PARTIAL EXCLUSION BETWEEN T-EVEN BACTERIOPHAGES; AN INCIPIENT GENETIC ISOLATION MECHANISM[1]

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ABSTRACT

Conditional lethal mutant systems developed in T-even bacteriophages T2, T4 and T6 have been used to study the partial exclusion which characterizes mixed infections of these phages. In bacteria mixedly infected with T2 and T4, the dominant phage (T4) acts against localized exclusion sensitivity determinants in the genome of the excluded phage (T2). These determinants are clustered near genes controlling early functions; the determinants themselves do not appear among the progeny, but markers located close to them appear infrequently, by recombination. The excluding action of T4 does not depend on the action of any gene so far identified by conditional lethal mutations, nor does it depend on differences in DNA glucosylation between infecting phages. Regardless of mechanism, the genetic consequence of this partial exclusion is to limit genetic exchange between T2 and T4 in the region of the genome controlling early functions, while retaining the capacity for extensive exchange in other regions; in short, partial exclusion constitutes a localized genetic isolating mechanism. Related forms of partial exclusion characterize mixed infections of other T-even phages, including those of some phages newly isolated from nature.

THE demonstrated similarity of the T-even bacteriophages (ADAMS 1952; BRENNER *et al.* 1959; SCHILDKRAUT *et al.* 1962; EISERLING and BOY DE LA TOUR 1965; COWIE, AVERY and CHAMPE 1971) together with recent evidence that they do possess some discrete genetic differences (BECKENDORF and WILSON 1972; BECKENDORF, KIM and LIELAUSIS 1973; KIM and DAVIDSON 1974; RUSSELL 1974) suggests that these phages are anologous to races of higher organisms. In other species, races frequently show isolating mechanisms by which genetic exchange between them is limited, and it is through these isolating mechanisms that divergence to discrete species is thought to occur (DOBZHANSKY 1951). Whether such isolating mechanisms operate to limit genetic exchange between bacteriophage races was heretofore unknown, but the phenomenon called partial exclusion (DELBRÜCK 1945; STREISINGER 1956a, b, c; STREISINGER and WEIGLE 1956) seemed likely to play such a role. In this phenomenon, an infecting phage

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grossly reduces the number of progeny produced by a related but radically distinct phage which happens to infect the same bacterium. Because of this phenomenon, the number of recombinant progeny emerging from such a mixed infection is markedly reduced, despite the fact that rather extensive genetic exchange between the two parental phages is otherwise possible.

This genetic-exchange-limiting phenomenon is of particular interest because it must involve, in essence, the interaction of two initial DNA molecules (the infecting phage genomes) in a common cytoplasm. The mechanism by which genetic exchange between these molecules is limited must therefore be free of more complex features (such as differences in mating behavior or in fertility timing) often found in higher organisms. In consequence, the bacteriophage mechanism may be a reasonable model of the kind of mechanism by which some of the earliest genetic divergences (of primordial microbes) occurred.

We report below some genetic studies of partial exclusion between T-even bacteriophages. The central conclusion of these studies is that exclusion operates by some action of the excluding phage against localized exclusion-sensitivity-determinants in the genome of the excluded phage.

MATERIALS AND METHODS

Phages: T4D wild type, T2L wild type, T6 wild type, and all mutants of T4D were from the collection of Dr. Robert S. Edgar. Amber mutants of T2L and T6 are described in the accompanying paper (Russell 1974). The new T-even-like phages denoted by the prefix RB followed by a number were obtained from Rosina O. Berry, who isolated them from six Long Island sewage treatment plant inlets during her tenure as an Undergraduate Research Participant at the Cold Spring Harbor Laboratories in the summer of 1964. These phages were selected for their ability to form plaques on *Escherichia coli* strain B/5 on LT plates, and were subsequently shown to be inactivated by anti-T4 and/or anti-T6 antisera. Non-glucosylated T2 and T4 phages were prepared by one cycle of growth on either W4597 (for T4) or $B/4_0$ (for T2). The resulting phages plated normally on Shigella strains, but plated with efficiencies of only $1\text{–}4 \times 10^{-3}$ on *E. coli* strain S/6/5.

Bacteria: The bacterial strain $B/4_0$ of *E. coli* and $Sh/4_0$ and Sh15 of *Shigella dysenteriae* were obtained from Dr. Stanley Hattman. The Shigella strains accept T-even phages which lack the normal glucose residues on their DNA, and $B/4_0$ and $Sh/4_0$ produce only such phages. The strain W4597 of *E. coli*, obtained from Dr. Sydney Brenner, resembles $B/4_0$ in these respects. All other bacterial strains were from the collections of Dr. Robert S. Edgar and the late Dr. Jean J. Weigle.

Media: K medium contained 7 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.6 g $MgSO_4$, 2 g dextrose (glucose), 15 g DIFCO Casamino Acids, and 1000 ml H_2O. Other media are described in the accompanying paper (Russell 1974).

Chemicals: Scintillation fluid contained 15.16 g 2,5-diphenyloxazole (PPO), 0.19 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 8 pt. toluene. ^3H-thymidine was from New England Nuclear Corporation. All other chemicals were standard reagent grades.

Antisera: The antisera used in this study were gifts from various people, as follows: anti-T2, J. J. Weigle; anti-T4, R. S. Edgar and J. E. Flatgaard; anti-T6, R. S. Edgar.

Methods: Methods for maintaining bacterial strains and for preparing stock cultures, plating bacteria, bacteria for infections, and phage stocks are described in the accompanying paper (Russell 1974). Exclusion experiments to measure gene transmission frequency or functional capacity of excluded phage genes were performed as described for crosses in the accompanying paper (Russell 1974), except that (1) a mixture of appropriate antisera (usually anti-T2 and

anti-T4) was used to inactivate unadsorbed phage; (2) experiments with parental input ratios of greater than 3:2 or less than 2:3 were not accepted, and (3) experiments with an excluding phage (usually T4) multiplicity of less than 4 were not accepted (to avoid the occurrence of significant numbers of cells infected by chance with only the excluded phage).

Spot testing for exclusion sensitivity properties was accomplished as follows. The tested phages were always the *am*+ *ts* progeny of crosses between T2 wild type and a T4 *am ts* double mutant containing an amber (*am*) mutation in an early gene and a temperature-sensitive (*ts*) mutation in a late one; they were identified as the small, sharp-edged plaques formed when such progeny are plated on the restrictive host S/6/5 and incubated successively for 5 hours at 25° and then overnight at 42°. For testing, these plaques were picked, resuspended in Hershey broth, and subsequently spotted successively in combination with appropriate concentrations (usually 5×10^7 per ml) of two different tester phages, (1) a T4 mutant carrying an amber mutation in the same early gene as the original T4 *am ts* double mutant, and (2) a T2 mutant carrying an amber mutation in the homologous early gene of T2. These spottings were carried out on the restrictive host S/6/5 at 42°; in order for numerous *am*+ *ts*+ progeny to appear and lyse the spot, the *am*+ allele of the tested phage must appear efficiently among the progeny of mixed infections: Because this *am*+ allele lies in an early region, it will be particularly sensitive to exclusion (as explained in the RESULTS section) if the tested phage has the exclusion sensitivity of T2, so much so that the number of *am*+ *ts*+ progeny will be small in the test with the T4 early *am* tester. If, on the other hand, the tested phage is exclusion-resistant, like T4, the number of *am*+ *ts*+ progeny in the same test will be very much greater and the spot will lyse. What ever the exclusion sensitivity of the tested phage, it should be able to lyse the spot in the test with the T2 early amber mutant, where it cannot be excluded; therefore this test serves as a control. As expected the test distinguishes clearly between T2 and T4 mutants with *ts* mutations in homologous late genes.

Spot testing for non-excluding mutants of T4 was according to a similar rationale. Plaques from a highly mutagenized stock of T4 *am*269 (an amber mutation in the early gene 42) were picked and successively spotted with T4 *am*B20 and T2 *am*93 (both carry mutations in the late gene 14). Concentrations of the latter were adjusted (to $\sim 5 \times 10^7$/ml) so that plaques of unmutagenized T4 *am*269 showed a clear difference in the two tests, due to strong exclusion of the T2 *am*+ allele of *am*269; under these conditions, the T2 mutant *am*3 (like *am*269, a mutation in gene 42) gave the same result in both tests, and the same behavior would be expected for a non-excluding mutant of T4 *am*269.

