POLYNUCLEOTIDE LIGASE IN BACTERIOPHAGE T4D RECOMBINATION

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ABSTRACT

Following infection of *E. coli* B with ligase-deficient *rII* bacteriophage T4D recombination between linked markers is increased 4.2 fold and heterozygote frequency increased 2.3 fold. In such infection recombination occurs at a rapid rate for an extended period. This is in contrast to the time course of recombination observed in wild-type, lysis-inhibited, or lysis-defective (gene *t* defective) infection. In all of these cases recombination under standard cross conditions occurs early in the vegetative cycle. The increased recombination in ligase-deficient *rII* infection is reduced in a bacterial strain which produces greater than normal levels of host ligase. These results indicate that ligase has a crucial role not only in the replication of DNA but also in recombination. The level of ligase may determine whether DNA replication occurs with or without concomitant recombination.

FOLLOWING infection of *Escherichia coli* with bacteriophage T4D a number of early genes are expressed which are primarily concerned with the replication of the viral chromosome. BERNSTEIN (1968) and BERGER, WARREN and FRY (1969) have observed that limitation of expression of many of these functions produces marked effects on genetic recombination. We have examined in detail genetic recombination following infection with T4D which synthesizes DNA abnormally due to a deficiency of polynucleotide ligase. The aim of these experiments is to provide information about DNA structures which are precursors to formation of recombinants and to identify the gene products involved in generating these precursors.

Studies by several groups of workers have demonstrated that the insertion of an *rII* mutation into a T4D strain deficient in polynucleotide ligase (gene 30) restores its viability (KARAM 1969; BERGER and KOZINSKI 1969). Experiments on DNA replication and genetic recombination after infection of *E. coli* with ligase-deficient *rII* phage (KRISCH, SHAH and BERGER 1971) have shown that: (1) Newly synthesized DNA contains numerous discontinuities. (2) These discontinuities are more slowly repaired than in wild-type infection. (3) Host ligase is essential to produce viable progeny. (4) Genetic recombination is increased.

The correlation between the increased genetic recombination and the per-

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sistence of replicative intermediates which contain numerous single-stranded discontinuities suggested that these structures could facilitate break-reunion recombination. More evidence is reported here which lends support to this interpretation of the mechanism by which ligase-deficient *rII* phage increase recombination. A model of recombination will be presented incorporating these findings as well as other results which indicate that extensive DNA replication can occur without producing new recombinants. A possible regulatory role for polynucleotide ligase in recombination is suggested.

MATERIALS AND METHODS

Bacterial strains: *Escherichia coli* B was used as the host for all genetic crosses unless otherwise indicated. *E. coli* CR63 was used to prepare phage stocks and as the permissive host for phage containing *amber* mutations. The non-permissive host for *rII* mutants was *E. coli* CR63 (λh). As indicated in the appropriate figure legends, *E. coli* B40 SUI (obtained from E. GOLDBERG) was used as the indicator strain for phage containing both temperature-sensitive and *amber* mutations. *E. coli* W3110 AB24 SUA (obtained from C. YANOFSKY), which is restrictive for the late function *amber* mutants used, but is permissive for ligase-deficient *rII* phage, was employed as the indicator strain to determine the frequency of wild-type recombinants between late function *amber* markers. For studies on the effect of host ligase on recombination, M. GELLERT kindly provided *E. coli* strains N953 (wild type), and its derivatives N1323 (lop 8) and N1325 (lop 8-lig 2) (GELLERT and BULLOCK 1970). *E. coli* W3110 AB24 SU- (obtained from C. YANOFSKY) was used as the non-permissive host for determination of the DNA synthesis rates by phage strains containing *amber* mutations.

Phages: All of the phages used in the experiments described here are mutants of T4D. The *amber* and temperature-sensitive mutants were obtained from R. S. EDGAR and W. WOOD. The late function mutants used as genetic markers in the crosses are described and mapped in BERNSTEIN, EDGAR and DENHARDT (1965) and EPSTEIN *et al.* (1963). The gene *t* mutant *amtA3* (JOSSLIN 1970, 1971) was obtained via Dr. R. S. EDGAR. The wild-type T4D used in this laboratory, the *rIIA* mutant, *r59*, and the *rI* mutant, *r48*, were all originally obtained from A. H. DOERMANN. Phage stocks containing combinations of these mutations were constructed by recombination. The identification of these complex genotypes was accomplished following the methods described by DOERMANN and BOEHNER (1970).

