GENETIC RECOMBINATION BETWEEN CLOSELY LINKED MARKERS OF BACTERIOPHAGE T4. I. A DUAL MECHANISM FOR RECOMBINANT FORMATION

MASAAKI HONDA AND HISAO UCHIDA

*The Institute of Medical Science, The University of Tokyo,
P. O. Takanawa, Tokyo, Japan*

Received June 17, 1968

HERSHEY and CHASE (1951) discovered that about two percent of the progeny particles recovered from crosses of bacteriophage T2 were heterozygous for any specified marker, and that particles heterozygous for one marker were usually homozygous for other, unlinked markers. They suggested that the formation of heterozygotes and the formation of recombinants might be related processes.

By performing three-factor crosses with distantly linked markers, LEVINTHAL (1954) showed that phages heterozygous for the central marker produce progeny which are usually homozygous recombinant for the outside markers. He proposed that the heterozygotes are formed and segregate in the mating pool, and that each heterozygote contains two long parental segments separated by a short segment of biparental origin. He calculated that the frequency of heterozygous particles is enough to account for all recombinants—assuming that a heterozygote is the first product of recombination and that all homozygous recombinants arise by segregation of heterozygotes. It has generally been assumed that the partial diploidy of the phage genome specified in this model reflects the duplex nature of the DNA molecule, and that the heterozygote is structurally a “heteroduplex” in which the heterozygous region is a DNA segment, each strand of which derives from a different parent. Heterozygotes are also generated as a result of terminal redundancy (STREISINGER *et al.* 1967; FRANKEL 1968), and care must be taken to distinguish the two types.

The heterozygous region often includes two or more markers if they are very closely linked. When crosses are performed between parents multiply marked within a sufficiently short map interval, it is generally found that multiple exchanges are far more frequent than would be expected if exchanges were *statistically* independent. This phenomenon is called “high negative interference” (hereafter referred to as HNI—CHASE and DOERMANN 1959). HNI originates from a single act of mating (EDGAR and STEINBERG 1958), and may be attributable to segregation of heterozygotes (EDGAR 1961). EDGAR proposed a unified mechanism of recombination for all genetic distances, which assumes that a heterozygote is always an intermediate in the process of recombinant formation. STEINBERG and EDGAR (1962) devised a critical test of this hypothesis and applied it to phage T4. The principle of the test is as follows: Consider a biparental cross involving a group of very closely linked markers, and in addition, outside markers

loosely linked on either side. STEINBERG and EDGAR reasoned that most of the progeny particles recombinant for the closely linked markers should also be recombinant for the outside markers, regardless of how many exchanges are required to produce the selected recombinant class. Contrary to expectation, however, they found that markers outside a selected close double exchange are usually in the *nonrecombinant* configuration. They therefore concluded that the current theory of genetic recombination in bacteriophage is inadequate.

STEINBERG and EDGAR deliberately assumed the existence of only one type of heterozygote. However, if there exist *two* kinds of heterozygotes, the structure envisaged by LEVINthal (which produces recombinants between distantly linked markers by a single exchange) and a second kind of heterozygote (which produces HNI), then there would be no paradox. Our problem, therefore, becomes one of characterizing the second kind of heterozygous structure.

NOMURA and BENZER (1961) found that the frequency of r^+/r heterozygous particles incorporating a T4 *rII* deletion was only about 0.4%, instead of the value of 1.4% usually found in crosses involving point mutations. SÉCHAUD *et al.* (1965) explained this finding by assuming two kinds of heterozygous particles; one kind contains heteroduplex heterozygosity (internal heterozygotes), and the other is made possible by the terminal redundancy of the DNA molecule (terminal redundancy heterozygotes). The terminal regions of infecting chromosomes are claimed to show increased frequencies of recombination, the increase being sufficient to account for HNI (MOSIG 1963; DOERMANN and PARMA 1967). This conclusion was based on the observation that in single bursts of crosses involving partial T4 phage particles, each containing a *circularly permuted* DNA fragment two-thirds of the normal length, recombination frequencies were usually high in the "terminal regions" of the parental genomes. However, in order to identify recombinations from single bursts as having occurred at a terminus of the parental genome, the entire partial genome had to be recovered in the same burst. It is therefore crucial to know whether the partial genome can multiply without first recombining into a helper genome at the "terminus". However, there is no experimental evidence that DNA fragments replicate before they are integrated into complete chromosomes. If only those exceptional bursts had been examined in which an early recombination event had to occur within the terminal region of the parental partial genome, then the observed high recombination frequencies do not guarantee that similar high frequencies of recombination would also occur at the ends of freely replicating complete genomes.