Measurements of DNA synthesis were done by preparing B/5 bacteria for infection in K medium at a final concentration of 4×10^9/ml, and phages at a final total concentration of 4×10^{10}/ml. Equal volumes of phage and bacteria were mixed and adsorption was allowed to proceed for 5 minutes. The adsorption mixture was then diluted 20-fold into K medium containing ^{3}H-thymidine, 5 μg/ml, 0.1 μc/μg, and aerated. Samples of 0.5 ml withdrawn at 5 minute intervals were transferred to tubes on ice containing 0.05 ml of 1.0 M NaN_3, and 2.5 ml of 6% TCA was added and allowed to stand for 15 minutes. The contents were then filtered onto 0.45 μ pore size millipore filters, washed three times with 10 ml of 5% TCA, and counted in a liquid scintillation counter.

RESULTS

a) *Preferential exclusion of two regions of the T2 genome by T4*: Previous studies of partial exclusion between T2 and T4 had suggested that the excluded phage (T2) might contribute several different genetic markers to the progeny of a mixed infection, although at differing efficiencies (STREISINGER 1956a, b, c; STREISINGER and WEIGLE 1956). The genetic markers available at the time of these studies were rather limited, but subsequently a much more comprehensive set of markers has become available through the work of EDGAR and EPSTEIN (EPSTEIN *et al.* 1963; EDGAR, DENHARDT and EPSTEIN 1964; STAHL, EDGAR and

STEINBERG 1964; EDGAR and LIELAUSIS 1964; EDGAR and EPSTEIN 1965). These new conditional lethal markers have made possible a much more systematic study of the phenomenon of partial exclusion; in particular, they have permitted an assessment of the extent to which exclusion operates against genetic markers at any of a large number of defined positions in the excluded T2 genome. The logic of such an approach is straightforward; since the genetic map of T2 is nearly identical to that of T4 (RUSSELL 1974), one can assess how frequently a given T2 gene appears among the progeny of a T2-T4 mixed infection simply by crossing a T4 amber mutant in the homologous T4 gene with T2 wild type. Progeny which do not carry the T4 amber mutation must of necessity carry the homologous (wild-type) gene from T2, and their relative number is easily assessed by a plating on a restrictive bacterial host. Since T4 amber mutants span nearly the entire genetic map, nearly all known T2 genes can be tested in this fashion.

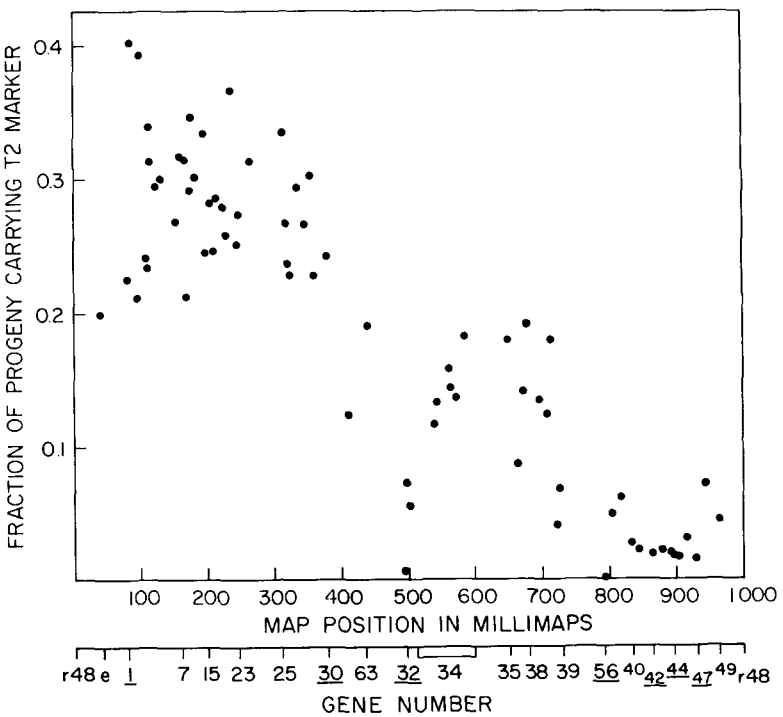

FIGURE 1.—Fraction of progeny carrying various T2 markers. The conditions used are as described in MATERIALS AND METHODS, with the permissive strain CR63 as the host. At least one mutant defective in each identified gene of T4 was crossed with T2 wild type, and each mutant is represented by a point in the figure. Each point is the average of from 1 to 13 determinations; the average number of determinations per point is 3.1. The abscissa is constructed by cutting the T4 map at the top (at mutant r 48) and opening it out into a line so that the blockwise direction is from left to right. The map position is reckoned in thousandths of the total map (millimaps), proceeding clockwise from r 48. The numbers of early genes are underlined. The values for the ordinate of each point are corrected for slight inequalities in the input of the relevant crosses.

The results of such a study are presented in Figure 1. Although the data exhibit some scatter, it is clear that different genetic markers from the excluded T2 parent appear with vastly different frequencies among the progeny, as expected from previous studies. Moreover, it appears that those markers which appear least frequently are grouped into two regions (one around gene 32, the other from gene 39 to gene 49). These "preferentially excluded" regions contain between them most of the early genes and very few late genes, but their preferential exclusion cannot be explained by this fact alone; the early genes 1 and 30 which lie outside these regions are not preferentially excluded, and the quasi-late gene 40 which lies within one of the regions is.

b) *The recombinant nature of progeny carrying T2 markers*: Since most T2-T4 progeny carry the T4 alleles of T2 markers from the preferentially excluded regions, those which carry T2 markers from other regions should be mostly T2-T4 recombinants. This implication was checked by measuring the fraction of such progeny at various times in the latent period; since the frequency of recombinants inceases during the latent period (LEVINTHAL and VISCONTI 1953), this fraction should also rise. The results presented in Figure 2 show that this is the case.

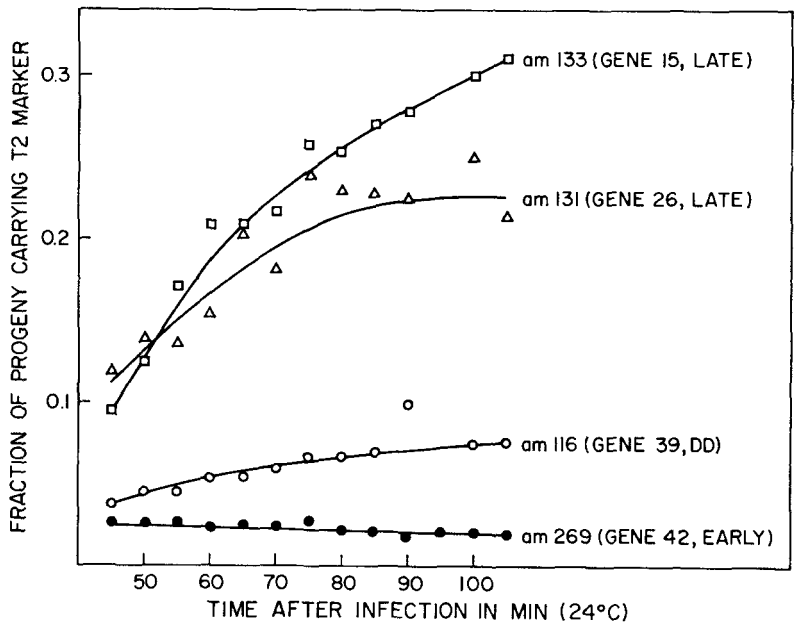

FIGURE 2.—Fraction of progeny carrying T2 markers as a function of time in the latent period. Conditions were described in MATERIALS AND METHODS, with CR63 as host, and each experiment was a mixed infection between T2 wild type and the indicated mutant of T4. After dilution, samples were withdrawn at the indicated times and chloroformed; the fraction of wild types in each of these samples was determined. The results of the cross of am269 with T2 wild type may not be representative of the kinetics of the mixedly infected cells, since some of the wild-type progeny in this case come from cells infected with T2 alone.

The progeny which carry T2 markers from the preferentially excluded regions might also be recombinants, or they might be entirely T2 in composition. To distinguish between these possibilities is technically difficult because of the background of entirely T2 progeny arising from rare cells which escape infection with T4. However, other experiments suggest that a significant fraction of these progeny from mixedly infected cells are not entirely T2 in composition, since their frequency responds to a treatment which enhances recombination. Another study (FRANKEL 1966) had indicated that the frequency of recombination between closely linked mutants in T4 could be greatly increased by a judiciously applied period of interruption in DNA synthesis. A T2-T4 cross was performed in which the T4 parent carried an amber mutation in an early gene and both parents carried temperature-sensitive mutations in gene 42; since gene 42 controls the dCMP hydroxymethylase, DNA synthesis in this cross could be interrupted at will by raising the temperature, and the fraction of progeny carrying the preferentially excluded T2 am^+ marker could be measured. The effects of a series of high temperature pulses are shown in Figure 3, together with the effects of a similar series of pulses on recombination frequencies in T4, for comparison. The similarity suggests that a significant fraction of the progeny carrying a T2 marker from the preferentially excluded regions may also be recombinants. This suggestion is borne out by further experiments, described below, which show that virtually all such progeny are indeed recombinants.

c) *The absence of progeny with T2 exclusion sensitivity*: The recombinant nature of progeny carrying T2 markers can be accounted for by assuming localized *exclusion sensitivity determinants* in the T2 genome which, because of some action exerted against them by T4, are transmitted very infrequently to the progeny. Most progeny phages carrying a given T2 marker would then necessarily arise through recombination events separating the marker from such determinants. Markers close to determinants would, of course, undergo fewer such recombination events and thus appear less frequently among the progeny, and the preferential exclusion of the two regions of the T2 genome could be rationalized by assuming that the determinants are clustered in these regions.