Media and bacterial growth: P-broth (plain broth), H-broth (Hershey broth), bottom-layer agar (H agar) and top-layer agar (H-soft agar) were prepared according to CHASE and DOERMANN (1958). The recipe for bottom layer agar was modified by altering the sodium citrate dihydrate concentration to 1 g/l of medium. P-broth was used as the growth medium for *E. coli* B which was to be used as the host in genetic crosses. H-broth was used as the growth medium for other *E. coli* strains used as host cells and also for the preparation of indicator bacteria. All phage and bacterial dilutions were made in H-broth. Phage stocks were prepared in M-9 medium (ADAMS 1959) to which 5 g/l Difco Casamino Acids was added (M-9+).

Exponential host cells were prepared by making a 1000-fold dilution of an overnight broth culture into 100 ml of fresh medium and aerating at 30°C for 2.5 hr. The cells were then concentrated by centrifugation and resuspended in fresh medium at a concentration of 2×10^8 cells/ml. Indicator strains were prepared similarly except a 50-fold dilution from an overnight culture was made. After growth at 30°C for 2.5 hrs, the cells were concentrated 4 fold by centrifugation and resuspension in fresh H-broth. Indicator strains were prepared daily and stored on ice until used.

Preparation of phage stocks: An overnight broth culture of *E. coli* CR63 was diluted by 100 fold into M-9+ medium, grown with aeration at 30°C for 2.5 hr. The culture was then inoculated with a phage plaque as described by EDGAR and LIELAUSIS (1964) and incubated 12 hr. Chloroform was added and the lysates were centrifuged at $5,000 \times g$ for 30 min to remove bacterial debris. Such stocks were stable when stored over chloroform at 4°C.

Standard cross procedure: Our procedure follows a modification of that of CHASE and DOERMANN (1958). The entire procedure was performed at 30°C. Exponential host *E. coli* B cells at density of 2×10^8 /ml were added to the adsorption tube with aeration. KCN (M/500 in the bacterial suspension) was added 5 min prior to the addition of phage. An equal volume of the phage mixture containing 1.5×10^9 of each parent per ml (a multiplicity of 7.5 of each type) was added to the adsorption tube at time (t) = 0. At t = 9.5 min an aliquot was taken to assay unadsorbed phage; and at t = 10 min T4-specific antiserum was added to neutralize remaining unadsorbed phage. At t = 15 min a 5,000-fold dilution of the adsorption tube was made for the growth tube and a subsequent dilution made from this to determine infected bacteria. At t = 100 min chloroform was added to the growth tube to complete lysis. Premature lysates were obtained by adding chloroform to aliquots of the growth tube at various times.

Cross lysates were plated on *E. coli* CR63 to determine total progeny. Wild-type recombinants were scored on selective indicator plates. Bacterial and phage assays and plating techniques are described by ADAMS (1959).

Heterozygote frequency: The methods employed to determine the frequency of heterozygotes for the r48 marker were the same as those of BERGER, WARREN and FRY (1969).

³H-Thymidine incorporation: An overnight broth culture of *E. coli* W3110 AB24 SU⁻ was diluted 50-fold into M-9+ medium containing 20 µg/ml of L-tryptophan. After growth for 3 hr at 37°C, the cells were centrifuged at $5,000 \times g$ for 10 min and resuspended at a cell density of 10^9 /ml in fresh medium which contained in addition 500 µg/ml of deoxyadenosine. After aeration for a few minutes the culture was infected with a multiplicity of 10 (zero time) and simultaneously the medium was supplemented with the indicated amount of non-radioactive thymidine. Four minutes later ³H-thymidine (7ci/mmol) was added to a final concentration of 10 µCi/ml. For determination of ³H-thymidine incorporation samples of the infected culture were precipitated in an equal volume of 20% ice-cold TCA, filtered on a glass fiber filter (Reeve-Angel 934AH, 2.4 cm), and washed six times with 6 ml aliquots of ice-cold water. Radioactivity was determined as described by SHAH and BERGER (1971).

RESULTS

Time course of recombinant production: The increased genetic recombination which occurs following infection with ligase-deficient rII phage (amX39-r59) has been previously described (KRISCH, SHAH and BERGER 1971). In order to characterize this altered recombination more completely the time course of recombinant production has been examined. Two-factor crosses were performed and samples were removed at regular intervals and prematurely lysed with chloroform. The results of crosses utilizing wild type, gene t defective, and ligase-deficient rII phage are illustrated in Figure 1. Recombinant production at early times in ligase-normal phage is shown in Table 1. Following wild-type T4

TABLE 1
Recombination at early times in crosses with ligase-normal phage

Time after infection (min)	Progeny per infected cell (Percent final value)		Recombination frequency (Percent final value)		Number of determinations
	$\bar{x}$	σ_x^2	$\bar{x}$	σ_x^2	
20	0.07	0.03	20.5	2.4	4 ^{a,b,c}
25	6.0	1.1	59.2	3.3	6 ^{a,b}
30	32.1	6.3	75.5	5.2	8 ^{a,b,c,d}

^a r70 × r2-20,

^b amN69 × amB255,

^c tsB81 × tsB105,

^d tsB32 × tsB70.