We will explore a different classification of heterozygous structures. A recombinant heterozygote will be called a "Levinthal structure", and a non-recombinant heterozygote will be called an "anti-Levinthal structure" (Figure 1). Because terminal redundancy heterozygotes are usually recombinant outside the overlap region, they are classified as Levinthal structures. The results of STEINBERG and EDGAR can now be restated: single exchanges are produced by Levinthal structures, whereas tight *double* exchange originates from anti-Levinthal structures. Therefore, either (a) all close *odd* exchanges are produced by

Levinthal structure:

anti-Levinthal structure:

FIGURE 1.—Possible genetic models of the heterozygotes according to LEVINTHAL (1954). The molecular model for the Levinthal structure is indicated in the parentheses.

Levinthal structures, whereas all close *even* exchanges are produced by anti-Levinthal structures, or (b) Levinthal structures produce only *single* exchanges, and all close *multiple* exchanges are in fact *even* exchanges produced by anti-Levinthal structures. A crucial experiment to discriminate between these alternatives would be to examine the outside marker configuration bracketing close *triple* exchanges. We present evidence which supports alternative (b), which can be restated as the following postulate: *Recombination among closely linked markers showing HNI proceeds via anti-Levinthal structures*. Preliminary accounts have already been published (HONDA and UCHIDA 1967; UCHIDA and HONDA 1968).

MATERIALS AND METHODS

Bacterial strains: *Escherichia coli* B, strain S (HERSHEY and ROTMAN 1949) was used as the host for phage crosses, and as a nonselective indicator for the determination of the total progeny. K12(λ) was supplied by Dr. L. BARNETT, and found to be free of amber suppressors. K12(λ) S26RIE, harboring *su 1+* suppressor, and K12(λ) D32, harboring *su III+* suppressor, originally isolated by GAREN and BRENNER, respectively, were obtained from Dr. K. HORIUCHI. CR63(λ) was also used as an amber-permissive indicator.

Phage strains: The wild type strain T4B and *ts rII* mutants 221, H88, 3093, and 596 were obtained from Dr. S. BENZER. T4B *rII* NB4777 was obtained from Dr. A. NAKATA. T4B *rII* mutants G178, N11, and EM20 were supplied by Dr. S. CHAMPE. Other *rII* mutants used in the experiments were isolated by one of us (M. H.) from T4B following nitrous acid treatment. All of the *rII* mutants used fall in the *A* cistron; 221 and H88 are deletion mutants. Their map positions are indicated in Figure 2. NB4777, M36, N11, and M35 are amber mutants. M36 is suppressible by *su III+*. Various double mutants were prepared by recombination.

Crosses: Unless otherwise stated, crosses were performed according to CHASE and DOERMANN (1958), except that the temperature was maintained at 37°. Chloroform was added 90 min after infection.

UV irradiation: One ml samples in buffered saline at 2×10^{10} phage per ml were irradiated at room temperature with a 19 watt Toshiba Electric germicidal lamp. One phage-lethal hit was delivered in 4.5 sec. **Fluorouracil deoxyriboside:** Fluorouracil deoxyriboside (FUDr) was a gift of Dr. J. TOMIZAWA.

FIGURE 2.—Map of the T4B *rIIA* cistron showing approximate linkage relationships of the markers used in the present experiments. Distances are expressed as twice the frequency of wild-type recombinants (in percent) in each interval.

RESULTS

Experiment 1. UV cross-reactivation:

The purpose of the experiments described here is to examine close *triple* exchanges to see if markers bracketing the selected overlap will appear in recombinant or parental configurations. It is first necessary, however, to show that the “outside” markers are really outside of the overlap region. Therefore, instead of actually introducing outside markers, one of the parental phages in the biparental cross was UV-irradiated. Thus, the experiments to be described are in effect UV cross-reactivation experiments (DOERMANN *et al.* 1955), and what we will try to show is that by selecting single and double exchange recombinants, we are selecting *different* classes of heterozygotes, whereas by selecting double and triple exchange recombinants, we are selecting the *same* class of heterozygotes.

Cross-reactivation proceeds by recombining away an undamaged marker from its surrounding hits, but UV hits need not cause recombination at the site of the hit itself, and the entire segment between two lethal hits is rarely rescued (KRIEG 1959). With increasing UV doses, the sensitive target of cross-reactivation becomes progressively smaller and smaller as hits are crowded nearer and nearer to the marker, until at doses of 300 phage-lethal hits the minimum target size is obtained, and practically every marker-inactivation may be caused by inactivation of the marker itself (DOERMANN 1961). In the present experiments, however, at most 50 lethal hits were administered per particle, and parental markers were lost mostly because recombination incorporated nearby UV hits together with the marker. It has been demonstrated that, under the conditions of the ex-

periment, damaged parental genomes may be incorporated into progeny particles, the majority of these particles being dead (HERSHEY and BURGI 1956).