This interpretation does not demand that the determinants be absolutely excluded from the progeny, only that they appear very infrequently. Any rare progeny which might carry the determinants, of course, should be distinguishable by the fact that they remain sensitive to exclusion by T4; to see whether any such progeny existed we scanned selected T2-T4 progeny for T2 exclusion sensitivity, in two steps. Firstly, 48 T2-T4 progeny, 24 selected to contain T2 markers from the region between genes 39 and 49, and 24 selected to contain T2 markers from the region near gene 32, were tested in mixed infections with T4 mutants. Each progeny phage was tested with 5 different T4 mutants (including two from the gene 32 region and two from the region between genes 39 and 49), and in no case was there any evidence that the progeny phages were excluded by T4; thus, if progeny phages carrying the determinants exist, they must be quite infrequent.

Secondly, a more intensive search for these progeny was conducted using a spot test developed for the purpose (see MATERIALS AND METHODS). This spot

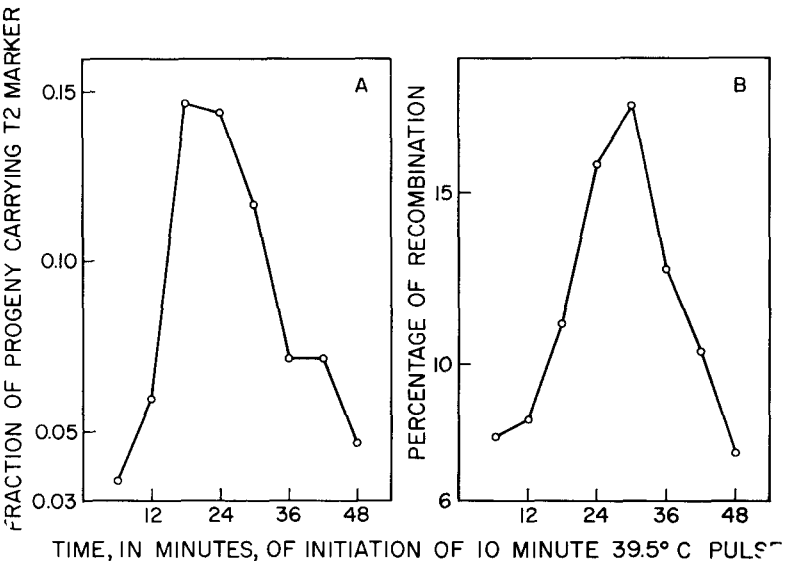

FIGURE 3.—Effects of interrupting DNA synthesis. (A) The experiment was a mixed infection between the T4 double mutant *am*82 *ts* LB-1 and T2 *ts*62; *am*82 is defective in gene 44, an early gene, and T4 *ts* LB-1 and T2 *ts*62 are both defective in gene 42, an early gene which controls the production of the dCMP hydroxymethylase. From the results of temperature-shift experiments, both T4 *ts* LB-1 and T2 *ts*62 lead to the formation of temperature-sensitive dCMP hydroxmethylases which are rapidly inactivated upon shifting infected cells to 39.5°. The mixedly infected cells were incubated at 25° and at 6-minute intervals samples were shifted to 39.5° for 10 minutes and then returned to 25°. When the cells had lysed, the fraction of progeny carrying the T2 *am*+ marker was determined. (B) The experiment was a cross between two T4 double mutants, T4 *ts* LB-3 *rIIa*41 and T4 *ts* LB-3 *rIIb*45. The mutant *ts*LB-3 is defective in gene 42; the two *rII* mutants are defective in A and B cistrons, respectively, and normally give about 6.3% recombination. The mixed infected cells were treated exactly as in part A, and the frequency of recombination between the *rII* mutants was measured in each sample. The high temperature pulses are intended to interrupt DNA synthesis by preventing the temperature-sensitive dCMP hydroxymethylases from acting; how effectively they do so has not been determined. The difference in timing of the peak effects has not been investigated further, but may result from such factors as different inactivation rates for the dCMP hydroxylases involved, different growth kinetics in T2-T4 mixed infections, or possible late degradation of T2 genomes, making them progressively unavailable for genetic exchange.

test, which depends on the preferential exclusion of the early region of the T2 genome, was designed to differentiate between phages with T2 and T4 exclusion sensitivities, and clearly differentiates between T2 and T4 themselves. From a T2-T4 cross 300 progeny phages, selected to contain T2 markers from a preferentially excluded region, were spot tested; none of them had T2 exclusion sensitivity. This result suggests that virtually none of the progeny carry the proposed determinants.

d) *Finer localization of determinants in the T2 genome:* Since the determinants do not appear among the progeny, linkage between T2 markers on either side of them should disappear, and this affords a way of determining their location more precisely. Crosses were performed between T2 wild type and a number of T4

double mutants, each carrying an amber mutation in gene 44 and a temperature-sensitive mutation in a nearby gene. For each cross, the fraction of the progeny carrying the T2 wild-type allele of the T4 amber mutation and the fraction carrying the T2 wild-type allele of the T4 temperature-sensitive mutation were determined, and the fraction expected to carry both T2 alleles by chance was calculated. The fraction actually carrying both T2 alleles was compared to this calculated fraction, and any excess was taken as evidence of linkage between the two alleles. A similar series of crosses was performed with T4 double mutants

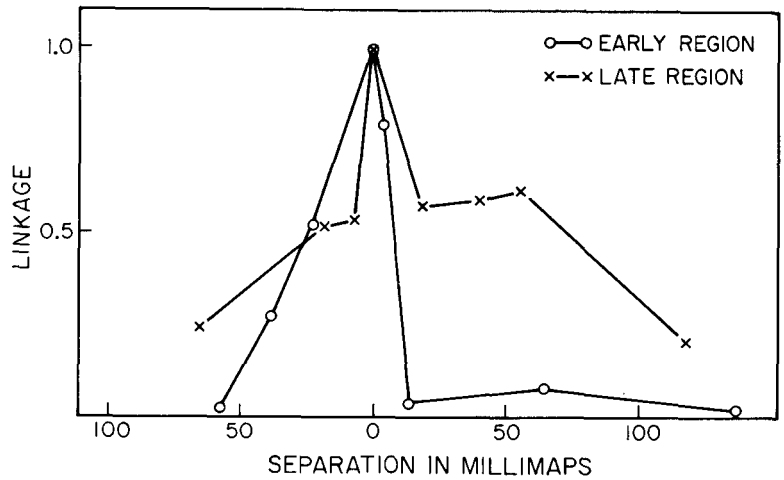

FIGURE 4.—Linkage of T2 early and late markers in T2-T4 mixed infections. Crosses were performed as described in the text between T2 wild type and two series of T4 double mutants, the members of one series carrying *am*82 (gene 44) and *ts* mutations in neighboring genes, the members of the other series carrying *am*B20 (gene 14) and *ts* mutations in neighboring genes. To avoid the contribution of T2 progeny from rare cells infected with the T2 parent alone, only progeny with T4 host-range were examined. The ordinate of each point is determined by separately measuring the number of am^+ts^+, am^+, ts^+, and total progeny and then calculating the value

$$\frac{\frac{am^+ts^+}{am^+} - \frac{ts^+}{\text{total}}}{1 - \frac{am^+}{\text{total}}}$$

. This value becomes 1.0 if the ts^+ is so tightly linked to the am^+ as to be identical with it, and it becomes 0 if there is no linkage between the two; it is therefore a measure of linkage between the am^+ and ts^+ markers of the T2 parent. The abscissa of each point is the map position of the *ts* mutant used, relative to that of the amber mutant used, with the clockwise orientation plotted left to right. The results from crosses involving T4 double mutants in the early and late regions are plotted separately. The central point in each curve (at 0 millimaps separation and 1.0 linkage) is theoretical. For the late region, the *ts* mutations used were A28 (gene 5), A10 (gene 10), A13 (gene 12), N3 (gene 19), L65 (gene 23), N29 (gene 24), and A82 (gene 51). For the early region they were A14 (gene 41), L13 (gene 42), S9 (gene 43), L159 (gene 45), L166 (gene 46), C9 (gene 49), and eC103 (gene e). The loss of linkage which occurs between genes 45 and 46 in the early region suggests the existence there of a determinant of the postulated type. Although the evidence is considerably weaker, there may be another determinant between genes 41 and 42. No determinants were detected in the late region, as expected.

of the late region, for comparison. The results are presented in Figure 4; the loss of linkage which occurs between genes 45 and 46 in the early region suggests the presence there of a determinant of the proposed type, and a similar loss of linkage between genes 41 and 42, although less pronounced, suggests a possible location for another determinant. (Although a rigorous demonstration of determinant positions would require a more extensive series of experiments of this sort, using several different central amber markers, the positions arrived at in this way agree with positions determined by a detailed examination of the gene 39–gene 49 region, using a set of 22 different temperature-sensitive mutants distributed throughout the region for experiments of the sort presented in Figure 1 [RUSSELL and HUSKEY, unpublished]). As expected, no determinants were detected in the late region.