FIGURE 1.—Time course of recombinant production in wild-type, gene *t*-defective, and ligase-deficient *rII* infection of *E. coli* B. Standard cross procedure, premature lysis with chloroform. Times are after dilution from KCN. Total progeny were determined utilizing *E. coli* CR63 as the indicator strain and incubating the plates at 25°C. The frequency of wild-type recombinants for the temperature-sensitive mutations was determined by incubation of plates at 43.5°C, the percentage recombination was calculated by multiplying by two the frequency of wild-type recombinants.

Symbols: ○ wild-type cross—*tsB105* × *tsB81*. △ ligase-deficient *rII* cross—*amX39-r59-tsB105* × *amX39-r59-tsB81*. □ gene *t*-defective cross—*amtA3-tsB105* × *amtA3-tsB81*.

infection, much of recombination in a standard cross occurs early in vegetative growth, and subsequent progeny merely reflect this level of recombination. Approximately 60% of the final recombination values are seen 25 min after infection, when only 6% of the final phage yields are present intracellularly. This result for wild-type infection is in good agreement with the data of DOERMANN (1953) which indicate that in non-lysis inhibited cells new recombinants are primarily generated early, and the latter portion of the latent period shows only small increases in the frequency of recombinants.

As shown in Figure 1, the time course of recombinant production in ligase-deficient *rII* infection differs from wild-type infection in two significant aspects: (1) Viable progeny (and consequently recombinants) cannot be detected until 20 min later than in wild-type infection and (2) new recombinants are produced at times when recombination has ceased in ligase-normal infection. A cross was also carried out with T4D strains which contained an *amber* mutation in gene *t* (Figure 1). With gene *t* mutants infection appears to be normal except that the shut-off of macromolecular synthesis and lysis of infected cells is much delayed

FIGURE 2.—DNA synthesis-³H-thymidine incorporation by wild-type and gene *t*-defective infected cells. Carried out as described in detail in MATERIALS AND METHODS. Carrier thymidine concentration, 2 μ g/ml.

Symbols: $\circ$ T4D⁺ (lysis inhibited). $\square$ *amtA3* (gene *t*-defective).

(JOSSLIN 1970, 1971). The ability of gene *t* mutant-infected cells to continue to synthesize DNA (Figure 2) and produce progeny makes it possible to examine the level of recombination in progeny produced at times long after phage production in wild type has ceased. It can be concluded from this data that the progeny produced at late times after infection have identical levels of recombination to those produced soon after the end of the eclipse period. These results indicate that in both wild type and gene *t*-defective infection recombination occurs relatively early and is followed by a period in which DNA continues to replicate, but little new recombination occurs. Thus the fact that ligase-deficient ϕ II phage infection produces additional recombinants cannot be merely attributed to the extended latent period.

This finding that the level of genetic recombination in wild type and gene *t*-defective infection does not vary greatly with time disagrees with LEVINthal and VISCONTI (1953). In their experiments with phage T2 lysis inhibition was achieved by carrying out the cross at an infected cell concentration of 10⁸/ml. Lysis was presumably inhibited because some bacteria liberated phage which were adsorbed on the other bacteria not yet lysed (DOERMANN 1948). Under these conditions there is a period of time which is long compared to the normal latent period during which phage-infected bacteria produce and accumulate phage at a constant rate. However, unlike our results with the *t* mutant, they reported a linear increase in the recombinant frequency during this extended period. We have repeated their experiment with phage T4. Figure 3 shows that under the conditions employed by LEVINthal and VISCONTI, T4, like T2, shows

FIGURE 3.—Time course of recombination in lysis-inhibited cells. Times are after addition of phage (KCN was omitted in this experiment). Chloroform was added to achieve premature lysis. Frequency of recombination was determined as in Figure 1, except that *E. coli* B4OSUI was used as the indicator strain.

Symbols: ● *tsB105* × *tsB81* under standard cross conditions; ○ *tsB105* × *tsB81*, adsorption at 10^8 cells/ml, secondary phage (MOI = 10) added at 10 min, antiserum added at 15 min, dilution to 2×10^4 cells/ml at 20 min. Δ *tsB105* × *tsB81* maintained at 10^8 cells/ml throughout the experiment.

an increase in the frequency of recombinants with time. In order to examine this phenomenon under better defined conditions, recombination was measured in cells which were lysis-inhibited by addition of phage 10 min after the primary infection. The culture was then diluted to prevent readsorption of liberated phage and to remove any possible effect of high cell concentration. As shown in Figure 3, this procedure establishes lysis inhibition but the effect on recombination is unlike deficient *rII* phage are illustrated in Figure 1. Recombinant production at early that reported by LEVINTHAL and VISCONTI. Recombinant frequency under these conditions of lysis inhibition, as in gene *t* defective infection, does not change with time.

