ON THE MECHANISM OF SPONTANEOUS REVERSION AND GENETIC RECOMBINATION IN BACTERIOPHAGE T4

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RECENT studies have distinguished two types of reversible mutations with respect to the type of change in the DNA molecule, in viruses and probably also in higher organisms (BRENNER, BARNETT, CRICK, and ORGEL 1961; MAGNI, personal communication). One type, involving substitution of one base for another and including transitions and possibly transversions, will be referred to here as "substitutions"; the other one, including deletions and/or additions of a few bases resulting in a shift of the reading frame, as "shift" mutations.

A large part of the evidence concerning the two distinct mutation types has arisen from studies of the mutagenic effects of base analogues and acridines on the *rII* region of bacteriophage T4. It appears that the reversion of a given type of mutation is generally produced by a change of the same type that originally produced the mutation itself and this property has provided a means of distinguishing between the two types. Thus a substitution may be corrected by another possibly inverse substitution (FREESE 1959) mostly in the same codon (HELINSKI and YANOFSKY 1962) and a shift by an opposite shift, which generally occurs at a site close to, but distinct from the site of the original mutation (CRICK, BARNETT, BRENNER, and WATTS-TOBIN 1961). Most spontaneous mutants in T4, as a matter of fact, were shown to behave like acridine induced, namely shift, mutants (FREESE 1959; ORGEL and BRENNER 1961).

Though the evidence concerning shift mutations is purely genetic (CRICK *et al.* 1961), a physical basis for their occurrence was provided by the structure of acridine-DNA complexes (LERMAN 1961, 1963, 1964). Since shift mutations are induced by acridines, LERMAN pointed out that a mutagenic effect of acridines has been reported only in recombining organisms, and he proposed a model which postulates that acridine molecules intercalated between bases in DNA would interfere with homologous pairing over short regions. According to this model, a crossover will be unequal if it occurs in a region in which base sequences of paired double helices are out of register as a result of structural changes produced by intercalated acridine. Local extension and untwisting of the paired double helices might also occur in the absence of acridine, and this might account for the majority of spontaneous mutations. An unequal crossing over at the molecular level may produce two opposite shifts in the daughter molecules (Figure 1), so accounting for both signs (additions and deletions) of shift mutants (thereafter called also sign mutants).

wild type	ABC ABC ABC ABC ABC ABC ABC ABC ABC
mutant	ABC ABC AB $\overline{A}$ BCA BCA BCA BCA BCA BCA
revertant	ABC ABC AB $\overline{A}$ BCA BCA BCA B $\overline{X}$ C ABC ABC
parents	ABC ABC AB $\overline{A}$ BCA BCA BCA BCA BCA BCA
	ABC ABC AB $\overline{A}$ BCA BCA BCA BCA BCA BCA
recombinants	ABC ABC AB $\overline{A}$ BCA BCA BCA B $\overline{X}$ C ABC ABC
	ABC ABC AB $\overline{A}$ BCA BCA BCA C $\overline{A}$ B CAB CAB

FIGURE 1.—LERMAN's (1963) model for unequal crossing over. Part of the base sequence in a DNA molecule is represented as it is read in the wild type, in a shift mutant and in a (possibly) revertant state. A triplet comma-free code for proteins is assumed. ABC indicates whatever group of three bases acts as codon in the wild type, X, whatever base intercalated in the sequences. The reading frame is symbolized by a vertical bar.—Unequal crossing over is indicated by the symbol $\rightleftharpoons$, where the arrows ($\rightarrow$ and $\leftarrow$) symbolize a shift in the reading frame. The two parent (both mutants) sequences and the two recombinant (one of them being a revertant) sequences produced by unequal exchange are represented.

The occurrence of spontaneous unequal crossing over was postulated by MAGNI and VON BORSTEL (1962) to account for the increased rate of spontaneous mutations in meiosis as compared with mitosis in *Saccharomyces*. While the present work was in progress, MAGNI (1963) further showed that revertant ascospores (for a given point-mutation) had undergone a genetic exchange of outside markers in diploid heterozygous yeast.

