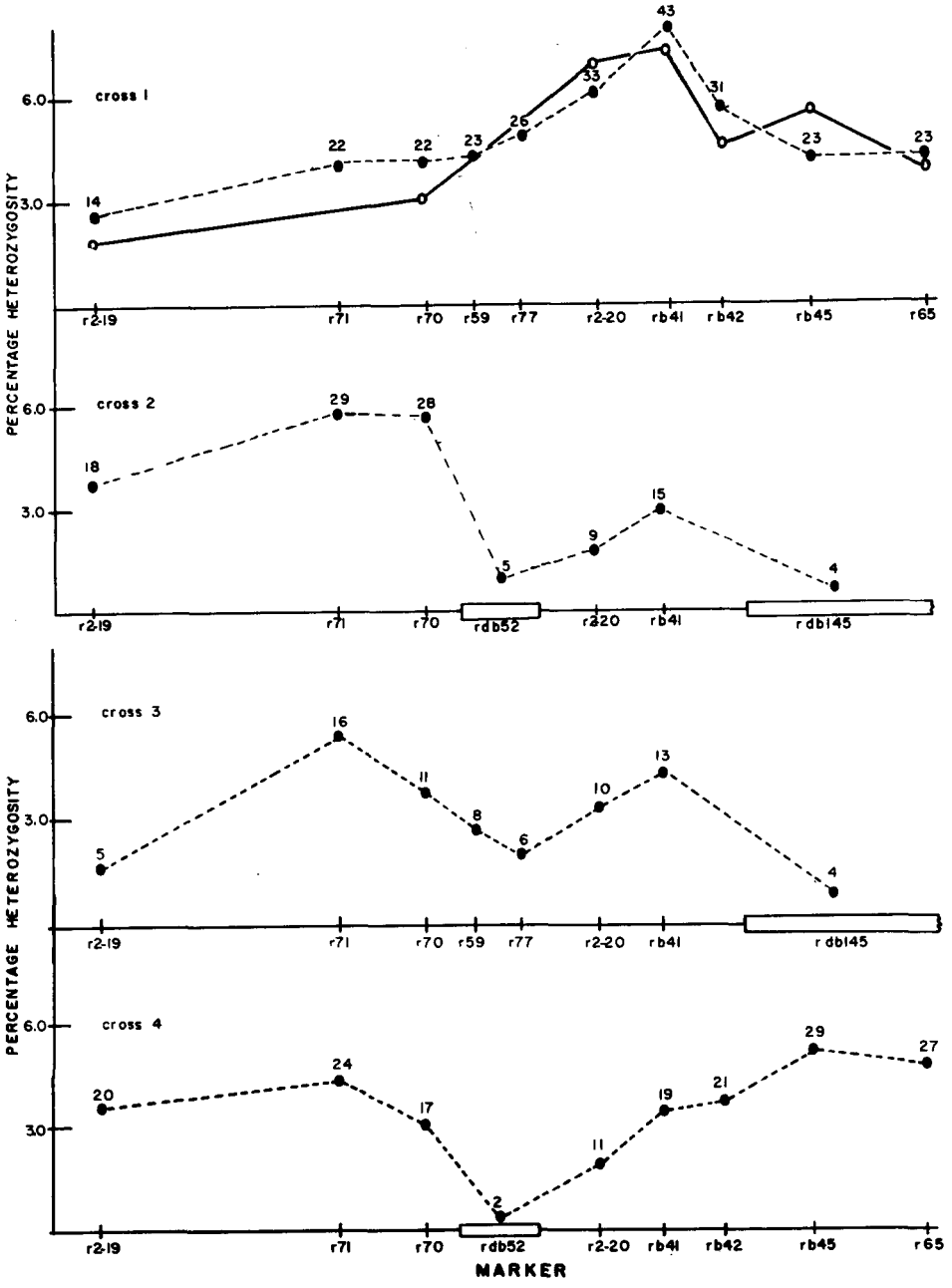

FIGURE 4.—The frequency of heterozygosity for various *rII* markers in FUDR crosses. The numbers above each point are the total number of HETs which were found for the specific marker. The solid line in Cross 1 represents the results obtained from scoring the frequency of mottled plaques among the progeny of individual single-factor FUDR crosses.