Since superinfection at 10 min in dilute culture did not result in increased recombination, the effect observed by LEVINTHAL and VISCONTI must be due to either (1) a peculiar time course of secondary infection which we failed to duplicate or (2) some change in intracellular conditions due to high cell density. In order to distinguish these possibilities, crosses were performed at high cell density

FIGURE 4.—Time course of recombination with a gene *t* mutant at high cell concentration. Infected cell concentration was maintained at 10^8 cells/ml throughout the experiment. Times are after addition of phage (KCN was omitted in this experiment). Chloroform was added to achieve premature lysis. Frequency of recombination was determined as in Figure 1, except *E. coli* B40SUI was used as the indicator strain. The dashed line shows the level of recombinants in a standard cross of *amtA3-tsB105* $\times$ *amtA3-tsB81*.

Symbols: ● *amtA3-tsB105* $\times$ *amtA3-tsB81*. ○ *amtA3-tsB105* $\times$ *amtA3-tsB81*, T4 antiserum added at 10 min.

between two strains carrying mutations in gene *t* in addition to the temperature-sensitive markers. Under these conditions recombination in gene *t*-defective infection, as in wild type, continues to increase with time (Figure 4). Since few phage are released in gene *t*-defective infection (JOSLIN 1970, 1971), the increased recombination is probably not due to superinfection. In order to eliminate this possibility rigorously, T4 antiserum was added to one cross to prevent readsorption of any phage that might have been liberated. A parallel cross with wild type established that the amount of antiserum used was sufficient to prevent lysis inhibition. As also shown in Figure 4, recombination still increased in the cross with antiserum added. These results indicate that the continued increase in recombinant frequency observed by LEVINTHAL and VISCONTI is not caused by superinfection, but is due to the high cell density at which their crosses were carried out.

The effect of a delay in DNA synthesis on genetic recombination: The experiments above indicate that the effect on recombination of limitation of ligase is not merely due to extending the time in which vegetative DNA molecules have an opportunity to recombine. The experiments on the time course of recombinant formation following infection with wild type, lysis-inhibited wild type, or gene *t* mutants indicate that under standard cross conditions phage made very late show no more recombination than the average of those made at the time of normal

lysis. Since recombination frequency continues to increase with time after ligase-deficient *rII* infection, it appears that DNA produced by ligase-deficient *rII* phage must have a greater likelihood of generating recombinants than analogous molecules in wild-type infection.

The possibility exists, however, that the delay in DNA synthesis which occurs following ligase-deficient *rII* infection (KRISCH *et al.* 1971) is responsible for the generation of intracellular conditions which are more recombinogenic. For example, the failure to initiate DNA synthesis at the normal rate has been shown to result in overproduction of early functions (KUTTER 1968). It is possible that some of these functions are involved in the generation of recombinants and that their overproduction could have an effect on recombination.

Although the delay in the initiation of DNA synthesis following infection with ligase-deficient *rII* phage is rather pronounced at 30°C (KARAM 1969; KRISCH *et al.* 1971), this delay is temperature dependent and is much less apparent at either 37°C or 42°C (Figure 5). Table 2 indicates, however, that even under these conditions, where the initiation and rate of DNA synthesis is essentially normal, the effect on recombination is still equally pronounced. It is concluded from this experiment that the DNA-delay aspect of the ligase-deficient *rII* infection is not essential to the observed effects on recombination.

Stimulation of recombination: We previously indicated (KRISCH *et al.* 1971) that preliminary experiments suggested the stimulatory effect of ligase deficiency on recombination varied depending on the interval between the recombining markers. Much more extensive experiments have been carried out addressed to this question. Although there is some scatter in the data, the results indicate that the effect of the ligase-deficient *rII* phenotype is to increase the frequency of recombination between two markers 4.2 fold over that observed between the same two markers in wild-type infection, irrespective of the interval between the markers. The crosses illustrated in Figure 6 utilized late function *amber* and temperature-sensitive mutants (BERNSTEIN, EDGAR and DENHARDT 1965) in a variety of genes in several different regions of the T4 map; thus, we can also conclude that the effect is uniform throughout the T4 chromosome.

Heterozygote frequency: Another measure of the recombination in phage-infected cells is the frequency of heterozygotes. BERGER, WARREN and FRY (1969)

TABLE 2
Recombinant production at various temperatures

Cross	Percent recombination† between <i>amB255</i> and <i>amN69</i>			
	30°C*		37.5°C*	42°C*
1. <i>r59-amB255</i> × <i>r59-amN69</i> (control)	4.7,	4.3	4.2,	3.8
2. <i>amX39-r59-amB255</i> × <i>amX39-r59-amN69</i> (ligase-deficient)	21.7,	22.4	22.1,	21.0
				19.8

* Standard cross procedure carried out at indicated temperature.