The occurrence of unequal crossing over was also proposed by DEMEREC (1962) to account for the stimulation of reversion with some auxotrophic mutants in *Salmonella* by transduction with homologous phage LT-22. This explanation, however, was subsequently discarded by him, since stimulation had also been observed when either the donor or the recipient strain carried a deletion covering the mutant concerned (DEMEREC 1963). Unequal crossing over is also proposed by BAYLOR, SYMONDS and HESSLER (1965) to explain some observations on recombination amongst *h* mutants in phage T2.

Working hypothesis: Various lines of both theoretical and experimental arguments indicate that genetic exchange does not occur in a single step, but rather involves (1) pairing and breakage of parental DNA molecules, (2) reunion of the fragments in heterozygous structures (HETs), and (3) segregation of HET into recombinant and/or parental DNA molecules. Evidence supporting each of these points is being accumulated (EDGAR 1961; STEINBERG and STAHL 1961; MESELSON and WEIGLE 1961; KELLENBERGER, ZICHICHI, and WEIGLE 1961; KELLENBERGER, ZICHICHI, and EPSTEIN 1962; DOERMANN and BOEHNER 1963; KOZINSKI and KOZINSKI 1963; WOMACK 1963), though the precise mechanism of the entire process is not yet clear (STEINBERG and EDGAR 1962).

In spite of this uncertainty, the model proposed by LERMAN (1963) for acridine mutagenesis may be tested in the *rII* region of bacteriophage T4. Revertants of *rII* mutants may be selected and the correlation between recombination and

reversion may be studied in a phage cross in which the parental *rII* particles are appropriately marked on both sides of the *rII* region. According to this model (Figure 1), *r*⁺ revertants are expected to arise from an unequal crossover between two genomes carrying the same *r* mutation. If this involves recombination of outside markers, as many as 50% of the revertants will be heterozygous or recombinant for the outside markers (the remaining 50% being expected to arise from homoparental matings). This prediction should be fulfilled with sign mutants and should not with substitution mutants.

A strong correlation between spontaneous reversion of shift mutations and heterozygosity of outside markers was indeed found in the experiments reported here. Spontaneous revertants may be inferred to arise by (unequal) crossing over. The segregation of the revertant HETs, however, which involves the problem of the actual mechanism of genetic exchange, will be discussed in a subsequent paper.

MATERIALS AND METHODS

Bacterial strains: The following *E. coli* strains were used: BB to grow phage stocks; B as host for phage crosses; S/6 as standard indicator (Both *rII* and *rII*⁺ phages form plaques on S/6). The Geneva collection was the source of all of these and of a λ -sensitive K 112-12 strain. The latter was lysogenized with wild type λ (KAISER 1957) and the lysogenic derivative used as a selective indicator (only *rII*⁺ phages form plaques on it) which will be referred to hereafter as K. Its transmission and efficiency of plating (e.o.p.) proved to be quite similar to that of the K 112-12 (λ h8) strain of J. J. WEIGLE (EDGAR 1961).

Bacteriophages: The wild-type T4D and various mutant derivatives were used. Spontaneous *r* mutants were isolated from mottled plaques in a *r*⁺ stock containing *r* particles with a frequency of 3×10^{-4} : substitution *r* mutants were isolated from *r* plaques in a 2-aminopurine (AP) treated *r*⁺ lysate containing 10^{-2} *r/r*⁺. *rII* mutants were scored amongst *r* on K. The choice of the four mutants to be tested in a cross-reversion system was based on their reversion properties (see Table I), according to the following criteria. Two spontaneous mutants were chosen: *r3* because it reverted spontaneously at a high rate (subsequently, this was shown slightly but definitely to increase with proflavine), *r9* because it was reverted with proflavine and not with 5-bromodeoxyuridine (BUDR). Both the substitution mutants (*r105* and *r108*) were chosen on the basis of their origin and the positive response to 5-BUDR in spot-tests (FRESE 1959).