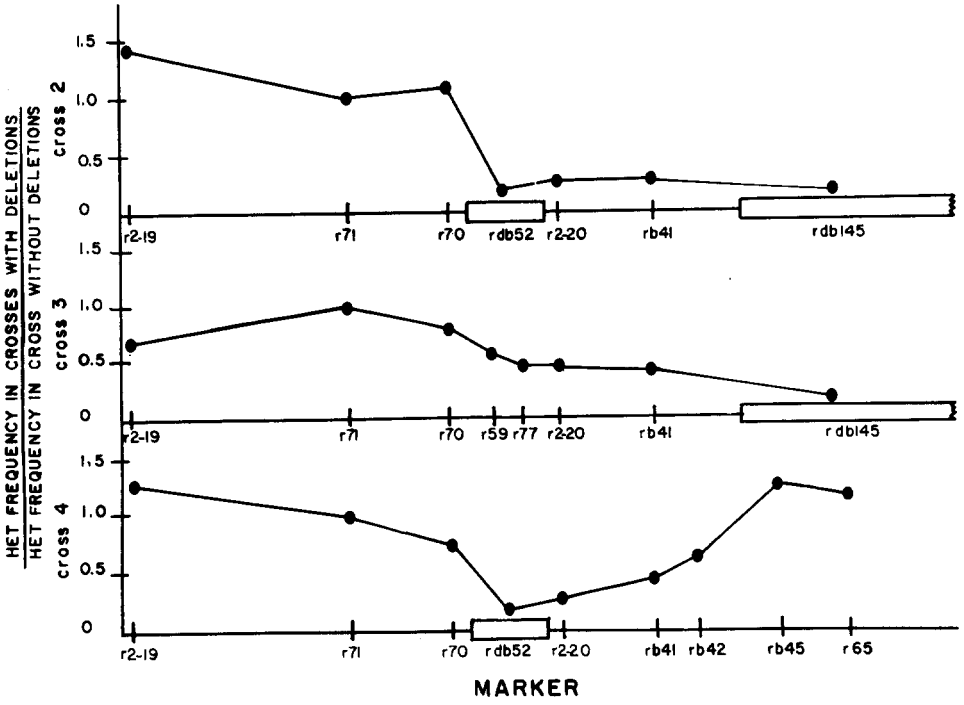

FIGURE 5.—Ratio of HET frequency with deletions to HET frequency in cross without deletions for FUDR multifactor crosses.

accumulation of HETs for markers located more than a few recombination units from the deletion site.

The results of Cross 1 (Figure 4), which contains only point-mutant markers, clearly show the gradient effect in the HET frequencies. The dotted curve is the result of a single multifactor cross; the solid curve is the result of scoring the mottler frequency among the progeny of single-factor crosses of individual *rII* point mutants by *rII*$^+$. Both results are quite similar, and in both cases a chi-square homogeneity analysis gives $P<0.01$ that the deviations from the average HET frequency are due to chance alone. Such heterogeneity is not observed in a normal cross. The results of HET frequency analysis of 1000 progeny from a single multifactor normal cross are shown in Figure 6. In this normal case the deviations from the average HET frequency may be attributed to chance alone (P from chi-square >0.3).

Tables 3 and 4 show the results of analyses to determine whether FUDR HETs are recombinant or parental for markers on opposite sides of the heterozygous region. Table 3 represents the combined results of the four FUDR crosses for markers outside the *rII* region, and also shows the percentage recombination between the outside markers as obtained from normal 2-factor mapping crosses and from analysis of the frequency of recombination observed in the multifactor FUDR crosses. Table 4 shows a similar analysis for the *rII* region markers in the