In the following experiments, recombinants requiring an odd number of exchanges between closely linked T4 *rII* markers were compared with recombinants requiring an even number of similar exchanges. It would be convenient, therefore, if the comparisons could be made among the progeny of the same cross. In order to accomplish this, one of the *rII* markers employed was amber and exhibited the wild type phenotype on an amber-permissive host. The experiment consisted of two parts. In the first part, the frequency of r^+ recombinants issuing from a biparental two-factor cross $r_a \times r_b$ was compared with the corresponding r^+ frequency among the progeny of a biparental three-factor cross $r_a r_c \times r_b$, which requires an additional exchange between r_b and r_c . Among the markers employed, r_c was an amber, so that when the progeny of the three-factor cross were plated on a nonpermissive indicator, only the wild type recombinants $r_a^+ r_b^+ r_c^+$ formed plaques. However, when the same progeny were plated on an amber-permissive indicator, recombinants of the type $r_a^+ r_b^+ r_c$ also formed plaques. In Figure 3, "Cross 1" indicates the markers used in the experiment, where *M13*, *M16*, and *M36* correspond to r_a , r_b , and r_c , respectively. In the second part of the experiment, the frequency of r^+ recombinants issuing from a biparental three-factor cross of the type $r_a r_c \times r_b$ was compared with the corresponding r^+ frequency issuing from the biparental four-factor cross, $r_a r_c \times r_b r_d$. One of the markers, r_d , was again an amber mutation. In Figure 3, "Cross 2" indicates the markers used in the cross, where *M8*, *G178*, *EM20*, and *M35* correspond to r_a , r_b , r_c , and r_d , respectively. For both Cross 1 and Cross 2, *E. coli* BS, the nonselective strain, was used as the host. One of the parental types, as indicated in Figure 3, was irradiated with various doses of UV. The progeny were

FIGURE 3.—Parental genotypes in multifactor crosses. Parents marked with arrows were UV-irradiated with varying doses.

FIGURE 4.—Effects of varying UV doses administered to one of the parental phages on the requirement for an extra act of exchange in the UV cross-reactivation of *rII* markers. The $n = 1$ curve compares single with double exchanges, and the $n = 2$ curve compares double with triple exchanges. The ordinate indicates the ratio of the frequencies of n exchange recombinants to $n + 1$ exchange recombinants. Multiplicity of infection was always adjusted to 5 particles of each parental type per bacterium, irrespective of UV dose administered.

plated on *E. coli* BS to measure the total yields, and were also plated on both nonpermissive and amber-permissive K12(λ) to measure recombinants. The recombinant frequencies were expressed in terms of R_n , which is twice the frequency of wild type recombinants, the subscript n indicating the number of exchanges required to produce the selected recombinants. In order to evaluate the effect of introducing one extra exchange, the ratio of $(R_n - R_{n+1})$ to R_{n+1} was calculated for each cross, and plotted against the UV dose administered to one of the parental phages (Figure 4). The $n = 1$ curve, which compares single exchanges with double exchanges, falls monotonically without a shoulder as the UV dose increases; at the highest UV dose employed, the ratio is only one-tenth of the zero-dose value. This indicates that a single-exchange recombinant molecule is more susceptible to UV damage than is a double-exchange recombinant molecule, and is interpreted to mean that single exchange recombinants incorporate parental UV lesions more easily than do double exchange recombinants. The requirement for a double exchange seems to have simultaneously eliminated UV lesions. Those hits which are unlinked to the *rII* region should be incorporated into both R_1 and R_2 recombinant molecules to the same extent; only the lesions in the immediate vicinity of the *rII* region should contribute to the differential UV sensitivity. Thus, the present experiment again manifests the "paradox" of

STEINBERG and EDGAR, in the sense that a single type of heterozygote structure cannot be responsible for both single- and double-exchange recombinants. A similar examination of double- and triple-exchange recombinants is summarized as curve $n = 2$ in Figure 4. This curve remains relatively flat as the UV dose increases. The result indicates that UV damages have the same effect on triple exchange as on double-exchange recombinant formation, or more explicitly, that close triple exchanges do not incorporate neighboring UV lesions into the recombinants. This result strongly contradicts the classical rules of crossing over, but is consistent with the idea that a special process of recombinant formation exists for multiple exchanges among closely linked markers. If we postulate that this process always involves an anti-Levinthal structure as an intermediate, regardless of the number of exchanges required, the experimental findings are well explained. According to our present model, close triple exchanges are in fact at least quadruple. Therefore, in four-factor crosses of the type $r_a r_c \times r_b r_d$, recombinant molecules are produced from both of the two alternative configurations of anti-Levinthal structures. Since one of them is liable to incorporate nearby UV lesions, the slight upward increase which may be noticed in the curve $n = 2$ of Figure 4 is quite reasonable. Because recombinations among closely linked markers always show HNI, we propose the above postulate as a generalization.

The Levinthal structure, which is mainly responsible for the production of single exchange recombinants, is currently visualized as a heteroduplex formed by the breakage and union of two parental DNA molecules (MESELSON and WEIGLE 1961; KELLENBERGER *et al.* 1961; TOMIZAWA and ANRAKU 1964). However, no exact model for the nature of the anti-Levinthal structure has yet been proposed. Therefore, its properties were further examined in the following experiments.