TABLE 1

Reversion properties of the rII mutants studied

Mutant	cistron	Origin	Reversion rate* (spontaneous)	Reversion index†		
				spontaneous	BUDR	proflavine
<i>r3</i>	A	spontaneous	2×10^{-4}	4×10^{-3}	3×10^{-3}	7×10^{-3}
<i>r9</i>	A	spontaneous	2×10^{-8}	5×10^{-7}	5×10^{-7}	2×10^{-5}
<i>r105</i>	A	2-AP	1×10^{-8}	4×10^{-7}	3×10^{-5}	7×10^{-7}
<i>r108</i>	A	2-AP	4×10^{-8}	8×10^{-7}	1×10^{-5}	6×10^{-7}

* Fraction of revertants per cycle (does not include clones). This was determined from the results obtained in 20 parallel lysates, each tube being infected with a number of phages such that the probability of introducing an *r*⁺ revertant was about 0.1 per tube. Jackpots were discarded before the mean *r*⁺/*r* ratio was calculated.

† Fraction of revertants in a lysate (includes clones). Infection at time 0 (high multiplicity of infection: cross-reversion system): after 12 minutes the mutagen (BUDR 100 μ g/ml or proflavine 4 μ g/ml) is added with chloramphenicol (50 μ g/ml). After 72 minutes infected cells are centrifuged and resuspended in M9s: lysis completed at 192 minutes with chloroform. Strain B3 was used as host with BUDR: reversion index (spontaneous and after proflavine) in B3 was the same as in B.

FIGURE 2.—Genetic map showing the relative position of the mutants used in this work. Double traits indicate the boundaries between different cistrons. The number below shows standard uncorrected recombination frequencies. Estimation of linkage was based on the frequency of r^+ arisen in crosses of the type $ts r_x ac \times + r_y +$, for the rII mutations. The relative position was established unambiguously, being based on the more frequent genotype of the r^+ with respect to the outside markers. For the outside markers (ts and ac) a sample of progeny phages from a cross $ts + ac \times r9 +$ was stabbed and tested phenotypically (see MATERIALS and METHODS). The mutant $rB48$ (from R. S. EDGAR) was included in the map as reference. No correction, except for e.o.p. and back-mutation (when needed), was introduced in the calculation of linkage.

The $ts A41$ (thermosensitive) and $ac41$ (acriflavin resistant) mutations were used as outside markers. Their sites probably lie in cistrons very close if not adjacent to the $rIIA$ and B cistrons (EDGAR, DENHARDT, and EPSTEIN 1964; EDGAR and EPSTEIN 1961). Phage strains carrying both a given rII mutation (r_x) and each one of the possible four combinations of outside markers were obtained by standard crosses. All strains carrying the same r_x in independent stocks from single plaques showed the same reversion index, within a factor of 3.

The map positions of the mutants are shown in Figure 2.

Chemicals—routine techniques: Proflavine, 5-BUDR (Calbiochem) and acriflavin (neutral NF IX, Nutritional Biochemical Co.) solutions were stored and used in the dark. A synthetic medium, M9, supplemented with 0.5% casamino-acids (Difco) filtered through activated charcoal (M9s), was used for phage crosses and stocks. A complete medium (EHA) served as top and bottom agar for standard plating; plaques were scored after 15 to 20 hours incubation at 30°C. For composition of media, preparation of phage stocks, preparation of bacterial indicators and control of their e.o.p., when not specified, see STEINBERG and EDGAR (1962).

Crosses: Some modifications were introduced to the basic procedure as described by STEINBERG and EDGAR (1962). A culture of *E. coli* B growing exponentially in M9s up to 5×10^7 cells/ml was centrifuged, adjusted to 10^9 in M9s plus L-tryptophan (20 μ g/ml) and infected at a total multiplicity of 8 to 10 at 30°C. No KCN was used. Under these conditions, more than 95% of the phages were adsorbed to bacteria within 2 min. Anti-T4 serum ($K = 1$) was added at 3 min. At 12 min the infection mixture was diluted $\times 100$ in M9s and samples plated on S/6 and K to measure total infective centres and infective centres yielding r^+ . At this time surviving free phages were less than 10^{-4} of the input. The growth tube contained thus about 10^7 infected cells/ml: it was vigorously aerated at 30°C and chloroformed 90 min after the infection.