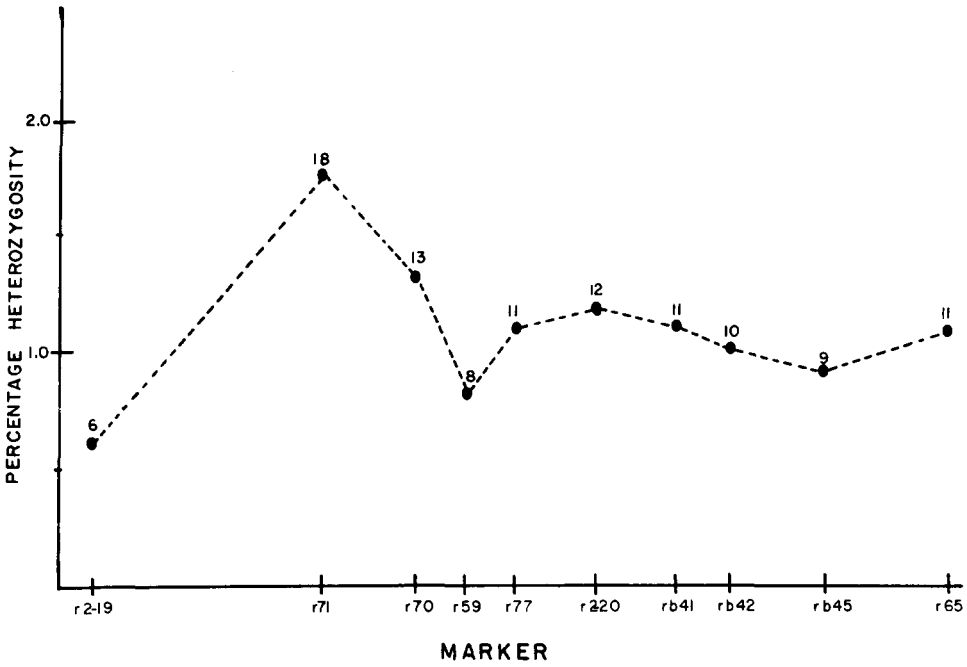

FIGURE 6.—HET frequency for *rII* point-mutant markers in a normal (non-FUDR) multifactor cross. The number above each point is the total number of HETs which were found for the specific marker.

TABLE 3

*Analysis of non-*rII *HETs to determine whether they are recombinant or parental for adjacent markers*

HET marker	Outside markers	Percent recombination between outside markers* (FUDR)	Percent recombination between outside markers† (normal)	No. of recombinant HETs	No. of parental HETs
*am*N6	*am*S60 *am*N85	10.0	7.5	18	22
*am*S60	*am*N131 *am*N6	11.5	5.2	12	22
*am*B17	*am*N53 *am*E355	18.0	7.4	32	36
*am*N131	*am*E355 *am*S60	24.0	13.5	20	23
*am*E355	*am*B17 *am*N131	25.0	9.8	25	27
*am*N85	*am*N6 *am*N54	25.0	18.7	31	27
*am*N82	*am*N81 *am*N130	29.0	10.8	27	33
			Total	165	190

* Results obtained from testing data by scoring all cases in which recombination had occurred and in which neither marker was heterozygous.

† Data from 2-factor mass lysate crosses; the wild-type recombinants were scored by plating on S/6 bacteria. Crosses were done with B host bacteria.

TABLE 4

Number of accumulated rII *region HETs which are recombinant or parental for adjacent markers in FUDR and normal crosses*

Cross*	Total phage tested	No. of HETs recombinant for adjacent markers	No. of HETs parental for adjacent markers	Terminal
1-FUDR	545	30	24	40
2-FUDR	503	11	28	19
3-FUDR	293	8	15	7
4-FUDR	551	12	31	40
Total FUDR	1892	61	98	106
1-normal	1000	14	11	14

* See Figure 3 for parental genotypes.

FUDR and normal crosses. Since only *rII* markers were used for this analysis, in no case is an adjacent marker greater than 4 normal-cross recombination units from an end of a heterozygous region. In most cases, the nearest marker was much closer. The terminal class consists of all HETs which included either or both of the terminal *rII* markers—*r*2–19 and *r*65. In these cases it was not possible to determine whether the HET was parental or recombinant. However, there is no obvious reason to believe that they would have preferentially fallen into one of the two other classes had an outside marker been present.