† The percentages were calculated by multiplying by two the frequency of wild-type recombinants as determined by plating on the selective indicator W3110AB24 SUA. Values are given for two separate crosses.

F17Z85 5.—DNA synthesis- ^3H -thymidine incorporation by T4D^+ and ligase-deficient $r\text{II}$ (amX39-r59) infected cells at various temperatures. Procedure as described in MATERIALS AND METHODS, except cell concentration was increased to 2.5×10^9 cells/ml, carrier thymidine concentration was $10 \mu\text{g/ml}$.

Symbols: ○ T4D^+ , □ amX39-r59 (ligase-deficient $r\text{II}$).

have observed that alterations in the level of recombination result in altered heterozygote (HET) frequency. When the frequency of HETs was measured for the $r\text{I}$ point mutant, $r48$, it was found to be higher in ligase-deficient $r\text{II}$ infection than in wild-type infection (Table 3). It is necessary to take into account that approximately half of the mottled plaques one scores in wild-type infection are not internal HETs but terminal redundancy HETs (SÉCHAUD *et al.* 1965; SHALITIN and STAHL 1965) whose frequency should be relatively insensitive to variations in genetic recombination. Making this correction in order to consider only internal heterozygotes, the frequency is increased approximately 4 fold.

TABLE 3

Effect of ligase-deficient $r\text{II}$ genotype on $r48$ heterozygote frequency

Cross*	Total plaques examined	Number of mottlers	Percentage mottlers
$\text{T4D}^+ \times r48$ (control)			
Experiment 1	2738	41	1.5
Experiment 2	5769	81	1.4
$\text{amX39-r59} \times \text{amX39-r59-r48}$ (ligase-deficient)			
Experiment 1	2093	67	3.2
Experiment 2	3903	138	3.5

* Standard cross procedure.

FIGURE 6.—Stimulation of recombination by the ligase-deficient *rII* genotype. Parallel crosses were performed to measure the frequency of recombination between two late function markers in either a wild-type or a ligase-deficient *rII* genetic background. The circled numbers refer to the particular set of markers used in that pair of crosses. The standard cross procedures were employed; total progeny were determined by plating on the indicator strain *E. coli* CR63 incubated at 25°C; the frequency of wild-type recombinants for the late function markers was determined by plating on an appropriate selective indicator strain (for late function *amber* mutations—*E. coli* W3110 AB24 SUA, for temperature-sensitive mutations—*E. coli* CR63 incubated at 43.5°C). The thin line is the slope expected if the ligase-deficient *rII* genotype had no effect on recombination. Genetic equilibrium under these conditions is 30–35% recombination.

Symbols: 1 *tsB81* × *amN69* both in gene 12. 2 *amN69* × *tsB105* both in gene 12. 3 *tsB32* × *tsN2* both in gene 37. 4 *tsB32* × *tsN31* both in gene 37. 5 *tsB70* × *tsB72* both in gene 37. 6 *tsB105* × *tsB81* both in gene 12. 7 *tsN3* × *tsB31* both in gene 19. 8 *amN69* × *tsB113* both in gene 12. 9 *tsB67* × *tsB70* both in gene 37. 10 *tsB113* × *tsB105* both in gene 12. 11 *tsB67* × *tsB72* both in gene 37. 12 *tsA18* × *amB225* both in gene 10. 13 *amN128* × *amN69* gene 11, gene 12. 14 *tsB113* × *tsB81* both in gene 12. 15 *amE17* × *amB255* gene 9, gene 10. 16 *amE17* × *tsA18* gene 9, gene 10. 17 *amB255* × *amN128* gene 10, gene 11. 18 *amB255* × *amN69* gene 10, gene 12. 19 *tsB71* × *tsB81* gene 10, gene 12. 20 *amE17* × *amN128* gene 9, gene 11. 21 *amE17* × *amN69* gene 9, gene 12. 22 *tsB105* × *tsB71* gene 10, gene 12. 23 *tsB32* × *tsB67* both in gene 37. 24 *tsB32* × *tsB70* both in gene 37. 25 *tsB32* × *tsB72* both in gene 37.

TABLE 4

Heterozygote frequency with normal and premature lysis

Cross*	Progeny per infected bacterium	Total plaques examined	Numbers of mottlers	Percentage mottlers
T4D ⁺ × r48 (control)				
1a. premature lysis	1.6	3994	59	1.5
1b. normal lysis	100.1	3826	50	1.3
<i>amX39-r59</i> × <i>amX39-r59-r48</i> (ligase-deficient)				
2a. premature lysis	4.0	4000	68	1.7
2b. normal lysis	28.3	3593	95	2.6

* Standard cross procedure.