Single-burst of revertants: Before lysis, 0.1 ml aliquots from a suitably diluted sample of the growth tube were distributed with a sterile automatic syringe to the wells of a large transparent tray (Disposo Tray from Linbro Chemical Co.). Dilution was adjusted so as to have 0.2 to 0.4 of the wells productive (of r^+). The infected cells were incubated for 90 min at 30°C, then .3 to .5 ml of EHA top agar containing K indicator were added with a syringe to each well and incubation continued for 15 to 20 days. At this time r^+ plaques in the progeny population from single cells (individual clones of revertants) could be counted and stabbed. All the plaques from small bursts, and two plaques each from a number of large bursts, were analysed as described below.

In each experiment the e.o.p. was controlled, using a standard phage suspension and freshly prepared indicators. Different phage strains used showed the same e.o.p. Experiments in which the relative e.o.p. of K vs. S/6 was less than .80 were discarded: the latter was quite reproducible, and assumed as an estimate of the absolute titer (KELLENBERGER and ARBER 1957 and unpublished; EISERLING, personal communication).

Loss of infected cells occurred, mostly through the mechanical manipulations, in the single-burst wells. This involved 30 to 80% of the infective centres, as compared with the standard platings, irrespective of the actual cell concentration in the wells, which varied by a factor of 10^4 with the different mutants used. This loss affected infective centres equally on K and S/6; by contrast, r^+ and r phages plated directly into the wells had an e.o.p. close to 1. It is now possible also to recover quantitatively the infected cells by an improved technique. Most of the experiments reported here, however, were made in conditions in which only a fraction of the infected cells was recovered. The above considerations prove that this fraction may be considered an unselected sample of the infected cells and that the phages liberated by them were quantitatively recovered. Moreover, the average burst size of r^+ was estimated both from the single burst data (assuming a Poisson distribution) and from the standard platings of the infected cells and the lysate on K, in each experiment. These two estimates were in good agreement within 10% (see, for an example, the legend to Figure 3).

Scoring of genotype for outside markers and HETs: Progeny phages produced mostly r plaques on S/6, and only r^+ plaques on K. Plaques to be analyzed (either random plaques from the mass lysate or selected plaques from single bursts of a given size) were picked with a sterile wire and suspended in chloroform saturated broth. Appropriate dilutions were plated on S/6 at 30°C to obtain samples of plaques derived from each isolate. Of the latter samples, usually 18 plaques were tested by stab transfer to two selective plates (see below) seeded with S/6.

The first plate contained EHA supplemented with 25 $\mu\text{g/ml}$ of acriflavin in the bottom agar: only the *ac* (resistant) genotype makes a spot after 15 to 20 hours incubation at 30°C. The second one (EHA with no addition) was well dried: incubation of 8 to 15 hours at 39°C allows an unambiguous classification of the *ts*⁺ and *ts* genotypes based on the size and the halo of the lytic area.

When isolates are stabbed directly (no resuspension and sampling), the apparent genotype depends mostly upon the presence of resistant alleles (*ac* and *ts*⁺) amongst phages sampled from the plaque: this simplified procedure (referred to as phenotypic analysis) was only applied to populations in which previous complete analysis had shown HETs to be very rare.

Isolates were scored as HETs when at least one of the 18 plaques tested showed a different genotype (in 20 cases, in which HETs were defined as such on the basis of only 1 plaque out of 18, an additional test confirmed the previous classification). The efficiency of this procedure in detecting HETs is probably less than one: it may be estimated empirically—in the same way as DOERMANN (1963) did in a similar system—by scoring the number of isolates which should be classified as HETs as a function of the number of plaques tested for each isolate (Table 2).

TABLE 2

Efficiency of detection of HETs

Number of plaques scored*	Number of HETs detected	
	DOERMANN†	Present work‡
5	172	124
10	190	171
15	194	192
18	...	200
20	199	...
40	200	...

* Eighteen plaques were actually tested from each isolate and 200 HETs were thus detected. Tests were kept in sequence. After only 5, 10, 15, etc. plaques (first column) had been scored, a number of HETs would have been detected: this is reported in the second and third columns.

† Cross $r \times r^+$: selection for mottled plaques on S/6, the marked region is about 8 units long (uncorrected value) and carries six r markers. Data from DOERMANN and BOEHNER (1963).

‡ Cross $r \times r$: selection for r^+ revertants on K occurring in small bursts (see text). The marked region is about 20 units long (uncorrected) and carries only two markers (besides the r).