Although an exact determination of the size of HETs is made difficult by the limited concentration of marked sites, several size comparisons can nevertheless be made. Three distinct analyses were used for the *rII* region HETs obtained in FUDR and normal crosses. Only HETs obtained from the cross without *rII* deletions were used for this study.

As described by DOERMANN and BOEHNER (1963), a rough comparative measure of the length of HETs may be obtained from the average number of *rII* markers included in the HET regions. Among the FUDR HETs there was an average of 2.8 markers per HET based on a total of 94 HETs. In the non-FUDR cross, with the same set of markers, the average was 2.7 markers per HET based on a total of 40 HETs. A somewhat more direct estimate of the HET length was obtained by measuring the map distance between markers included in individual HETs (see Figure 2b). The average length estimate obtained in this way was 2.1 recombination units for the 94 FUDR HETs and 2.1 recombination units for the 40 HETs formed in a normal cross. It should be noted that this calculation is probably a large underestimate of the true size since all single-marker HETs are arbitrarily assigned a zero map length for this calculation. Finally, the size distribution of the *rII* region HETs measured by the procedure followed by DOERMANN and BOEHNER (1963), is shown in Figure 7. The figures were obtained by plotting the percentage of HETs which have a value greater than or equal to a value (x) versus map length (x). It appears that the length distribution is not appreciably different for the FUDR and normal HETs. It should be noted that the average HET size from both FUDR and normal crosses is appreciably smaller than the values reported by DOERMANN and BOEHNER (1963) for

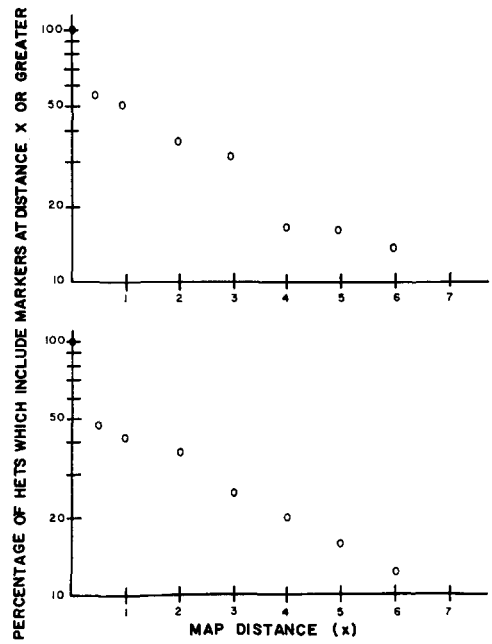

FIGURE 7.—Length distribution of HETs obtained from FUDR and normal crosses. The upper figure is the distribution from a normal cross; the lower from an FUDR cross.

HETs selected for their ability to produce mottled plaques. However, as they pointed out, their study involved several nonreverting *rII* markers which may have influenced the results. It is possible that their size estimate, which was based on the length distribution of HETs, may have been overly large because of the requirement that the HET include either *r*61 or *r*73 which are both possible deletion markers (they do not revert to wild type). In addition, the total map distance used by DOERMANN and BOEHNER was approximately 40% larger than in this study. This difference can be attributed to the greater distance between their outside markers and to their use of map values which were corrected for high negative interference.

The allele frequency or segregation pattern for the *rII* region HETs observed in the FUDR and normal crosses was also determined from the data obtained from the testing of ten segregants from individual progeny phages. The results indicate that the large majority of FUDR HETs exhibit nonpolarized segregation; i.e., the frequency of segregation of the alleles from one parent is approximately the same for a group of markers included in the same heterozygous region [see DOERMANN and BOEHNER (1963) and WOMACK (1963)]. Of the 260 FUDR HETs which were found among the progeny of the FUDR crosses, there were 125 which were heterozygous for more than a single marker. Of these, 91 did not show any evidence of polarity. Among the remaining 34, 21 appeared to show polarity and 13 could not be classified. These results are shown in Table 5 in which the data is presented individually for each of the FUDR crosses. The table