Experiment 2. Three-factor biparental crosses requiring double exchanges bracketing a deletion mutation:

Experiment 1 described above supported an idea that close multiple-exchange recombinants are produced by the anti-Levinthal structure. Then one should examine whether the postulate is always true, even in an unfavorable situation. It is generally assumed that extended deletions cannot form internal heterozygotes since deletions are *forbidden* to form heteroduplexes in the Watson-Crick structure, and that only the terminal redundancy heterozygote is available for deletion mutations (SÉCHAUD *et al.* 1965). On the other hand, anti-Levinthal structures may be considered as internal heterozygotes. Therefore, properties of three-factor biparental crosses requiring double exchanges bracketing a deletion mutation to yield wild-type recombinants were investigated in the following. Similar studies were also carried out by DOERMANN and PARMA (1967), but it was noted that deletion mutations employed in their experiments were much shorter than those used in the present study. In order to eliminate one class of terminal redundancy heterozygotes, the complementation heterozygotes (STAHL *et al.* 1965), all of the markers employed in the present study belonged to a single cistron, *rIIA*.

TABLE 1

T4B rII crosses in which two recombinational events are required to produce a wild-type recombinant

Cross	Inside marker	Percent recombinants		wild/total	$\frac{2 \times \text{wild/total}}{y \times z}$
		x	y + z		
<i>M36 M13</i> $\times$ <i>221</i>	deletion	2.6	2.3	3.5×10^{-4}	5.8
<i>NB4777 596</i> $\times$ <i>H88</i>	deletion	4.4	1.3	1.0×10^{-4}	4.7
<i>3093 596</i> $\times$ <i>H88</i>	deletion	4.7	2.0	1.9×10^{-4}	4.4
<i>M36 M13</i> $\times$ <i>M16</i>	point	2.6	2.8	1.5×10^{-3}	14.9
<i>M36 M13</i> $\times$ <i>G178 N11</i>	double	2.6	2.7	5.7×10^{-4}	7.0
<i>3093 596</i> $\times$ <i>M36 M13</i>	double	4.7	4.2	6.5×10^{-4}	3.1
<i>3093 596</i> $\times$ <i>221</i>	deletion	4.7	4.7	8.1×10^{-4}	3.0
<i>205 287</i> $\times$ <i>320 114</i> *	double	4.4	2.1	6.5×10^{-5}	1.7
<i>205 287</i> $\times$ <i>320 163</i> *	double	4.4	1.2	2.8×10^{-5}	1.8

* Data from CHASE and DOERMANN (1958).

When such crosses were carried out, the occurrence of HNI was noticed for a long deletion *H88* which covers 117 recombinable point-mutant sites as well as for a short deletion *221* which covers 16 known sites (BENZER 1961) as the central marker in the cross (Table 1). In order to see whether the observed HNI was due to correlated exchanges in a single mating act, or to a concentration of exchanges in the same region in successive matings, a test devised by EDGAR and STEINBERG (1958) was applied to the present case: The test consisted of measuring recombination values in a series of crosses in which the relative frequencies of the two parental phages were systematically varied. Thus, in a cross $+r+$ with $r+r$, if the wild-type recombinant is produced by only two successive mating events, both of which involve the $+r+$ parent, the frequency of $+++$ recombinants is expected to change in an asymmetrical manner, giving much more recombinants when $+r+$ is the majority parent in crosses with an unequal input. However, as can be seen in Figure 5, the maximum number of wild-type recombinant phage was produced at an equal parental input, which indicates that the recombinants were formed mostly in a single mating event. Therefore, at least qualitatively, deletion mutations behave in the same manner as point mutations in similar crosses. According to our postulate, recombinations requiring close multiple exchanges are always produced from intermediates having anti-Levinthal structures. In order to apply this postulate to the present situa-

FIGURE 5.—The frequency of wild-type recombinants as a function of the frequency of the $+r+$ parent for a biparental cross $r+r \times +r+$. The central marker, 221, was a deletion mutation. Different symbols refer to independent experiments. Total input multiplicity of infection was always adjusted to 10 per bacterium.