RESULTS

Detection of revertants arising in a growth cycle: The basic experiment includes two reciprocal phage crosses of the type $r_x ac \times + r_x +$ and $ts r_x + \times + r_x ac$, in which the same alleles as outside markers are introduced either in coupling or in repulsion. This will be referred to as the cross-reversion system, since the revertants are selected as r^+ and then the genotype (unselected) of outside markers is determined in both r^+ and r progeny. The opposite procedure, namely to select recombinants and then to determine the frequency of r^+ amongst them, was not feasible for reasons that will become clear now.

Since rII mutants to be studied in a cross-reversion system must revert, a number of r^+ phages will be always present in the stocks of both r_x parents used for a cross. These *old* r^+ are not expected to differ from the bulk of r phages in their recombining ability (for the outside markers : about 21% of recombinants). In contrast, the *new* r^+ (newly arisen in cells infected only with r) according to the working hypothesis, need a crossover to become r^+ (up to 50% recombinants). Thus the frequency of recombination in the r^+ progeny phages is expected to be the weighted mean of the two different values and will depend on the relative weight of the two populations of r^+ . An estimate of the number of *new* r^+ clones may be obtained by comparing the fraction of cells which receive r^+ (K/B in the input phages multiplied by the multiplicity of infection) and will then give *old* r^+ clones, with the fraction of cells which liberate r^+ (K/B in the infective centres) including both *old* and *new* r^+ clones. Data from such experiments are reported in Table 3. In the reconstruction experiment a number of wild-type phages is added to the same infection mixture (as *old* r^+) so that the fraction of *new* r^+ becomes negligible.

The mean number ($\overline{CS}$) of progeny phages derived from a single input particle may be calculated from the mean burst size ($\overline{BS}$), which is estimated as usual as the ratio of progeny phages to infective centres, both on S/6 and K. Then the $\overline{CS}$ of r is equal to the $\overline{BS}$ on S/6 divided by the multiplicity of infection, whereas the $\overline{CS}$ of r^+ is equal to the $\overline{BS}$ on K (in the latter case multiplicity is irrelevant, since each cell received only one r^+ if any). Since $\overline{CS}$ of r^+ is roughly equal to $\overline{CS}$ of r in the reconstruction, no strong selection seems to occur in a growth cycle, and one may conclude that *old* r^+ clones are similar in size to r clones. However, *new* r^+ clones should be much smaller as an average to account for the small $\overline{CS}$ of r^+ in all the other experiments.

The former conclusion could also be anticipated on theoretical grounds, and can in any case be tested by studying the BS distribution in a single-burst experiment. The BS distribution of r^+ found in a cross-reversion experiment as compared with a reconstruction is shown in Figure 3. It is actually nonhomogeneous and may be considered bimodal, with a fraction of large clones which correspond to the *old* r^+ in the reconstruction, and a fraction of small clones which are not found among *old* r^+ . Though considerable overlap occurs between these two populations, clones of size 1 and 2 may be assumed to be mostly *new* r^+ , and clones of size >5 mostly *old* r^+ .

TABLE 3

 Old and new r^+

Input phages		Infective centers		Progeny phages		
Cross	r^+/r (A)	m.o.i. [†] (B)	old r^+ calculated (A × B)	old and new r^+ found r^+/r	r^+/r	yield [‡] r^+
<i>ts r3 ac</i> × + <i>r3</i> +	5.3×10^{-3} *	8	4.2×10^{-2}	9.3×10^{-2}	5.5×10^{-3}	14
<i>ts r105 ac</i> × + <i>r105</i> +	4.9×10^{-7}	8	4.0×10^{-6}	8.7×10^{-6}	5.1×10^{-7}	15
<i>ts r108 ac</i> × + <i>r108</i> +	8.1×10^{-7}	9	7.3×10^{-6}	11.8×10^{-6}	8.3×10^{-7}	13
<i>ts r9 ac</i> × + <i>r9</i> +	4.2×10^{-7}	10	4.2×10^{-6}	7.8×10^{-6}	4.2×10^{-7}	13
§ <i>ts r9 ac</i> × + <i>r9</i> +	4.2×10^{-7}	10	4.2×10^{-6}			..
<i>ts r⁺ ac</i> added	5.0×10^{-3}	(.005)	5.0×10^{-3}	5.1×10^{-3}	5.5×10^{-4}	13
						14

* r^+/r figures are based on counts of at least 500 plaques of each type, correction for efficiency of plating was made.