TABLE 5

Number of polarized, nonpolarized, and single-marker rII *HETs in FUDR crosses*

Cross*	Total phage tested	Single-marker HETs	Polarized HETs	Nonpolarized HETs
1	545	49 (10)	2 (4)	39 (8)
2	503	27 (9)	5 (4)	16 (7)
3	293	15 (5)	3 (2)	12 (4)
4	551	44 (10)	11 (4)	24 (8)
Totals	1892	135 (34)	21 (14)	91 (27)

* See Figure 3 for parental genotypes.
The numbers in parentheses are the expected number of HETs, calculated from the results of testing 1000 progeny from a normal cross, using the same markers as in Cross 1 (Figure 3). In this case 18 single-marker HETs, 7 polarized HETs, and 14 nonpolarized HETs were observed.

also gives the expected number of HETs in each category based on the results obtained from the testing of 1000 progeny from a single *normal* cross using the same parental stocks as in Cross 1 (see Figure 3). In the normal cross, there were 22 out of 40 total HETs which covered more than a single marker. Again based on testing ten segregants, 14 of these HETs appeared to be nonpolarized, 7 showed polarity, and 1 could not be classified.

Finally, Table 6 indicates the frequency of mottlers which resulted from crosses in *E. coli* strain B3 of an *rII* point mutant (*r*71) by *rII*$^+$ and of an *rII* deletion mutant (*r*db52) by *rII*$^+$—in each cross the parental stocks also contained the *td*8 mutation. The point to be noted is simply that the results are qualitatively similar to those obtained in FUDR crosses—namely, among the progeny there is an accumulation of HETs for a point mutant but not for a deletion mutant. This result would tend to eliminate the possibility that results obtained with FUDR are due to any unanticipated toxic effect of the drug which is unrelated to the inhibition of DNA synthesis.

Discussion

The results indicate that HET formation in the presence of FUDR may occur by a mechanism that differs in some respects from the break-reunion model suggested by Séchaud *et al.* The observed large frequency of FUDR HETs which

TABLE 6

HET frequency for point and deletion markers

Cross	Total phage	Burst size	Number of mottlers	Percentage mottlers
*r*71 × *rII*+				
—thymidine	877	4.2	53	6.1
+thymidine	12,140	445	67	0.6
*r*db52 × *rII*+				
—thymidine	820	2.2	2	0.2
+thymidine	16,340	640	21	0.1

Stocks all contained the *td*8 mutation and crosses were done with the thymidine-requiring strain *E. coli* B3.

are parental for nearby outside markers suggests that many HETs result from the insertion of regions of heterozygosity without changing the sources of the genetic information on either side of the region of insertion. If the structure of FUDR HETs were as shown in Figure 1–1, a simple assumption would be that all HETs would be recombinant with the exception of those which arose from the interaction of genomes which had previously undergone recombination (see LEVINTHAL 1954). If it is assumed that the recombination frequency between markers is a direct measure of the frequency of recombinant genomes, a measure of the frequency of HETs which *appear* to be parental for outside markers should be given by the frequency of recombination between markers on opposite sides of a heterozygous region. It is clear from the data in Tables 3 and 4 that the number of parental HETs is in large excess of the value predicted by this simple assumption, even when the increased recombination resulting from prolonged incubation in the presence of FUDR is taken into consideration. These results are in contrast to those of LEVINTHAL (1954) and the limited normal cross data shown in Table 4 which indicate that the majority of HETs arising from *normal* crosses are recombinant for outside markers.

An additional observation that should be considered is the gradient effect which is observed in the frequency of HETs for different *rII* point-mutant markers spanning the *rII* region of the phage genome. Since FUDR prevents DNA synthesis by limiting the availability of thymine-containing nucleotides, this distinct HET frequency pattern may suggest that HET formation is related to the thymidylate requirement for new DNA synthesis in specific portions of the genome.