In either wild-type or ligase-deficient *rII* infection the level of internal HETs found in the progeny represents a balance between their rate of generation via recombination and their loss via replication (SÉCHAUD *et al.* 1965). Since DNA replication rates, once initiated, are the same in both cases (Figure 5), then the increased HETs observed following ligase-deficient *rII* infection must reflect increased rates of generation by recombination.

Since genetic recombination following ligase-deficient *rII* infection is occurring for a prolonged period at a high rate (Figure 1), one would expect the frequency of HETs to be elevated throughout the period of progeny production. Such a result would rule out the possibility that the additional HETs were generated during the extended early period of slow DNA synthesis in ligase-deficient *rII* infection. If these HETs were generated during this slow phase of DNA synthesis early in infection then the proportion of mottled plaques would be expected to be greater after premature lysis than with normal lysis. Table 4 shows this not to be the case; indeed the frequency of HETs increases with time following infection with ligase-deficient *rII* phage. In the control experiment, a cross between T4D⁺ and r48, the level of HETs for the *rI* marker is the same with either premature or normal lysis. This result with wild-type infection is identical to that found by HERSHEY and CHASE (1951) in a similar experiment utilizing T2H.

In view of the above results, it seems unlikely that the increased frequency of heterozygosity results from the production of substantial numbers of HETs during the slow initial phase of DNA synthesis.

Effect of host ligase on recombination: The failure to produce phage ligase following infection with ligase-deficient *rII* phage results in the synthesis of DNA which contains numerous and persisting discontinuities (KRISCH *et al.* 1971). Presumably normal host ligase levels are insufficient to effect repair at the rates seen in wild-type infection. It is possible that these discontinuities resulting from ligase limitation are responsible for the stimulation of recombination seen in ligase-deficient *rII* infection. To test this possibility we carried out crosses in host bacteria containing mutations which alter the levels of bacterial polynucleotide ligase (GELLERT and BULLOCK 1970). Hosts containing five-fold reduced, normal, and four-fold increased levels of ligase were used.

TABLE 5

Effect of host ligase levels on recombination in ligase-deficient rII phage

Cross*	Percent recombination† between markers		
	Host-N953 (normal ligase)	Host-N1323 (high ligase)	Host-N1325 (low ligase)
1a. <i>amB255</i> × <i>amN128</i>	6.7†	8.1†	8.1
1b. <i>amX39-r59-amB255</i> × <i>amX39-r59-amN128</i>	21.6†	10.1†	30.5
(ratio) = $\frac{\text{ligase}^{-}\text{-rII}^{-}\text{ recombination}}{\text{ligase}^{+}\text{-rII}^{+}\text{ recombination}}$	(3.2)	(1.2)	(3.8)
2a. <i>amB255</i> × <i>amN69</i>	7.4†	8.1†	10.8
2b. <i>amX39-r59-amB255</i> × <i>amX39-r59-amN69</i>	20.8†	12.4†	34.8
(ratio) = $\frac{\text{ligase}^{-}\text{-rII}^{-}\text{ recombination}}{\text{ligase}^{+}\text{-rII}^{+}\text{ recombination}}$	(2.8)	(1.5)	(3.2)
3a. <i>amN128</i> × <i>amN69</i>	4.2†	5.1†	—
3b. <i>amX39-r59-amN128</i> × <i>amX39-r59-amN69</i>	11.6†	6.4†	—
(ratio) = $\frac{\text{ligase}^{-}\text{-rII}^{-}\text{ recombination}}{\text{ligase}^{+}\text{-rII}^{+}\text{ recombination}}$	(2.8)	(1.3)	—

* Standard cross procedure except that host bacterial strain as indicated and that the multiplicity of each parent in the crosses was 5.

† Values given represent an average of two separate experiments.

‡ The percentages were calculated by multiplying by two the frequency of recombinants as determined by plating on the selective indicator *E. coli* W3110AB24 SUA.

It can be seen from the results of crosses with three sets of markers (Table 5) that in the host with normal ligase the stimulation of recombination is similar in magnitude to that observed in *E. coli* B. However, in the host which has four times the normal amount of ligase this stimulation is nearly eliminated. Presumably following infection of this host the discontinuities in the phage DNA are being repaired by the bacterial enzyme at nearly normal rates; consequently the level of recombination is closer to that observed following wild-type infection. This is in contrast to the results obtained in the host which has much reduced levels of ligase. In this bacterial strain ligase-deficient *rII* phage viability is greatly reduced (GELLERT and BULLOCK 1970; KRISCH *et al.* 1971); nevertheless, among those progeny which are produced, recombination is stimulated even more than in the normal host. These experiments indicate that the amount of recombination occurring following ligase-deficient *rII* infection can be altered by varying host ligase levels.