tion, it is convenient to assume that the formation of a loop-out structure is allowed within the overlap structure, in order to circumvent the base-pairing difficulty caused by a deletion singularity. An alternative to loop-out formation would be a discontinuity produced in the deletion mutant DNA which lies in apposition to the wild-type allele. Discrimination between the two models, loop-out or discontinuity, may be possible if we could show that the distance between two appropriate mutations shrinks when the double mutant containing the two mutations is mated with an inner deletion. By "distance," we do not mean here a contour length along the DNA strands, but the effective separation between the two loci to be included within an anti-Levinthal structure. In this connection, the observation of CHASE and DOERMANN (1958), a part of which is quoted in Table 1, is pertinent. They found that in crosses $r_a r_d \times r_b r_c$, the HNI gradually disappears as the r_b to r_c interval becomes longer. For example, one of their crosses, $r_{205} r_{287} \times r_{320} r_{114}$ showed no HNI in excess of what can be accounted for from the mating kinetics, indicating that the interval r_{320} to r_{114} is too long to allow the occurrence of two correlated exchanges at both the ends of the interval. According to BENZER (1961), segmentation mapping shows that there are 122 ± 13 recombinable point-mutant sites between the inner markers r_{320} and r_{114} , and there are 207 ± 7 sites known between the outer markers r_{205} and r_{287} . Fortunately, this geometry can be readily compared with that of the present cross, $3093\ 596 \times H88$ which showed HNI; $H88$ covers 117 known sites, and the distance between 3093 and 596 is 192 ± 2 . Therefore, we may infer that the sub-

stitution of the double mutant $r_b r_c$ by a deletion of that interval allowed the HNI to occur, because the loop-out circumventing the deletion enabled the inclusion of both r_a and r_d within a single overlap. If we accept this inference, two colloraries will follow:

(a) The length of the overlap is short enough to cover 75 recombinable point-mutant sites but not 200 sites. DRAKE (1967) estimated the size of the homologous pairing region as being larger than the deletion $r196$ which covers about 26 sites, but probably smaller than the deletion $r1272$ which covers at least the whole rII region.

(b) The "short fragment" of the anti-Levinthal structure may be longer than the overlap length, because in crosses $r_a r_c \times r_b$, the double mutation $r_a r_c$ should be in the short segment and form loop-out in case r_b is a deletion, since we are presently selecting for wild-type recombinants.

Experiment 3. UV cross-reactivation with a deletion mutant:

A UV cross-reactivation experiment similar to that described in Experiment 1 was carried out using $M36 M13$ and 221 (a deletion which covers $M16$ used in Experiment 1). Figure 6 shows the results in which the ratio $(R_1 - R_2)/R_2$ is plotted against the UV dose administered to the parental $M36 M13$, together with a reproduction of the $n = 1$ curve of Figure 4, with which the present results

FIGURE 6.—UV cross-reactivation experiment with a deletion mutant. UV-irradiated double mutant, $M36 M13$ was crossed with a nonirradiated, centrally located deletion mutant, 221 . The lower curve is a reproduction of the $n = 1$ curve of Figure 4, to which the upper curve can be compared.

can be compared. As can be seen from the figure, the two curves are very similar. This is interpreted to mean that the mechanisms of recombinant formation are also very similar, at least as far as the configuration of the adjacent regions are concerned, regardless of whether the central marker was a point mutation or a deletion. Therefore, the present experiment again suggests that, in the biparental three-factor cross with a central deletion, the recombinants are produced from the anti-Levinthal structure.

Experiment 4. Effect of FUdR on three-factor biparental crosses:

SÉCHAUD *et al.* (1965) discovered that in the presence of fluorouracil deoxyriboside (FUdR), which inhibits the replication of DNA but allows recombinations to proceed, the frequency of r^+/r heterozygous particles incorporating deletions stays constant, whereas heterozygous particles involving point-mutations accumulate. The finding was interpreted to suggest that the ability to form internal heteroduplex heterozygotes is the privilege of point-mutations. Experiments of WIEMANN (1965) also confirm this interpretation. However, if the loop-out structure is possible, this privilege of point-mutations will disappear, and the heteroduplex heterozygotes involving deletions should also accumulate in the presence of FUdR. Therefore, effects of the presence of FUdR on three-factor biparental crosses were examined in the following.

First, the effect of FUdR on the wild-type recombinant frequency of a cross $r_a r_c \times r_b$, where the central marker r_b was a deletion, was examined. The results are summarized in Figure 7, from which one can see that the recombinant frequency increases with the time of FUdR treatment, exactly in a similar manner as when the central marker was a point-mutation. However, since the frequency of heterozygous particles incorporating the deletion does not change with the time of FUdR treatment, it follows that the structure which was incorporated within the heterozygous *particle*, or the terminal redundancy heterozygote, is *not* the direct precursor of the wild-type recombinants. It may be that deletion mutations form internal heterozygotes within the DNA pool, but they are later repaired by a mechanism leading to homozygosity before maturation into infective progeny-particles. When the size of a deletion is small, loop-out structures may escape repair, and mature into infective particles (DRAKE 1966).