The picture which emerges from these studies may be briefly summarized as follows. In mixed infections between T2 and T4, T4 acts on determinants in the T2 genome, and as a result these determinants appear in a very few, if any, progeny. These determinants are inferred to be at several positions in the early region of T2, including one between genes 45 and 46 and another between genes 41 and 42, and at one or more positions in the vicinity of gene 32. Markers from

TABLE 1

Functional capacity of T2 genes near determinants

Gene containing T4 amber mutation	Burst size as percentage of $T2^{++} \times T4^{++}$ control
63	125
32	162
59	149
33	105
52	70
rIIA	70
60	113
39	99
56	192
58	140
61	91
40	72
41	51
42	80
43	165
62	106
44	150
45	97
46	25
47	23
Average	104

Crosses were performed between T2 wild type and the indicated T4 amber mutants in the restrictive host B/5. The burst sizes were computed and compared to those of simultaneously performed T2 wild type by T4 wild type controls. These burst sizes are measures of the extent to which the excluded T2 can provide the functions which the T4 mutants lack, and thus they measure the functional capacities of the corresponding T2 gene.

the T2 parent appear among the progeny by virtue of recombination events which separate them from these determinants.

e) *The nature of the excluding action of T4*: The nature of the action exerted by T4 against the T2 exclusion sensitivity determinants is unclear, but at least one possibility is that the determinants are sites for specific nucleases. If so, DNA degradation initiating from such sites might be sufficiently rapid and extensive to eliminate effective action of neighboring genes. To test this possibility we performed the crosses listed in Table 1, in which progeny production is dependent on the functional capacity of one or another T2 early gene in the near vicinity of a determinant. In nearly all cases the functional capacities of these genes are high; consequently, if DNA degradation does occur in the vicinity of determinants, it is not reflected in a measurable decline in gene activity. The somewhat low functional capacity of genes 46 and 47 might possibly reflect the expected consequences of degradation, but other possibilities (particularly the possibility that the products of genes 46 and 47 may not be entirely cross-compatible between T2 and T4) have certainly not been ruled out.

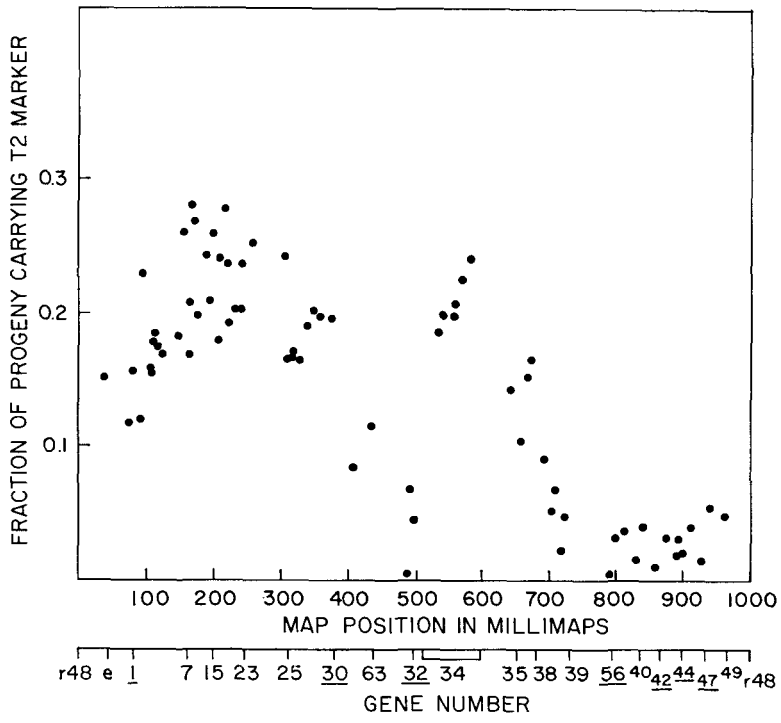

FIGURE 5.—Fraction of progeny carrying various T2 markers, restrictive host. The conditions are the same as in Figure 1, except that the crosses were performed in the restrictive host, B/5, so that each cross is performed in the absence of one particular T4 gene function. Each point is the average of from one to six determinations; the average number of determinations per point in the figure is 2.1. There is a striking similarity to Figure 1, as demonstrated further in Figure 6.

We next asked whether any known T4 genes determined the specificity of the T4 action. The crosses of Figure 1 were repeated in the restrictive host B/5, and the genetic composition of the progeny was compared to that of progeny from the permissive host (see Figure 5). If any of the T4 amber mutants had been defective in a gene controlling the specificity of T4 action against a closely linked determinant in T2, the results in the two hosts should have differed markedly; as Figure 6 shows, none did. However these results do not rule out the possibility that some of the tested T4 genes may control the specificity of action against more distantly located T2 determinants.

Only essential genes of T4 could be examined in this way, since only those genes yielded conditional lethal mutants. Reasoning that perhaps the T4 genes required for exclusion might be non-essential, we attempted to isolate a (non-conditional-lethal) T4 mutant defective in excluding ability. Using the spot test described in MATERIALS AND METHODS, we examined 1800 plaques from a highly mutagenized stock of T4 *am*269; none had lost the ability to exclude T2.

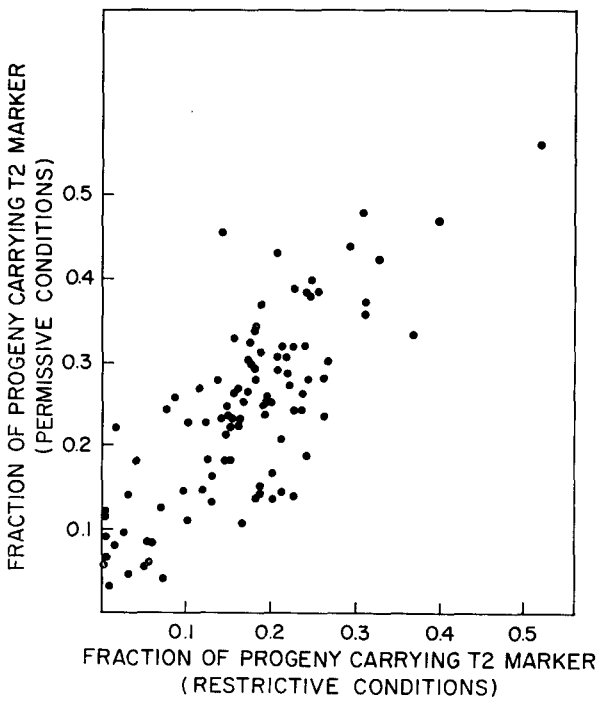

FIGURE 6.—Comparative exclusion of T2 in the presence and absence of various T4 gene functions. The points in this figure are drawn from Figures 1 and 5, and from some other crosses not included in those figures. The fraction of the progeny carrying a given T2 marker is compared for the case in which the corresponding gene function of T4 is performed (permissive conditions, Figure 1) and the case in which it is not performed (restrictive conditions, Figure 5). The similarity of the results of the two cases is indicated by the fact that the results scatter about a line with a slope of nearly 1. If any of the T4 genes tested had been specifically required for exclusion of T2, a point should have appeared in the lower right-hand corner of the figure; clearly none did.