The recessiveness of ligase deficiency: The results of the preceding experiments suggest that genetic recombination is favored by low levels of ligase. Induction of wild-type levels of phage ligase results in lower recombination frequencies. Thus, the effect of the ligase-deficient *rII* phenotype on recombination should be recessive to the wild-type allele for phage ligase. This is shown in Table 6. If either parent in the cross has a functional gene 30 the frequency of recombinants is reduced.

Effect of rII mutations on recombination: The preceding analysis of the altered recombination in ligase-deficient *rII* infection has emphasized the importance of

TABLE 6

The recessiveness of ligase deficiency

Cross*	Percent recombination† between markers
1. <i>tsB81</i> × <i>tsB105</i>	1.7
2. <i>tsB81</i> × <i>amX39-r59-tsB105</i>	1.3
3. <i>amX39-r59-tsB81</i> × <i>tsB105</i>	1.2
4. <i>amX39-r59-tsB81</i> × <i>amX39-r59-tsB105</i>	5.5

* Standard cross procedure.

† The percentages were calculated by multiplying by two the frequency of wild-type recombinants for the temperature sensitive markers as determined by plating on *E. coli* CR63 incubated at 43.5°C.

the reduced levels of ligase. It is important to examine the contribution that the *rII* mutation is making to this phenotype. In previous reports on the mechanism by which an *rII* mutation restores viability to ligase-deficient phage (BERGER and KOZINSKI 1969; KRISCH *et al.* 1971) it was suggested that the *rII* mutation removes the requirement for phage-induced ligase by directly or indirectly reducing "nickase" activity. Apparently the early endonucleolytic attack of injected DNA molecules in ligase-deficient infection cannot be repaired adequately by host ligase alone. With the reduction of this nucleolytic activity by the *rII* mutation the host enzyme presumably provides sufficient ligase activity for phage DNA replication and recombination to proceed although in an abnormal fashion. It should be noted that RUTBERG and RUTBERG (1966) have reported that deoxyribonuclease activity in extracts of *rII*-infected *E. coli* B is lower than in wild type.

Even in the presence of phage-induced ligase, reduction of early nucleolytic activity by an *rII* mutation might be expected to have measurable effects on genetic recombination. As shown in Table 7, the absence of *rII* function reduces genetic recombination, albeit slightly. These results are in agreement with the findings of CARLSON and KOZINSKI (1969). Their experiments, comparing molecular recombination in *rII* and wild-type infection, indicate aberrant and reduced molecular recombination caused by the *rII* mutation. These findings are consistent with the hypothesis that recombinant frequency reflects, at least in part, the balance between nuclease and ligase activities.

DISCUSSION

We have examined in detail the genetic recombination in a bacteriophage T4D strain which is incapable of synthesizing either functional phage-induced ligase or the *rII* gene product. In this situation recombination between linked markers is increased 4.2 fold and heterozygosity for a point mutant is increased at least 2.3 fold. We have performed a series of experiments which indicate that these increases are not merely a consequence of the delay in DNA synthesis or the delay in the production of mature progeny. Following infection with ligase-deficient *rII* phage, not only is the level of recombination altered, but also its

TABLE 7

Effect of rII mutations on genetic recombination

Cross*	Percent recombination† between markers					
	Exp. 1	Host <i>E. coli</i> B Exp. 2	Exp. 3	Host <i>E. coli</i> K12-N953 Exp. 4	Exp. 5	Exp. 6
1a. <i>amB255</i> × <i>amN128</i>	4.9	5.9	—	6.8	6.3	6.5
1b. <i>r59-amB255</i> × <i>r59-amN128</i>	3.6	3.7	—	5.0	4.4	4.0
(ratio)‡ = $\frac{rII^- \text{ recombination}}{rII^+ \text{ recombination}}$	(.74)	(.63)	—	(.74)	(.70)	(.62)
2a. <i>amN128</i> × <i>amN69</i>	4.0	3.4	3.7	4.4	4.2	—
2b. <i>r59-amN128</i> × <i>r59-amN69</i>	3.7	2.6	3.0	3.9	4.0	—
(ratio)‡ = $\frac{rII^- \text{ recombination}}{rII^+ \text{ recombination}}$	(.92)	(.76)	(.81)	(.89)	(.95)	—
3a. <i>amB255</i> × <i>amN69</i>	5.9	5.8	6.2	7.2	8.3	—
3b. <i>r59-amB255</i> × <i>r59-amN69</i>	4.4	4.2	5.1	5.1	5.7	—
(ratio)‡ = $\frac{rII^- \text{ recombination}}{rII^+ \text{ recombination}}$	(.75)	(.72)	(.82)	(.71)	(.69)	—

* Standard cross procedure except host bacterial strain as indicated.