† m.o.i. = multiplicity of infection.

‡ Average burst size divided by the multiplicity of infection.

§ This reconstruction experiment was made in parallel with the preceding one. Two samples of the same bacterial culture were infected (1) with a mixture of *r9* phages and (2) with the same mixture plus a small fraction of r^+ . In case (2) the fraction of revertant r^+ is negligible as compared with the input r^+ , and therefore clone size and recombination for outside markers (see Figure 3 and Table 4) amongst r^+ refers only to old r^+ .

FIGURE 3.—Clone-size distribution of new and old r^+ . Burst-size distribution of r^+ in a cross-reversion system (including both old and new r^+ : thick line in the figure) and in a reconstruction experiment (only including old r^+ : narrow line in the figure) are compared. The size of the experiments was chosen such as to have about 100 to 60 r^+ bursts in the two cases, according to the proportion of total to old r^+ infective centers (Table 3). Actually, 864 and 480 fractions were distributed from the last cross-reversion and the reconstruction experiments listed in Table 3: 324 and 197 r^+ bursts were detected, respectively. According to a Poisson distribution, about $\frac{1}{4}$ of the bursts were in fact mixed (containing more than one clone of r^+ : no correction for pure clones was made in the figure). The 324 r^+ bursts in the cross-reversion system are assumed to include about 60% old r^+ clones (corresponding to the 197 r^+ bursts analysed in the reconstruction), plus about 40% new r^+ clones (see Table 3). Then the excess of small clones which was found in the cross-reversion system may be reasonably ascribed to new r^+ arisen in cells infected only with r particles.—The average clone size of r^+ (calculated from data of single bursts) was 6 for the cross-reversion and 11 for the reconstruction (here correction for mixed bursts was made) as compared with 7 and 14 in Table 3 (calculated from data of mass lysates). However, the number of plaques (and then the clone size) in very large bursts scored in small wells were probably underestimated.

Since the single burst enables newly arisen r^+ to be distinguished from the pre-existent ones, the correlation between reversion and crossing over must be searched in the former population, selected and defined as small bursts of r^+ (size 1 and 2), in a cross-reversion system with sign mutants. Two comparable populations will provide an internal and external control for this correlation: (1) large bursts of r^+ (size >5) in the same cross-reversion system, and (2) small and large burst of r^+ in a similar system with substitution mutants. Both of them are expected to show no correlation between reversion and crossing over.

Genotype of revertants: In the first experiments (STRIGINI 1964) with sign mutants, "new" r^+ were reported to contain more recombinants for the outside markers than both "old" r^+ and r phage in the same cross progeny when the classification was entirely based on the phenotypic analysis (see MATERIALS and METHODS). An apparent excess of *one* recombinant class was observed, and moreover a high frequency of HETs was found amongst new r^+ . Since it was established that HETs build up a considerable fraction of the revertants, a complete analysis of heterozygosity was needed to define unambiguously the genotype of revertants. It will be shown that HET revertants may be considered as structures

TABLE 4
Genotypes of new and old r^+ with respect to outside markers

Combination of the outside markers	Selection* and mutants	Total scored r^+	Genotype			H H+R+P	R R+P	H+R H+R+P
			Recom- binant (R)	Hetero- zygote (H)	Parental (P)			
$ts\ ac \times + +$		15	1	6	8	..	..	..
$ts + \times + ac$		8	1	3	4	..	..	..
	$r3$	23	2	9	12	..	..	..
$ts\ ac \times + +$		38	5	15	18	..	..	..
$ts + \times + ac$		16	2	5	9	..	..	..
$ts\ ac \times + +$		30	3	15	12	..	..	..
$ts + \times + ac$		41	4	15	22	..	..	..
	$r9$	125	14	50	61	..	..	..
	$B_1\ r3, r9$	148	16	59	73	.40	.18	.51
$ts\ ac \times + +$		22	3	4	15	..	..	..
$ts + \times + ac$		30	5	7	18	..	..	..
	$r3$	52	8	11	33	..	..	..
$ts\ ac \times + +$		30	5	8	17	..	..	..
$ts + \times + ac$		46	8	9	29	..	..	..
	$r9$	76	13	17	46	..	..	..
	$B_2\ r3, r9$	128	21	28	79	.22	.21	.38
$ts\ ac \times + +$		12	2	..	10	..	..	..
$ts + \times + ac$		50	10	3	37	..	..	..
	$r3$	62	12	3	47	..	..	..
$ts + \times + ac$		42	8	2	32	..	..	..
	$B_{>5}\ r3, r9$	104	20	5	79	.05	.20	.24
mass r^+		500	111	..	389	(.06)	.22	(.25)
mass r		498	99	..	399	(.04)	.20	(.22)
reconstruction r^+		190	30	..	160	(.04)	.16	(.18)