If the exclusion sensitivity determinants of T2 contained any glucosylated HMC residues, it might be that the homologous regions of T4 are rendered insensitive to exclusion by virtue of a difference in the degree or nature of glucosylation of the homologous HMC residues (T2 and T4 are known to differ overall in degree and kind of glucosylation [SINSHEIMER 1960; REVEL and LURIA 1970]). To test this possibility, exclusion experiments were performed between T2 and T4 phages whose degree of glucosylation had been markedly altered by growth in hosts defective in the synthesis of UDP-glucose (HATTMAN and FUKASAWA 1963; SHEDLOVSKY and BRENNER 1963; FUKASAWA and SAITO 1964). The results are presented in Table 2; it appears that alterations in the glucosylation of either parent or of the progeny do not affect the extent of exclusion. However, since the "unglucosylated" phages used probably still contained small amounts of glucose in their DNA, these experiments cannot completely rule out the possibility that glucosylation plays some role in specifying the T2 determinants.

f) *Mixed infections between T2 and T6*: The T2-T6 mixed infection had not previously been studied. In the accompanying paper, however, evidence from T2-T6 complementation tests suggested that T6 excludes T2 (RUSSELL 1974). This suggestion was confirmed by crosses between various T6 amber mutants and T2 wild type; the results appear in Table 3. As in the T2-T4 case, markers from early regions of the T2 genome are somewhat preferentially excluded, but the effect is much less marked; "preferentially excluded" markers appear in more than 10% of the progeny. This evidence suggests that T6 may act against the

TABLE 2

Effect of glucosylation differences on T2–T4 exclusion

		Fraction of progeny carrying T2 marker for cross in			
		B/5	$B/4_0$	Sh15	$SH/4_0$
Acceptance of non-glucosylated phages		—	—	+	+
Ability to glucosylate		+	—	+	—
T4 parent*	T2 parent				
Glucosylated	Glucosylated	.063	.021	.049	.001
Glucosylated	Unglucosylated	—	—	.030	.001
Unglucosylated	Glucosylated	—	—	.065	.0001
Unglucosylated	Unglucosylated	—	—	.042	.0001

* Since some of the hosts used were resistant to T4, it was necessary to use as the T4 parent a T2–T4 hybrid with the host range of T2 but the exclusion properties of T4. Such a hybrid was obtained from a cross of T4 tsL13 (gene 42) by T2 wild type, by selecting progeny with the host range of T2 and the temperature-sensitive marker from the T4 parent. One such progeny phage was selected, and its exclusion properties were tested in mixed infections with T2 and T4 wild type; since it excluded T2 and was not excluded by T4, it was judged to have the exclusion properties of T4.

Each number in the table is the fraction of progeny carrying a T2 marker from a preferentially excluded region, in a cross between the indicated parents in the indicated host. Unglucosylated phages were prepared by one cycle of growth in UDPG-defective hosts, as described in MATERIALS AND METHODS. A slight correction has been applied to each number in the table for the difference in burst sizes of the two parents on the indicated host. The average burst sizes obtained from the different hosts were: B/5, 60; $B/4_0$, 5.8; Sh15, 327; and $Sh4_0$, 22. The reason for the very low values for crosses performed in $Sh/4_0$ is not known.

TABLE 3

Comparative exclusion of T2 by T4 and T6

Gene containing amber mutant	Fraction of progeny carrying T2 marker in T4am × T2^{++} cross	Fraction of progeny carrying T2 marker in T6am × T2^{++} cross
5	0.30	0.14
6	0.27	0.20
7	0.32	0.29
27	0.23	0.40
32	0.009	0.12
34	0.13	0.21
35	0.18	0.20
37	0.14	0.20
42	0.019	0.10
43	0.021	0.16
44	0.018	0.12
45	0.016	0.11
46	0.018	0.11

All crosses were performed in CR63, a permissive host.

same T2 determinants as T4, but if so, its action is considerably weaker. If this interpretation is correct, some of the progeny of T2-T6 infections should retain the exclusion sensitivity determinants of T2, a point which has not been checked.

g) *Mixed infections between T4 and T6*: Mixed infections between T4 and T6 had been previously shown to exhibit a "depressor effect", by which the yield of the mixed infection is markedly reduced relative to infection with either phage alone (DELBRUCK 1945, and see RUSSELL 1974). Because this effect complicates study of the exclusion relations of T4 and T6, it was examined further. Measurements of the rate of DNA synthesis in the T4-T6 mixed infection and in infections with each phage separately are presented in Figure 7; in the mixed infection the rate is more than 4-fold lower. This reduction alone would probably be sufficient to reduce the burst size by about a factor of 4, but other effects must be invoked to account for the observed 30-fold reduction. The nature of these additional effects was not investigated, but it may be of importance that the depressor effect is less marked in CR63 than in B/5 (T4-T6 mixed infections give burst sizes of about 15 in CR63, as opposed to about 3 in B/5).

If CR63 is used as host, the genetic composition of the progeny of T4-T6 mixed infections can be reliably determined. Since the results of T4-T6 complementation tests had suggested that T4 excludes T6 (see accompanying paper, RUSSELL 1974), crosses were performed between T6 wild type and a number of T4 mutants and the progeny were analyzed as in Figure 1. The results are presented in Table 4, along with the results of equivalent T2-T4 crosses for comparison; the similarity between the two argues that T4 also excludes T6 by acting against localized exclusion sensitivity determinants in the T6 genome. Since the T6 map resembles those of T2 and T4 (RUSSELL 1974) these determinants fall in the same map positions as in T2.

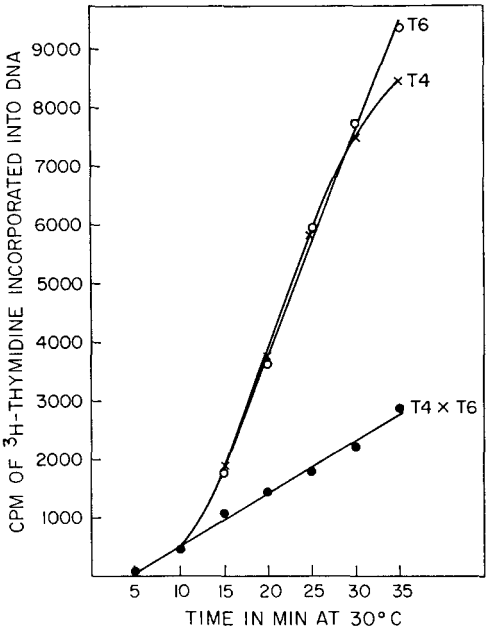

FIGURE 7.—DNA synthesis in T4 and T6 infections. The rates of DNA synthesis were measured as described in MATERIALS AND METHODS. The host for all infections was B/5, and the total multiplicity for all infections was 10 (in the mixed infection, a multiplicity of 5 of each parent was used).

TABLE 4

Comparative exclusion of T2 and T6 by T4

Gene containing T4 amber mutation	Fraction of progeny carrying T2 marker in T4am × T2⁺⁺ cross	Fraction of progeny carrying T6 marker in T4am × T6⁺⁺ cross
1	0.22	0.33
9	0.32	0.50
14	0.24	0.44
20	0.26	0.16
23	0.27	0.26
31	0.12	0.15
33	0.055	0.12
35	0.18	0.19
36	0.087	0.16
39	0.068	0.071
41	0.022	0.097
42	0.019	0.035
43	0.021	0.017
44	0.018	0.028
46	0.018	0.038
47	0.015	0.050

All crosses were performed in CR63, a permissive host.

TABLE 5

Properties of RB phages

Phage	Source	Plaque size	Inactivation by		Plating group	Sensitivity to to citrate
			Anti-T4	Anti-T6		
T16	Hershey	Medium	?	?	I D	—
RB1	Huntington	Medium	++	++	I A	—
RB2	Huntington	Medium	++	++	I B	—
RB3	Huntington	Medium	+	++	I A	—
RB5	Huntington	Medium	+	++	I A	—
RB6	Huntington	Medium	+	++	I A	—
RB7	Huntington	Medium	+	++	I A	—
RB8	Huntington	Medium	+	++	I A	—
RB9	Huntington	Medium	++	+++	I A	—
RB10	Huntington	Medium	++	+++	I A	—
RB14*	Oyster Bay	Medium	+++	+++	I A	—
RB15	Oyster Bay	Medium	+++	+++	I A	—
RB17	Oyster Bay	Medium	++	++	I A	—
RB18	Oyster Bay	Medium	+++	+++	I C	—
RB19*	Oyster Bay	Medium	++	+	II B	—
RB23	Flushing	Medium	++	++	I D	—
RB26	Flushing	Small	+++	+++	I A	—
RB27	Flushing	Medium	+++	++	I A	—
RB30	Flushing	Medium	+++	++	I A	—
RB46	Glen Cove	Medium	++	++	II A	—
RB51*	Great Neck	Medium	+	+	II A	—
RB52	Great Neck	Medium	++	++	II A	—
RB53	Great Neck	Medium	++	+	II C	—
RB57	Great Neck	Medium	++	+	II D	—
RB58	Great Neck	Medium	+++	++	I A	—
RB60	Great Neck	Small	+++	+++	I E	—
RB61*	Long Beach	Medium	?	?	II A	—
RB62	Long Beach	Large	+	+	II A	—
RB68	Long Beach	Medium	++	++	II A	—
RB69*	Long Beach	Medium	+	0	II E	—
RB70	Long Beach	Medium	+	0	II A	—