Secondly, FUdR was found to affect the relative frequency of single- and double-exchange recombinants. As can be seen from the results listed in Table 2, the ratio of single- to double-exchange recombinants decreases in the presence of FUdR when chloramphenicol was added 9 min after phage infection. The decrease was not observed when chloramphenicol was added 3 min after infection, nor when FUdR was not present. Since double-exchange recombinants are products of anti-Levinthal structures, we may infer from the experiments that FUdR induces a reaction which enriches the frequency of anti-Levinthal structures. In this connection, it is pertinent to cite two observations of BERGER (1965) and of WIEMANN (1965) who found that a large percentage of the heterozygous particles arising from T4 crosses in the presence of FUdR are parental for markers immediately adjacent to the region heterozygous for point mutation(s), and that

FIGURE 7.—The effect of FUDr presence for various periods on the frequency of wild-type recombinants. *E. coli* BS was grown to a concentration of 2×10^8 cells/ml in M9 medium supplemented with 0.1% casamino acids and 20 $\mu\text{g}/\text{ml}$ tryptophan. FUDr and uracil were added to final concentrations of 8 $\mu\text{g}/\text{ml}$ and 16 $\mu\text{g}/\text{ml}$, respectively. 3 minutes later, a mixture of input phage, M36 M13 and 221, was added with an average of five phage of each type per bacterium. 9 minutes after the phage infection, 200 $\mu\text{g}/\text{ml}$ of chloramphenicol was added, and at various times after that, aliquots of the culture were diluted with an equal volume of cold M9, washed twice by centrifugation to remove chloramphenicol, and resuspended with M9 medium containing casamino acids, FUDr, and uracil. The cultures were then further incubated with aeration for 60 min, lysed by the addition of chloroform, and plated on both BS and K12(λ)*su*⁻. Abscissa indicates time after infection to chloramphenicol removal, and different symbols refer to independent experiments. The filled square represents a control experiment in which FUDr addition was omitted, but incubation with chloramphenicol continued until 130 min after infection.

the frequency of heterozygosity for individual *rII* point-mutation sites exhibits a distinct gradient effect which is not observed in normal crosses without FUDr treatment. The former finding, of course, supports our inference, and the second finding was interpreted to be due to a nonrandom requirement for thymine-containing nucleotides in different regions of the genome, which leads to local slowing-down of the DNA replication in the presence of FUDr, and thus causes a longer availability of the single-stranded regions for pairing with the postulated single-stranded fragments.

DISCUSSION

DOERMANN and BOEHNER (1963) have analyzed the phage present in mottled plaques arising from particles heterozygous for several fairly closely linked *rII* markers, and found that the two-ended heterozygous structures occur with a

TABLE 2

The effect of FUdR addition on recombination between closely linked T4B rII markers

FUdR	Time of CMP addition (min)	R ₁	R ₂	$\frac{R_1 - R_2}{R_2}$
				R ₂
PRESENT	3	0.76%	0.024%	31
		0.98	0.027	35
		0.88	0.027	32 average = 32
		1.12	0.036	30
		1.04	0.037	27
		1.22	0.032	37
	9	3.50	0.27	12
		3.36	0.29	11
		3.94	0.38	9 average = 11
		3.78	0.30	12
		3.80	0.30	12
		4.60	0.40	10
ABSENT	3	0.88	0.032	27
		0.84	0.034	24 average = 29
		0.84	0.025	33
		0.94	0.029	31
	9	1.02	0.029	34
		1.16	0.030	38 average = 37
		1.28	0.036	35
		1.10	0.027	40

E. coli BS were grown to a concentration of 2×10^8 cells/ml in aerated M9 medium supplemented with 0.1% casamino acids and 20 $\mu\text{g/ml}$ tryptophan. The culture was divided into two parts, and one of them received 8 $\mu\text{g/ml}$ FUdR and 16 $\mu\text{g/ml}$ uracil. 3 minutes later, a mixture of input phages, M36 M13 and 221, was added with an average of five phages of each type per bacterium. 3 or 9 minutes after the phage infection, 200 $\mu\text{g/ml}$ of chloramphenicol was added, and at 55 min after infection, chloramphenicol was removed by centrifugation. The cells were resuspended in M9 medium containing casamino acids, uracil, and 10 $\mu\text{g/ml}$ thymidine, and further incubated with aeration for another 60 min and then lysed by the addition of chloroform. Progenies were plated on BS, K12(λ)*su*⁻ and K12(λ)D32 *su III*⁺.

considerable frequency. In spite of this observation, they refrained from assuming two basically different kinds of heterozygotes as we have done here, but instead visualized the two-ended heterozygotes as a structure in which a short replica patches over what would constitute a small discontinuity in an otherwise continuous genome, thus forming two discrete overlapping areas, each one of which can be classified as the Levinthal structure according to the present terminology (Figure 1). Although their model may explain tight double exchanges, if it were applied to a case of, for example, four close exchanges, one would have to imagine a very complicated situation in which a number of very short replicas partially overlap, in succession, to patch over discontinuities of the genome. Moreover, the assumption for any discontinuity in one or both of the strands of matured T4 phage DNA was rejected on the basis of band centrifugation studies of DNA extracted from T4 phage particles in an alkaline caesium chloride solution (TOMIZAWA and ANRAKU 1965).