Pertinent to the question of the mechanism of progeny formation in FUDR crosses are the experiments of VOLKIN and RUFFILLI (1962), KOZINSKI and KOZINSKI (1963, 1964), and ANRAKU and TOMIZAWA (1965a,b). VOLKIN and RUFFILLI have shown that the large majority of the DNA of T2 progeny phage produced in the presence of FUDR consists of nucleotides derived from the infecting parental phage. KOZINSKI and KOZINSKI using T4 phage have concluded that progeny phage made in the presence of FUDR contain parental segments of DNA which are integrated as discrete single-stranded subunits. Furthermore, they concluded that in their experiments, some new DNA synthesis had occurred during incubation with FUDR, using nucleotides from a source other than the infecting parental phage. ANRAKU and TOMIZAWA also studied T4 produced in FUDR-inhibited cells. While some of their experiments confirmed the findings of KOZINSKI and KOZINSKI, one of their experiments led them to postulate a somewhat different mechanism for the origin of the recombinant DNA molecules. In repeated experiments in which the multiplicity of infection was ten phage per bacterium, they found quite variable mean burst sizes, and they presented data on the analysis of DNA from progeny phage for the experiment with the highest yield (four progeny phage per infected cell) and for that with the lowest yield (0.2 progeny phage per infected cell). In both cases, as in the experiments of KOZINSKI and KOZINSKI, they found many phage with DNA derived predominantly from one parent type, and some phage with molecules evidently formed by a break-reunion mechanism in which fragments of strands from one parent

were covalently linked to fragments of strands from another parent. Their results from the experiment with the lowest yield, however, led them to conclude that the break-reunion process appeared to involve predominantly double-stranded pieces of parental DNA. They then interpreted the results from the experiment with the larger progeny yield to indicate that the break-reunion recombination process was often preceded or followed by DNA synthesis that resulted in a large fraction of the progeny molecules having new DNA in strands paired to parental material. From other experiments in which the phage DNA in infected cells was examined at various times during and after the eclipse period, they concluded that the break-reunion processes involved first an association of parental molecules only by hydrogen-bonding of complementary single strands, and then several minutes elapsed before the strands of different parental origin became covalently linked. The break-reunion mechanism inferred by ANRAKU and TOMIZAWA (1965a,b) is evidently in complete agreement with that inferred by MESELSON (1964) from experiments with phage λ crosses in which no FUDR had been used. Moreover, the cross progeny both in the T4 and λ cases were found to contain DNA molecules with no interruptions in the polynucleotide continuity.

Employing the observations of the above workers and the genetic results described in this paper, the process of HET formation in FUDR is pictured as occurring by some kind of break-reunion process leading to progeny DNA molecules containing single-stranded segments of parental DNA. It must be noted, however, that the break-reunion process postulated in the preceding paragraph cannot, unless it is modified in some way, account for the results reported in this paper.

The experimental results of this genetic analysis may be summarized as follows: (1) Approximately one half of the total FUDR HETS are parental for markers on opposite sides of the heterozygous region. (2) On the whole, FUDR HETS exhibit nonpolarized segregation. (3) FUDR HETS are on the average approximately 2 to 3 recombination units in length. (4) The frequency of heterozygosity for 10 *rII* point-mutant markers exhibits a gradient effect which is not seen in normal crosses. (5) The effect of deletions on the accumulation of point-mutation HETs extends only a short distance from the deletion site.

These observations lead to the following conclusions:

1. The frequent occurrence of HETs which have a parental genotype for outside markers is most readily explained by the production and utilization of short single-stranded fragments of parental DNA. The incorporation of these fragments into progeny molecules can be conceived according to any of several schemes:
   a. There also may be much longer single-stranded fragments, to which the short fragments become paired, and then DNA synthesis occurs which incorporates a short fragment into a strand copied on both sides from the long fragment.
   b. Single-stranded material may be excised from within a predominantly double-stranded molecule, and a short fragment may become paired to the resulting single-stranded region and ultimately become joined (perhaps through some limited new synthesis) to remaining preexisting material at the ends of the single-stranded region.

c. If the underlying mechanism is presumed to generally involve two complementary single-stranded regions at the ends of two double-stranded molecules, which then become associated into an "overlap" structure (MESELSON 1964), HETs with parental outside markers may be produced in a case where two molecules from a parent of the same genotype have formed an overlap of limited extent, leaving a portion of the single-stranded region of one of the molecules able to pair with a short single-stranded fragment from a parent of a distinguishable genotype. Then, as in the other models, the continuity of strands would eventually be achieved following some limited DNA synthesis on any remaining single-stranded regions.