* Selection was made according to the burst size of r^+ on K in a cross-reversion system (e.g. B_1 means : burst of size 1, etc.). Each line reports one independent experiment. In some experiments, only a sample of the r^+ bursts detected was analysed : in most cases only two plaques were analysed in bursts of size >5 . Sums for each individual mutant and for a given burst size are also reported. Only r^+ isolates wholly analysed for heterozygosity are listed in the single-burst data.

† For mass lysates only phenotypic analysis was made. Only 50 r plaques and 50 r^+ plaques were wholly analysed in the standard way (see MATERIALS and METHODS) and 1 and 3 HETs were detected, respectively. Actual fraction of HETs (right part of the table) is assumed to be .04 amongst r and .06 amongst r^+ . 50% of them are assumed to behave phenotypically like parents and 50% like recombinants (see MATERIALS and METHODS). The calculated values are given in parentheses.

able to segregate a recombinant and a parental genotype with respect to the outside markers. This may account for the apparent excess of recombinants in one of the two reciprocal classes, the double resistant $ts^+ ac$, previously found by phenotypic analysis.

Genotypes of new and old r^+ found in cross-reversion systems with two sign-mutants in different experiments are reported in Table 4: only r^+ isolates analysed for heterozygosity are listed. As both mutants show a similar pattern of recombination and heterozygosity, data are pooled together to compare bursts of size 1, 2 and >5 .

The reciprocal crosses gave quite consistent results.

The frequencies of both reciprocal recombinant classes were equal to each

other in bursts of size 1 and 2, and so were the frequencies of both parental classes. In bursts of size > 5 the two parental classes varied according to the fluctuations in reversion index occasionally found in different stocks (resulting in different input of the r^+ parents), whereas the two recombinant classes were still equal. For instance, in a particular experiment the ratio of $ts r^+ ac$ to $+ r^+ +$ revertants in the input stocks was 3:1 ($ts r ac$ to $+ r +$ being 1:1), and the same ratio was found in the large clones of revertants in the progeny; however, in the small clones the ratio was 1:1. This confirms the notion that small clones are mostly new r^+ and large clones old r^+ .

The most striking feature in Table 4 is the very high frequency of HETs found in new r^+ , especially in bursts of size 1. However, the actual frequency of HETs is likely to be even higher if about 10% of them were misclassified as pure parents or recombinants (see MATERIALS and METHODS). In contrast, the ratio of pure recombinants to pure (non-HET) progeny amongst new r^+ do not seem to exceed the overall figure in the mass lysate.

The frequency of HETs in old r^+ is close to the expected value for two loosely linked markers in a cross-lysate with T4, which should be about twice the average figure of 2% for any single marker (HERSHEY and CHASE 1951; EDGAR 1961; STREISINGER unpublished; WOMACK 1963). This indicates that no correlation exists between the r^+ genotype as such and heterozygosity (for the outside markers) in a cross-reversion system.