Plaque sizes were determined on strain B/5 at 30°; T2, T4 and T6 have medium plaque sizes on this scale. The extents of inactivation by anti-T4 and anti-T6 antisera were determined by R. O. BERRY, and T4 and T6 give +++ degrees of inactivation with their respective antisera. Plating groups were determined by the ability to plate on some standard laboratory strains of *E. coli* and on various resistant derivatives of B/5; phages in the same group could not be distinguished from one another by this test, and the ten groups thus identified could be divided into two sets of five each on the basis of rather broad similarities in their plating patterns (sets I and II). The sensitivity to citrate was determined by plating on EHA plates, which contain citrate; presumably such sensitivity would reflect a requirement for divalent cations, which are chelated by citrate. Phages indicated by asterisks (*) were examined in the electron microscope, and were T-even-like.

h) *Mixed infections between T4 and some new T-even phages*: To see whether the interactions observed between the classical T-even phages might occur among T-even phages in nature, mixed infections were performed between T4 and a group of newly isolated T-even-like phages. These RB phages have been described briefly in MATERIALS AND METHODS; they were isolated from six different

Long Island sewage treatment plants, and were selected for their ability to plate on *E. coli* strain B/5 and for their sensitivity to inactivation by anti-T4 and/or anti-T6 antisera. Further characterizations presented in Tables 5 and 6 suggest that they represent a set of reasonably close relatives which share certain properties in common but can be distinguished by others. Their burst sizes cover the range expected of T-even phages, and they adsorb well to B/5 under standard conditions. Their ability to plate efficiently at 42° allowed them to be treated as non-temperature-sensitive in crosses with T4, and their ability to plate efficiently

TABLE 6

Growth parameters of RB phages

Phage	Percent adsorption	Burst size		E.O.P. on CR63	E.O.P at 42° on B/5	E.O.P. on S/4
		25°	39.5°			
T16	82	434	349	0.33	0.97	1.08
RB1	75	84	1.3	0.30	0.81	0.68
RB2	82	105	166	0.51	0.92	1.26
RB3	78	88	1.4	0.40	0.66	1.01
RB5	87	125	1.4	0.50	0.26	1.09
RB6	71	93	8.5	0.43	1.40	0.78
RB7	65	90	7.6	0.32	1.35	0.82
RB8	78	32	6.3	0.43	1.30	0.97
RB9	76	76	4.3	0.25	0.86	1.14
RB10	72	78	4.5	0.35	1.59	1.05
RB14	51	303	330	0.04	1.18	1.07
RB15	82	325	212	0.81	1.09	1.33
RB17	86	132	34	0.26	0.89	0.94
RB18	91	427	386	0.65	1.15	0.93
RB19	97	293	336	0.52	1.19	0.99
RB23	85	234	51	0.49	0.94	1.15
RB26	85	54	8.9	1.49	0.96	1.13
RB27	77	255	86	0.61	1.03	1.09
RB30	82	223	101	0.56	1.22	1.12
RB46	97	314	95	0.34	1.30	0.87
RB51	99	443	119	0.28	1.12	0.96
RB52	97	360	122	0.29	0.94	0.96
RB53	98	324	94	0.22	1.02	0.84
RB57	93	307	99	0.22	0.88	1.12
RB58	91	202	51	0.49	0.97	1.00
RB60	88	476	141	0.34	0.95	1.26
RB61	99	340	124	0.28	0.96	0.92
RB62	88	339	92	0.26	0.96	0.75
RB68	96	277	66	0.21	0.97	0.90
RB69	99	181	30	1.54	0.82	1.67
RB70	99	284	94	0.23	1.04	0.73

Adsorption was carried out for 10 minutes to strain B/5 at a final concentration of 2×10^8/ml at 30°; under these conditions the classical T-even phages adsorb as follows: T2, 96%; T4, 93%; and T6, 75%. Burst sizes were determined on B/5 at the indicated temperatures; it is interesting to note that even the most severe reductions in burst size at 39.5° have remarkably small effects on the ability of the phages to form plaques at 42°. All efficiencies of plating (E.O.P.'s) are relative to strain B/5 at 30°.

on S/4 permitted their host ranges to be distinguished from that of T4 on mixed indicators composed of B/5 and S/4 (on such an indicator mixture, the RB phages form clear plaques, while those of T4 are turbid).

Crosses were performed between a T4 temperature-sensitive mutant defective in gene 42 (an early gene controlling the enzyme dCMP hydroxymethylase) and each of the new RB phages. Immediately after adsorption, the infected complexes were divided into two aliquots and one was incubated at 25°, a permissive temperature for the temperature-sensitive mutant, the other at 39.5°, a restrictive temperature. The infective centers from the 25° aliquot were plated on the mixed indicator to determine how many of them yielded phages with the host range of the RB parent. After lysis, the burst sizes for each high temperature and low temperature infection were determined, and the progeny of the low temperature infection were analyzed to determine how many contained the host range marker of the RB phage parent and how many contained the RB phage wild-type allele of the T4 temperature-sensitive mutation. The low temperature progeny were also plated on the mixed indicator at 42°, and the presence of turbid plaques on these plates, when it occurred, was taken as evidence for the formation of recombinants. The results of these crosses are summarized in Table 7.

In almost all of the mixed infections, a significant fraction of the mixedly infected cells yielded phages with the host range of the RB phage parent and in about two-thirds of the cases examined, T4-RB phage recombinants could be detected; both observations suggest that most of the RB phages can, to some extent, grow together with T4 in the same cell. Moreover, the relatively high burst sizes in the 39.5° aliquots suggest that these phages possess a gene for dCMP hydroxymethylase and that this gene can function in mixed infection with T4. The genetic composition of the progeny shows, however, that all but one of these phages (RB69) are strongly excluded by T4. Whether this exclusion also operates against localized sensitivity determinants in the genomes of the RB phages cannot be determined from these results. If indeed there are such determinants, localized to the early regions as in T2 and T6, then the extent of genetic exchange between T4 and the RB phages must be much more limited than that among the classical T-even phages; otherwise the fraction of progeny carrying the RB phage host range should have been higher than observed. An alternative explanation might be that determinants are distributed more evenly throughout the RB phage genomes so that no RB phage marker can appear frequently among the progeny. Whatever the interpretation, however, it appears that the RB phages are more distantly related to the classical T-even phages than the latter are to one another, and the possibilities for effective genetic exchange are correspondingly reduced.

i) *Mixed infections between RB69 and T2, T4 and T6*: RB69, in contrast to all the other RB phages, strongly excluded T4; this unusual exclusion property led to a further study of its interactions with the classical T-even phages. In order to proceed efficiently, it was necessary to have a set of conditional lethal mutants of RB69. These were isolated essentially as described for T2 and T6 in the accompanying paper (RUSSELL 1974b). The set of RB69 amber mutants so

TABLE 7

Mixed infections between T4 and the RB phages

RB phage phage	RB yielder frequency	Burst size		Fraction of 25° progeny carrying		Recombinants detected
		25°	39.5°	RB phage host range	RB phage ts+ allele	
T16	0.14	78	77	0.047	0.014	yes
RB1	0.30	119	107	0.009	0	no
RB2	0.05	222	286	0.015	0.003	yes
RB3	0.30	70	85	0.011	0	yes
RB5	0.21	90	93	0.010	0	yes
RB6	0.32	67	77	0.011	0.001	no
RB7	0.42	45	73	0.034	0	yes
RB8	0.57	34	67	0.039	0.016	yes
RB9	0.43	66	91	0.029	0	yes
RB10	0.46	32	75	0.037	0	no
RB14	0.03	283	68	0.003	0.002	yes
RB15	0.11	81	28	0.046	0	yes
RB18	0.10	32	169	0.072	0.061	no
RB19	0.36	24	30	0.157	0.069	no
RB23	0.44	180	?	0.033	0.013	—
RB26	0.93	89	?	0.055	0.005	—
RB27	0.68	206	43	0.038	0.011	—
RB30	0.66	196	56	0.041	0.021	—
RB46	0.58	107	30	0.041	0.041	—
RB51	0.51	91	34	0.040	0.033	—
RB52	0.43	125	29	0.043	0.022	—
RB53	0.51	98	31	0.059	0.032	—
RB57	0.51	133	37	0.044	0.034	—
RB58	0.45	224	54	0.025	0.004	—
RB60	0.68	145	76	0.054	0.043	—
RB61	0.62	73	33	0.075	0.051	—
RB62	0.50	82	29	0.075	0.044	—
RB68	0.56	80	25	0.059	0.035	—
RB69	0.96	172	23	0.988	0.990	—
RB70	0.56	113	35	0.059	0.040	—