We propose to classify the acts of recombination into two categories: the breakage and union of two parental DNA molecules is responsible only for the production of recombinants, by single exchange, via the Levinthal structure (Figure 1), whereas recombinants requiring close multiple exchanges are produced by the anti-Levinthal structure. This generalized statement of the above postulate is based mainly on the findings of the UV cross-reactivation experiment (Experiment 1), which suggested that *both* double and triple exchanges originated from a heterozygous structure, parental with respect to the adjacent outside regions (anti-Levinthal structure). The anti-Levinthal structure may also contribute to two-factor crosses, but only to a minor extent when markers are distantly linked. When markers are sufficiently close, the contribution of the anti-Levinthal structure is expected to be much larger. HERSHEY cited an observation of EDGAR that phage particles arising in a four-factor cross and selected for a rare recombination between the two closely linked central markers do not tend to be recombinant with respect to markers lying at some distance on either side of the selected region (HERSHEY 1958; STEINBERG and EDGAR 1962). This phenomenon obviously needs reexamination, and will be dealt with in a subsequent communication.

Our postulate of the dual mechanisms for recombinant formation may help to resolve some of the previously experienced inconsistencies: the classical "paradox" of STEINBERG and EDGAR (1962) is resolved in a manner as discussed in the Introduction. Perhaps the term "high negative interference" is misnamed, and the "positive correlation of exchanges within a short segment" must be understood as a characteristic of the function of the anti-Levinthal structure itself, which is unrelated to the recombinant formation by the Levinthal structure. Since we are postulating that close double exchanges are produced by a route basically different from the one which produces single exchanges, close double exchanges cannot be considered as successions of two single exchanges. Thus, the coefficient of coincidence frequently used to express the *degree* of HNI has only qualitative meaning—it indicates the presence or absence of the close, multiple exchanges.

Since the gene conversion (or muton conversion) model is inadequate to explain recombinant formation (CHASE and DOERMANN 1958), we suppose that any tight exchanges are always characterized by an even number of switches. A model in which a small, two-ended piece attached to the side of a normal chromosome (a material overlap, anti-Levinthal structure) may explain the requirement for the even number of switches. It should be stated here that the heterozygous structure to which we are referring here is a structure present within the mating pool, and not necessarily a structure incorporated into the mature particle. Therefore, the material overlap, anti-Levinthal structure may be visualized to represent a collision complex of two DNA molecules. The collisions are effective when identical parts of the genetic message are brought into apposition, with a possible allowance for loop-out formation (Experiment 2).

Another clue to the nature of the anti-Levinthal structure may be found in the effects of FUDR addition during mating, which leads to enrichment of the anti-Levinthal structure (Experiment 4). If we consider the anti-Levinthal structure as a collision complex, the enrichment of the latter may result from an increase in

the concentration of at least one of the colliding species. Since FUdR is known to inhibit DNA synthesis by inhibition of thymidylate synthetase (COHEN *et al.* 1958), and the same inhibitory action also affects the transformation of a joint molecule to a duplex molecule by polynucleotide synthesis (ANRAKU and TOMIZAWA 1965), not both of the colliding components can be intact DNA molecules, but at least one of them should be a derivative of an intact molecule. Short DNA fragments, single-stranded or duplex, long DNA molecules with partial exposure of single-stranded segments, and other structures can be conceived as candidates for the derivative structure.

We wish to thank Drs. S. BENZER, S. CHAMPE, A. NAKATA, L. BARNETT, and K. HORIUCHI for generously supplying stocks of T4B *rII* mutants and bacterial strains, Drs. GUNTHER S. STENT and K. YOSHINO for sympathetic help in the preparation of the manuscript, and Drs. J. TOMIZAWA and GISELA MOSIG for criticisms.

SUMMARY

Based on a comparative study of UV cross-reactivation behavior of T4 phage, a dual mechanism for recombinant formation in T4 genetic crosses is proposed: The breakage and union of two parental DNA molecules is responsible only for the production of recombinants by single exchange, whereas recombinants requiring close multiple exchanges are produced by a different route through a structure parental with respect to the adjacent regions outside a short material overlap. The model is useful to resolve some of the previously experienced inconsistencies in the current theory of genetic recombination in bacteriophage.