2. The fact that in an FUDR cross, HETs for different markers are found at different frequencies may be due to different proportions of thymine in different regions of the genome, such that the limited availability of thymidylate for DNA synthesis leads to slower replication of DNA strands where there is a relatively high thymidylate requirement, and thus a longer availability of the complementary single-stranded regions for pairing with the postulated short single-stranded fragments.
3. The size of the postulated short single-strand fragments may be estimated from the calculated length of the HET region which is about 2 to 3 recombination units in length. The mapping functions for T4 developed by STAHL, EDGAR and STEINBERG (1964) suggest that such a map distance corresponds to about 200 nucleotides. It seems not unlikely that there could be the postulated heterogeneity of thymine concentration within regions of DNA of such lengths.
4. It follows logically from this interpretation, that a heterozygote for a deletion mutant would not be accumulated in an FUDR cross, and that the effect of the deletion in reducing HET frequency would extend only to markers within a few map units on either side of the deletion.

Figure 8 shows a possible structure for a parental HET (utilizing the notion of single-stranded fragment incorporation) and the molecular structure of a HET which is recombinant for markers on opposite sides of the heterozygous region.

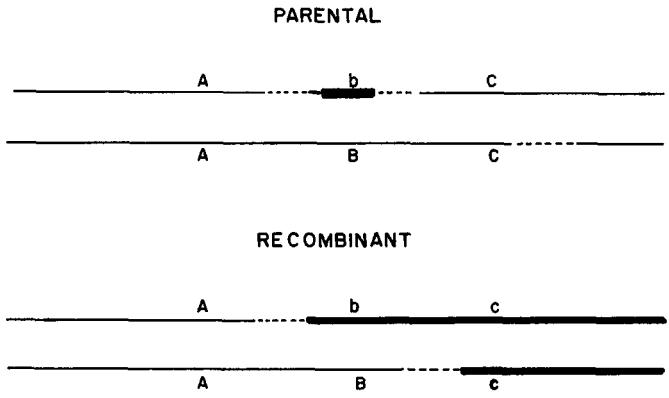

FIGURE 8.—Proposed molecular structure for the parental and recombinant HETs observed in multifactor FUDR crosses. The light and heavy solid lines represent DNA strands from the infecting parental genomes. The dotted lines indicate newly synthesized DNA.

The relationship between the results described in this investigation and recombination data obtained from non-FUDR crosses using parents containing deletions will be discussed in a forthcoming paper (BERGER, in preparation).

The author wishes to acknowledge the advice and encouragement of DR. A. H. DOERMANN during the course of this work. DR. F. C. WOMACK is also acknowledged for her many helpful discussions. Gratitude is extended to DR. H. ROMAN and the Department of Genetics, University of Washington, who generously provided facilities during the preparation of this manuscript.

SUMMARY

Experiments were designed to study the genetic structure of bacteriophage T4D heterozygotes (HETs) produced in crosses carried out in the presence of 5-fluorodeoxyuridine, which inhibits DNA synthesis. Multifactor crosses were performed with stocks containing up to ten *rII* markers and 16 other markers distributed around the genome. Individual progeny were tested to determine their genotype at each of the marked loci. (1) Individual point-mutant markers fluctuate widely in their frequency of heterozygosity. This result is clearest in the well marked *rII* region, where a distinct gradient effect is apparent. (2) Approximately one half of the HETs are parental for markers on opposite sides of the heterozygous region. (3) The majority of HETs exhibit nonpolarized segregation. (4) Deletion markers do not show an increased HET frequency in FUDR. (5) Deletions affect the accumulation of HETs only for point mutant markers located quite close to the deletion site. A model is proposed for the formation of HETs in the presence of FUDR which involves the incorporation of single-stranded parental DNA segments into progeny molecules.

LITERATURE CITED

ADAMS, M. H., 1959 *Bacteriophages*, Interscience Publishers, New York.