† Based on 15 determinations this ratio is significantly less than 1.0 at the 99.5% or greater confidence level by *t* test.‡ The percentages were calculated by multiplying by two the frequency of wild type recombinants for the *amber* markers as determined by plating on the selective indicator *E. coli* B.

time dependence. In a standard genetic cross (see MATERIALS AND METHODS) such infection results in an extended period of recombinant production. This is unlike the results obtained in similar crosses involving wild type, wild type which has been lysis-inhibited by superinfection at 10 min, or gene *t* (lysis defective) mutants. In all of these situations the appearance of new recombinants occurs predominantly in the early part of the period in which mature progeny are produced. Subsequent replication of the vegetative pool of DNA proceeds without the formation of appreciable numbers of newly recombinant molecules. This view of the time course of recombinant production differs from that of LEVINTHAL and VISCONTI (1953). While we are able to reproduce their results with lysis-inhibited T4-infected cells, this is only possible if the cross is carried out in concentrated culture. In a genetic cross involving lysis-inhibited cells which are diluted soon after superinfection recombination is not observed to increase with time. We suggest that LEVINTHAL and VISCONTI's results reflect alterations in the normal pattern of recombination brought about by the particular set of growth conditions in which their experiments were carried out.

In order to consolidate these observations on T4 recombination the following model is proposed. Immediately after infection with wild-type T4 the level of phage-induced and host ligase is adequate to counteract phage and/or host endonucleases which attack the T4 chromosome (KOZINSKI 1968). Although phage ligase levels are continually increasing during this early period of infection (WEISS *et al.* 1968), the initiation of DNA synthesis probably increases the

requirement for ligase substantially. The observation of OKAZAKI *et al.* (1968) that recently replicated DNA contains numerous discontinuities whose repair requires ligase could be the cause of this increased requirement.

It is suggested that during this early stage of DNA replication ligase levels are not adequate to catalyze the rapid repair of nicks in the DNA. Thus, replicative discontinuities as well as discontinuities caused by endonucleolytic activity are comparatively long-lived and consequently may have a much greater chance of participating in the formation of a recombinant. It is suggested that during this "ligase-deficient" phase of wild-type DNA synthesis much of the genetic recombination occurs. In the subsequent period of DNA synthesis, when phage-induced ligase levels have reached their maximum, it is suggested that replicative and/or endonucleolytic discontinuities in the DNA can be repaired more rapidly; thus, the likelihood of these molecules forming a recombinant is reduced. DNA synthesis can continue, nevertheless, at a linear rate making new copies of those molecules in the vegetative pool. Because molecules are being continually withdrawn from this pool by packaging and maturation the number of DNA growing points remains constant (WERNER 1968) and hence the requirement for ligase is also constant. This subsequent phase of replication results in the production of progeny whose frequency of recombination is essentially unchanged from that of the progeny produced early.

In ligase-deficient rII infection no phage-induced ligase is synthesized, and replication proceeds in the presence of the low and constant level of ligase available from the host cell. Consequently, the recently synthesized DNA contains numerous discontinuities which persist for long times (KRISCH *et al.* 1971). It is suggested that these highly nicked replicative intermediates have a much greater probability of participating in recombination than the more intact DNA produced by wild-type infection. Thus, a direct consequence of this aberrant DNA synthesis could be the production of recombinant molecules at a rapid rate. It is implicit in this model that in addition to the direct role of ligase in DNA synthesis, it has a concomitant function in controlling the level of recombination in the infected cell.

Several other aspects of the results reported here are also consistent with ligase levels controlling the rate of recombination in T4 infection. The variations in frequency of recombination obtained by altering the amount of available host ligase under conditions in which phage ligase is absent support this view. These results also point out that the source of ligase is irrelevant, since either the phage or the bacterial enzyme can produce the same effect.

In summary, this model of T4 recombination proposes that in the presence of low levels of ligase DNA synthesis generates new molecules which because of their highly nicked structure are recombinogenic. This contrasts to the case where high levels of ligase are available; such a situation results in the replication of DNA with very little concomitant recombination because the newly synthesized molecules contain comparatively few discontinuities which are presumably essential to the eventual formation of a recombinant.

In support of this model, SANDRI, KRISCH and BERGER (in preparation) have

shown that the average lifetime of an "OKAZAKI" fragment after wild-type infection is dependent on the time in the vegetative cycle when the measurement is made. At late times the lifetime of these fragments is reduced.

Other genes involved in T4 DNA replication (gene 43, gene 61) also produce effects on recombination; we are currently investigating the molecular basis of these effects.

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