LITERATURE CITED

- ANRAKU, N. and J. TOMIZAWA, 1965 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.
- BENZER, S., 1961 On the topography of the genetic fine structure. *Proc. Natl. Acad. Sci. U. S.* **47**: 403-415.
- BERGER, H., 1965 Genetic analysis of T4D phage heterozygotes produced in the presence of 5-fluorodeoxyuridine. *Genetics* **52**: 729-746.
- 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., 1961 The analysis of ultraviolet lesion in bacteriophage T4 by cross reactivation. *J. Cell. Comp. Physiol.* **58** (Suppl. 1): 79-93.
- DOERMANN, A. H. and L. BOEHNER, 1963 An experimental analysis of bacteriophage T4 heterozygotes. I. Mottled plaques from crosses involving six *rII* loci. *Virology* **21**: 551-567.
- DOERMANN, A. H., M. CHASE and F. W. STAHL, 1955 Genetic recombination and replication in bacteriophage. *J. Cell. Comp. Physiol.* **45** (Suppl. 2): 51-74.
- DOERMANN, A. H. and D. H. PARMA, 1967 Recombination in bacteriophage T4. *J. Cell. Physiol.* **70** (Suppl. 1): 147-164.
- DRAKE, J. W., 1966 Heteroduplex heterozygotes in bacteriophage T4 involving mutations of

- various dimensions. *Proc. Natl. Acad. Sci. U. S.* **55**: 506-512. —, 1967 The length of the homologous pairing region for genetic recombination in bacteriophage T4. *Proc. Natl. Acad. Sci. U. S.* **58**: 962-966.
- EDGAR, R. S. and C. M. STEINBERG, 1958 On the origin of high negative interference over short segments of the genetic structure of bacteriophage T4. *Virology* **6**: 115-128.
- FRANKEL, F. R., 1968 Evidence for long DNA strands in the replicating pool after T4 infection. *Proc. Natl. Acad. Sci. U. S.* **59**: 131-138.
- HERSHEY, A. D., 1958 The production of recombinants in phage crosses. *Cold Spring Harbor Symp. Quant. Biol.* **23**: 19-46.
- HERSHEY, A. D. and E. BURGI, 1956 Genetic significance of the transfer of nucleic acid from parental to offspring phage. *Cold Spring Harbor Symp. Quant. Biol.* **21**: 91-101.
- HERSHEY, A. D. and R. ROTMAN, 1949 Genetic recombination between host-range and plaque-type mutants of bacteriophage in single bacterial cells. *Genetics* **34**: 44-71.
- HONDA, M. and H. UCHIDA, 1967 Mechanism of T4 bacteriophage recombination involving deletion mutation. *Japan. J. Genetics* **42**: 411.
- KELLENBERGER, G. M., M. L. ZICHICHI and J. J. WEIGLE, 1961 Exchange of DNA in the recombination of bacteriophage λ . *Proc. Natl. Acad. Sci. U. S.* **47**: 869-878.
- KRIEG, D. R., 1959 A study of gene action in ultraviolet-irradiated bacteriophage T4. *Virology* **8**: 80-98.
- LEVINTHAL, C., 1954. Recombination in phage T2: Its relationship to heterozygosis and growth. *Genetics* **39**: 169-184.
- MESELSON, M. and J. WEIGLE, 1961 Chromosome breakage accompanying genetic recombination in bacteriophage. *Proc. Natl. Acad. Sci. U. S.* **47**: 857-868.
- MOSIG, G., 1963 Genetic recombination in bacteriophage T4 during replication of DNA fragments. *Cold Spring Harbor Symp. Quant. Biol.* **28**: 35-52.
- NOMURA, M. and S. BENZER, 1961 The nature of the "deletion" mutants in the *rII* region of phage T4. *J. Mol. Biol.* **3**: 684-692.
- SÉCHAUD, J., G. STREISINGER, J. EMRICH, J. NEWTON, H. LANFORD, H. REINHOLD and M. M. STAHL, 1965 Chromosome structure in phage T4. II. Terminal redundancy and heterozygosis. *Proc. Natl. Acad. Sci. U. S.* **54**: 1333-1339.
- STAHL, F. W., H. MODERSOHN, B. E. TERZAGHI and J. M. CRASEMANN, 1965 The genetic structure of complementation heterozygotes. *Proc. Natl. Acad. Sci. U. S.* **54**: 1342-1345.
- STEINBERG, C. M. and R. S. EDGAR, 1962 A critical test of a current theory of genetic recombination in bacteriophage. *Genetics* **47**: 187-208.
- STREISINGER, G., J. EMRICH and M. M. STAHL, 1967 Chromosome structure in phage T4. III. Terminal redundancy and length determination. *Proc. Natl. Acad. Sci. U. S.* **57**: 292-295.
- TOMIZAWA, J. and N. ANRAKU, 1964 Molecular mechanisms of genetic recombination in bacteriophage. II. Joining of parental DNA molecules of phage T4. *J. Mol. Biol.* **8**: 516-540. —, 1965 IV. Absence of polynucleotide interruption in DNA of T4 and λ phages, with special reference to heterozygosis. *J. Mol. Biol.* **11**: 509-527.
- UCHIDA, H. and M. HONDA, 1968 Heterozygosis and recombination of bacteriophage T4. *Proc. 12th Internatl. Congr. Genetics* **1**: 33.
- WIEMANN, J., 1965 Zur Charakterisierung von Heterozygoten des Phagen T4. *Z. Vererbl.* **97**: 81-101.