ANRAKU, N., and J. TOMIZAWA, 1965a Molecular mechanisms of genetic recombination in bacteriophage. III. Joining of parental polynucleotides of phage T4 in the presence of 5-fluorodeoxyuridine. J. Mol. Biol. **11**: 501–508. —— 1965b Molecular mechanisms of genetic recombination in bacteriophage. IV. Absence of polynucleotide interruption in DNA of T4 and λ phage particles, with special reference to heterozygosis. J. Mol. Biol. **11**: 509–527.

CHASE, M., and A. H. DOERMANN, 1958 High negative interference over short segments of the genetic structure of bacteriophage T4. Genetics **43**: 332–353.

COHEN, S. S., J. G. FLAKS, H. D. BARNER, M. R. LOEB, and J. LICHTENSTEIN, 1958 The mode of action of 5-fluorouracil and its derivatives. Proc. Natl. Acad. Sci. U.S. **44**: 1004–1012.

DOERMANN, A. H., and L. BOEHNER, 1963 An experimental analysis of bacteriophage T4 heterozygotes. Virology **21**: 551–567.

DOERMANN, A. H., and M. B. HILL, 1953 Genetic structure of bacteriophage T4 as described by recombination studies of factors influencing plaque morphology. Genetics **38**: 79–90.

EDGAR, R. S., 1958 Mapping experiments with *rII* and *h* mutants of bacteriophage T4D. Virology **6**: 215–225.

EDGAR, R. S., R. P. FEYNMAN, S. KLEIN, I. LIELAUSIS, and C. M. STEINBERG, 1962 Mapping experiments with *r* mutants of bacteriophage T4D. Genetics **47**: 179–186.

EDGAR, R. S., and R. H. EPSTEIN, 1961 Inactivation by ultraviolet light of an acriflavine sensitive gene function in phage T4D. Science **134**: 327–328.

EPSTEIN, R. H., A. BOLLE, C. M. STEINBERG, E. KELLENBERGER, E. BOY DE LA TOUR, R. SHEVALLEY, R. S. EDGAR, M. SUSSMAN, G. H. DENHARDT, and A. LIELAUSIS, 1963 Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. **28**: 375–392.

KOZINSKI, A. W., and P. B. KOZINSKI, 1963 Fragmentary transfer of P^{32}-labelled parental DNA to progeny phage. Virology **20**: 213–229. —— 1964 Replicative fragmentation in T4 bacteriophage DNA. Proc. Natl. Acad. Sci. U.S. **52**: 211–218.

LEVINTHAL, C., 1954 Recombination in phage T2; its relation to heterozygosis and growth. Genetics **39**: 169–184. —— 1959 Bacteriophage genetics. Chapt. 8. *The Viruses*, Volume 2. Edited by F. M. BURNET and W. M. STANLEY. Academic Press, New York.

MESELSON, M., 1964 On the mechanism of genetic recombination between DNA molecules. J. Mol. Biol. **9**: 734–745.

NOMURA, M., and S. BENZER, 1961 The nature of the "deletion" mutants in the *rII* region of phage T4. J. Mol. Biol. **3**: 684–686.

SIMON, E. H., and I. TESSMAN, 1963 Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S. **50**: 526–532.

STAHL, F. W., R. S. EDGAR, and J. STEINBERG, 1964 The linkage map of bacteriophage T4. Genetics **50**: 539–552.

STREISINGER, G., R. S. EDGAR, and G. H. DENHARDT, 1964 The gross structure of the genome of phage T4: The circularity of the linkage map. Proc. Natl. Acad. Sci. U.S. **51**: 775–777.

TESSMAN, I., 1962 The induction of large deletions by nitrous acid. J. Mol. Biol. **5**: 442–444.

VOLKIN, E., and A. RUFFILLI, 1962 Dissociation of macromolecular synthetic processes in T2-infected *E. coli*. Proc. Natl. Acad. Sci. U.S. **48**: 2193–2200.

WOMACK, F. C., 1963 An analysis of single-burst progeny of bacteria singly infected with a bacteriophage heterozygote. Virology **2**: 232–241.