All infections were between a T4 temperature-sensitive mutant defective in gene 42 and the indicated RB phage. The RB yielder frequency is the fraction of the infected cells which yield phages with the RB host range. The reasonably high burst sizes of the 39.5° infections suggest that the RB phages can supply the dCMP hydroxymethylase which the T4 mutant lacks, even though they are strongly excluded in all but one case. Where recombinants between T4 and RB phage parent were looked for, they were found in about two-thirds of the cases.

derived could be complementation-tested against one another to identify a total of 37 complementation groups, but these could not be cross-tested with T4 mutants to identify homologous genes (the attempted cross tests failed, apparently because of the very strong exclusion of T4 by RB69). Instead it was necessary to characterize the mutant phenotypes of the RB69 mutants in order to distinguish early from late genes. This characterization was accomplished as already described for T4 (EPSTEIN *et al.* 1963) and yielded the same classes of mutant phenotypes. When representative mutants were crossed to provide a genetic map

TABLE 8

Exclusion of the classical T-even phages by RB69

RB69 amber mutant	Mutant phenotype	Fraction of wild type progeny in cross with T2 wild type	T4 wild type	T6 wild type
am18	DNA arrested	0.009	0.025	0.019
am65	DNA negative	0.007	0.021	0.018
am9	(Tail fiberless)	0.005	0.016	0.019
am24	DNA negative	0.008	0.026	0.018
am36	late	0.009	0.030	0.021
am94	late	0.006	0.016	0.020

All crosses were performed in CR63, a permissive host. Since the fraction of progeny bearing markers from the classical T-even phages is so low, most of these progeny may be coming from cells which, by chance, were not infected with RB69. In fact, only about 1–2% of the infected bacteria yielded wild type progeny in these crosses, indicating that the fraction of wild types among the progeny of truly mixedly infected cells may be considerably lower than the values shown.

for RB69, the general features of RB69 recombination proved very similar to those of T4, the early genes fell into two clear regions as in T4, and the overall structure of the map strongly resembled that of T4. For purposes of testing exclusion with the classical T-even phages, a few RB69 mutants were selected as representatives of early and late genes. Crosses were performed between these mutants and T2 wild type, T4 wild type, and T6 wild type, and the results are presented in Table 8. RB69 excludes T2, T4, and T6 so strongly that only 1–2% of the cells yielded phages with markers derived from them. Since some such cells are those which, by chance, escaped infection by RB69, the fraction of T-even phage yielders among truly mixedly infected cells may be even lower. Indeed, the exclusion is so strong that one cannot rule out the possibility that T2, T4, and T6 may not even have injected their DNA's in these infections.

To determine whether the T-even phages can function in mixed infections with RB69 despite their very strong exclusion, the crosses were repeated in the restrictive host. The results, presented in Table 9, show that the T-even phages

TABLE 9

Functional capacity of the classical T-even phages in mixed infection with RB69

RB69 amber mutant	Mutant phenotype	Burst size as percentage of control in mixed infection with T2 wild type	T4 wild type	T6 wild type
am18	DNA arrested	6.8	11.3	5.8
am65	DNA negative	2.6	8.4	11.9
am9	(Tail fiberless)	1.0	3.4	2.5
am24	DNA negative	12.1	19.0	12.7
am36	late	2.1	6.8	5.1
am94	late	5.7	38.4	28.9

All crosses were performed in B/5, a restrictive host. Controls were mixed infections between RB69 wild type and T2, T4, or T6 wild type. The control burst sizes were all in the range of 100–150 phage per cell. Control infections of the restrictive host with the RB69 mutants alone yield about 1 phage per cell.

can provide limited amounts of several functions lacking in different mutants of RB69. However, their inability to provide other functions well and their marked inability to form viable recombinants with RB69 mutants in spot tests suggest that their relationship to RB69 is quite distant indeed.

DISCUSSION

The results described above concern mixed infections involving a number of different T-even phages of differing degrees of relatedness. It is possible to view all of these infections as part of a common continuum, ranging from the infection between T2 and T6 through the infections between RB69 and the classical T-even phages. At one end of the continuum, the co-infecting phages differ relatively little (as is the case with T2 and T6) and both contribute significantly to the genetic composition of the progeny; there is a slight predominance of one phage (T6) over the other (T2) and the mild excluding action of the predominant phage may be directed against localized determinants in the excluded phage genome. With greater differences between the infecting phages (as in the case of T2 and T4) the excluding action is more marked and is more clearly directed against localized determinants. If the co-infecting phages are even more different (as in the case of T4 and the RB phages) the excluded phage contributes relatively little to the progeny, and if the difference is as great as that between RB69 and the classical T-even phages, even the ability of the excluded phage to function may be lost. It is tempting, of course, to suppose that this continuum may correspond to a series of stages in the process by which speciation occurs in bacteriophage evolution.

The most profitable part of this continuum to study has been the T2-T4 mixed infection; at this point partial exclusion is strong enough to be conveniently measurable, but the co-infecting phages retain sufficient genetic exchange so that the phenomenon can be genetically analyzed. The results described above show that in this infection some gene or genes of T4, as yet unidentified by conditional lethal mutations, act against localized exclusion-sensitivity determinants at several locations in the T2 genome, thereby preventing these determinants from appearing among the progeny. These determinants are found in the early regions of T2 (one between genes 45 and 46, one probably between genes 41 and 42, one or more in the vicinity of gene 32, and probably others); T2 markers appear among the progeny only by recombination away from these determinants, so that markers close to them are preferentially excluded from the progeny. Although little is known of the mechanism of exclusion, it does appear that DNA glucosylation has little to do either with specifying the sensitive determinants or with carrying out the T4 action against them.

These results confirm, extend, and in some cases rationalize previous observations on T2-T4 partial exclusion. In the first extensive series of experiments, STREISINGER and WEIGLE (1956) identified a series of T4 properties, called the "bar" properties, which were inherited by all the progeny of a T2-T4 mixed infection, to the exclusion of their T2 alternatives. These properties were (1) the 100% glucosylation of T4 hydroxymethyl cytosine residues (in contrast to 77%

glucosylation in T2), (2) the ability of T4 to exclude T2 mixed infection, and (3) the ability of T4 to plate with high efficiency on *E. coli* strain 112–12 (T2 plates poorly, forming small "pinpoint" plaques). According to the results described above, this is just the result to be expected if all these properties are controlled by early genes close to the sites of exclusion sensitivity determinants. In the case of glucosylation, the expected gene location has been subsequently confirmed; the gene for T4-induced β-glucosyl transferase, the enzyme which accounts for most of the glucosylation difference between T4 and T2, has been mapped between genes 41 and 42, very close to the site of a determinant (GEORGOPOULOS 1968). In the case of excluding ability, PEES (1970) has shown that a T4 gene controlling the exclusion of a particular determinant of T2 maps close to the site of that determinant, and it appears that T4 genes controlling the exclusion of other determinants are separate and may well map close to the sites of their respective determinants (PEES and DE GROOT 1970). And in the case of the plating efficiency difference between T2 and T4, REVEL (1967) has shown that this may be due to a difference in specificity between T2 and T4 α-glucosyl transferases, the genes for which map between genes 47 and 55 (GEORGOPOULOS 1968; MOLHOLT and DE GROOT 1969; and RUSSELL, unpublished) quite close to a determinant. In short, the previously confusing tendency of all T4 bar properties to remain together in crosses with T2 is easily rationalized; each maps sufficiently close to the site of a T2 exclusion sensitivity determinant so that it appears in a very high fraction of the progeny of a T2-T4 mixed infection, and consequently virtually all such progeny contain all three bar properties of T4.

The non-dissociability of the bar properties had led initially to the idea that all of them might be manifestations of a common phenomenon, or in short that glucosylation differences between T2 and T4 might account both for partial exclusion and for plating efficiency differences. The glucosylation studies reported above, however, suggest that glucosylation has little to do with exclusion, and the same conclusion is suggested by a number of other results obtained both previously and subsequently. STREISINGER and WEIGLE (1956) obtained a rare T2-T4 recombinant with the glucosylation and plating efficiency of T2 but the excluding ability of T4, showing that these properties could be separated. DE GROOT (1963, 1966a, b, 1967a, b) obtained several different recombinants; one had the plating efficiency, glucosylation, and exclusion resistance of T4, but the non-excluding character of T2, while others had only the plating efficiency of T4 and all other properties of T2. JESAITIS (1961) showed that glucosylation and plating efficiency differences between T2 and T6 could be separated by recombination.

All of these results demonstrate the genetic separability of the bar properties, but they are complicated by the difficulty of isolating the required recombinants; in each case rather stringent artificial measures, such as delayed superinfection or heavy ultraviolet irradiation of one parent, had to be used to circumvent the normal exclusion process and permit useful recombinants to be isolated. More recently, mutants defective in one or another bar property have clarified matters somewhat. Mutants of T4 defective in both α- and β-glucosyl transferase continue

to exclude T2 (whether or not it is α-glucosyl transferase-deficient) in appropriately receptive hosts, showing the independence of exclusion and glucosylation (GEORGOPOULOS and REVEL 1971). And a T4 mutant defective in T2 exclusion maps at a site different from those of the two glucosyl transferase genes (PEES 1970). These mutants, like the previously mentioned recombinants, provide strong evidence that glucosylation is not involved in exclusion.

The exclusion sensitivity determinants described above are inferred from the behavior of different T2 markers in mixed infections with T4; a most convincing demonstration of their reality would be afforded if they could be separated and counted, and progress in this direction has recently been made by PEES and DE GROOT (1970). By a series of crosses PEES and DE GROOT (1970) constructed a T2-T4 hybrid which behaves like T2 in some regions of its genome and like T4 in others; in particular, it appears to contain two standard T2 exclusion sensitivity determinants, one near gene 32 and one near gene 39, but it apparently lacks all the determinants in the large early region of T2, from genes 56 to 55. Its existence demonstrates the independence of the determinants. Furthermore, PEES (1970), by a selection technique analogous to the one tried above, has isolated a T4 mutant defective in exclusion of gene 56 of T2. In crosses of this mutant with T2, the region of the T2 genome around gene 56 is much less strongly excluded than normally, but other regions, close to other determinants, are excluded normally. This result not only shows the independence of the different determinants, but it also shows that different T4 genes are required for exclusion of different T2 determinants.

Interestingly, the T4 gene responsible for excluding the determinant near gene 56 of T2 maps very close to gene 56 of T4; if this were generally true, there might exist many small regions in which T2 and T4 differ, T4 containing a gene specifying an excluding activity of some sort, and T2 containing a site sensitive to that activity. How such a set of differences might have come to arise in evolution is not particularly clear. One intriguing possibility is based on the following observations: KIM and DAVIDSON (1974) have observed that T2 and T4, despite their extensive homology, show numerous small regions of non-homology in which the two phages differ sufficiently so that the two DNA strands fail to pair in T2-T4 DNA hybrids. The discreteness of these regions is somewhat surprising, and suggests rather strongly that their origin is not by a successive nucleotide-by-nucleotide divergence of the two phages, but rather by insertions and deletions of DNA in one phage relative to the other. In fact, in about half the regions, the non-homology arises because one phage possesses a stretch of genetic material which the other lacks, and in these cases insertion or deletion must have occurred. If, indeed, there are several regions in which T4 possesses a gene (or genes) for excluding activity, and T2 possesses instead a site of exclusion sensitivity, these regions would probably be non-homologous enough to show up as loops in DNA hybrids, and should have been seen in the relevant map position by KIM and DAVIDSON (1974). In fact, for each T2 exclusion sensitivity determinant so far localized, it is possible to find a corresponding loop in KIM and DAVIDSON's map. Moreover, most of these loops are not found in hybrids between T2 and T6, whose exclusion differences are much milder than those of T2 and T4.

If, as these considerations suggest, T2 and T4 differ by several "exclusion-related" loops, the evolutionary origin of these loops would be of interest. One possible origin would be for a T2-like progenitor to undergo an insertion to produce a T4-like product; if this were indeed the case, the insertion would have to eliminate an exclusion-sensitive site while at the same time generating a linked excluding activity directed against that site. A simple mechanism for accomplishing this is to hypothesize the existence of a circular episome-like element of the type shown in Figure 8. Such an element would possess a gene or genes specifying a protein with the capacity for recognition of specific DNA sequences (perhaps originating from specific recombinases). It would also possess a nucleotide sequence forming a part of the total sequence recognized by this protein, analogous to the attachment site of bacteriophage lambda (GOTTESMAN and WEISBERG 1971). The remaining part of the recognized sequence would be the original exclusion-sensitive site, still found in T2. Transition from a T2-like progenitor to a T4-like product could be accomplished in a rather simple step, by site-specific recombinational insertion of the element as shown in the figure. The sensitive site would be split and thus destroyed by the insertion, and if the two recombinant products differ from the original site (as in bacteriophage lambda) the resulting structure should be stable. In order for the newly included recognition protein to act as an excluding activity against the original site, it is only necessary to imagine that it initially possessed nuclease activity against the site as part of its recombinational function, or that relatively minor mutations could give it this activity.

Without precedent, this sort of possibility seems extremely *ad hoc*, and it would

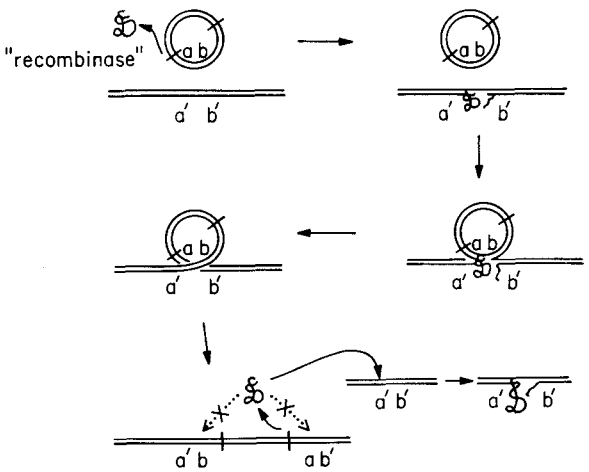

FIGURE 8.—A hypothetical episome-like element for converting a T2-like progenitor to a T4-like product. The element is presumed to code for a recombinase which recognizes the exclusion-sensitive site a'b' and nicks it as the first stage in a recombination event. The next stage involves approach of the complementary (but different) site ab on the element, followed by a specific integration-producing recombination event between the two sites. The resulting hybrid sites a'b and ab' are not nicked by the recombinase, but other a'b' sites on other genomes are nicked; in the absence of additional elements, these nicked sites cannot proceed further in the recombination process and are presumed to constitute replication blocks.

be well to consider a few additional points that support it. (1) Although the origins of such an element are unclear, it is clear that once created it would have tremendous selective advantage, since it would immediately confer on any DNA into which it inserted a strong excluding advantage over its DNA siblings. (2) Such an element would probably be of great use to bacteria as well, so that it might easily have been originated in a bacterial host and later picked up by bacteriophages. (3) The insertional properties of the element already have precedent in bacteriophage lambda insertion (GOTTESMAN and WEISBERG 1971). (4) Many episomes are already known to carry their own restriction mechanisms, analogous to the excluding mechanism postulated by this element (YOSHIMORI 1971; HEDGPETH, GOODMAN and BOYER 1972; BOYER *et al.* 1973; BIGGER, MURRAY and MURRAY 1973). (5) There is growing evidence that other defined sequences are inserted repeatedly in different positions of the *E. coli* genome (HIRSCH, SAEDLER and STARLINGER 1972; SAEDLER and HEISS 1974). Of course the possibility that such an element or its analogs might play a role in accelerating the process of speciation generally is intriguing.

Regardless of these speculations, it is clear that in the T2-T4 mixed infection, exclusion is directed primarily against early genes of T2. Although it is not clear why this should be so, it does appear that this observation is in agreement with those of KIM and DAVIDSON (1974), who find that the "early" and "non-essential" regions of the T-even phage genome are much more heterologous among the T-even phages T2, T4 and T6 than are the regions controlling the structure of the phage particle (with the exception of the tail fibers); both observations suggest that the process of speciation has proceeded much farther in the early and non-essential regions of the T- even phage genomes. It may be that these regions are subject to greater selection pressure for diversity, as appears to be the case for the tail fiber region (RUSSELL 1974), or it may simply be that these regions can better tolerate any diversity which arises by chance. In either case it appears that bacteriophage speciation, like that of higher organisms (DOBZHANSKY 1951) can proceed through a stage in which genetic exchange between subspecies is severely limited for some parts of the genome but not for others.

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