Table 5 shows detailed data obtained with two substitution mutants: pooled

TABLE 5

Genotypes of r^+ from substitution and shift mutations, with respect to outside markers

Combination of the outside markers	Selection* and mutants	Total scored r^+	Genotype			H H+R+P	R R+P	H+R H+R+P
			Recom- binant (R)	Hetero- zygote (H)	Parental (P)			
<i>ts ac</i> $\times$ $++$		33	6	6	21	..	..	..
<i>ts +</i> $\times$ $+ ac$		20	4	2	14	..	..	..
	<i>r105</i>	53	10	8	35	..	..	..
<i>ts ac</i> $\times$ $++$		47	10	4	33	..	..	..
<i>ts +</i> $\times$ $+ ac$		40	6	5	29	..	..	..
	<i>r108</i>	87	16	9	62	..	..	..
	<i>B₁ r105, r108</i>	140	26	17	97	.12	.21	.31
	<i>r3, r9</i>	148	16	59	73	.40	.18	.51
<i>ts ac</i> $\times$ $++$	<i>r105</i>	48	9	4	35	..	..	..
<i>ts ac</i> $\times$ $++$		40	8	2	30	..	..	..
<i>ts +</i> $\times$ $+ ac$		44	8	3	33	..	..	..
	<i>r108</i>	84	16	5	63	..	..	..
	<i>B₂ r105, r108</i>	132	25	9	98	.07	.20	.26
	<i>r3, r9</i>	128	21	28	79	.22	.21	.38
<i>ts +</i> $\times$ $+ ac$	<i>B_{>5} r108</i>	50	11	3	36	.06	.23	.28
	<i>r3, r9</i>	104	20	5	79	.05	.20	.24
mass r^+		258	53	..	205	(.04)	.21	(.23)
mass <i>r</i>		204	47	..	157	(.04)	.22	(.24)

* See legend to Table 4.

Pooled data for shift mutants from Table 4 are reported for comparison in italics.

data from Table 4 are also reported for comparison. Though some difference between the two mutants studied is visible in bursts of size 1, data are pooled together for purpose of comparison with the more homogeneous data of shift mutants. The frequency of HETs in bursts of size 1 is much lower than with shift mutants; however, it is definitely higher than the expected value (4%) in case of no correlation: this is true especially with *r*105 and only to a lesser extent with *r*108. In bursts of size 2 the frequency of HETs is close to the no-correlation value with both mutants.

The frequency of HETs in small *vs.* large clones of revertants might be related to some physiological events, as, for example, nonrandom maturation of HETs during the latent period. Alternatively, the loose correlation between reversion and heterozygosity with some substitution mutants may indicate that even in this case spontaneous reversion could sometimes occur through the same mechanism which seems to operate with shift mutants. If this is true, no correction is needed for the correlation found. Some more direct evidence favours this explanation, at least with *r*105: (1) Two types of spontaneous revertant plaques were observed on K: small plaques yielded a high fraction of recombinants for outside markers, whereas wild-type plaques did not; (2) a slight but definite increase of the reversion index was found with proflavine; (3) only wild-type plaques and no excess of recombinants amongst *r*⁺ were found with 5-BUDR, which increases the reversion index by a factor of 50 in a single cycle.

Genotype of revertant HETs: The HET region is defined by the occurrence of both the alleles, which were separately present in the parents, in a single progeny phage. All the revertant HETs from sign-mutants, whatever the combination of outside markers in the parents, may be considered together. They will be called *ts* HETs or *ac* HETs according to the location of the HET region.

For the sake of simplicity, *r*3 and *r*9, which are closely linked (see Figure 1), will be considered together in Tables 6 and 7. Most of the revertant HETs were single HETs with respect to the outside markers (Table 6). The HET region was found to cover the *ts* locus, which is closer to the *r* site, more frequently than the *ac* locus. The occurrence of double HETs is close to the random expectation based on single HET frequency in bursts of size 1; in bursts of size 2, double HETs seem to be more frequent than random, if such small numbers are significant.

In Table 7 the formal genotypic structures and the numbers found for the four

TABLE 6

Heterozygous region in revertant HETs from shift mutants

Selection and mutants	Markers included in the HET region			Ratio <i>ts</i> HETs : <i>ac</i> HETs
	<i>ts</i> 1/2*	<i>ac</i> 3/4	<i>ts</i> and <i>ac</i> 1/2, 3/4	
<i>r</i> 3, <i>r</i> 9 B ₁	37	27	5(6.7)†	1.3(1.3)‡
B ₂	18	12	6(2.2)	1.4(1.3)

* 1/2 represent the alleles at the *ts* locus, and 3/4 the alleles at the *ac* locus (see Table 7).

† In brackets : expected value for random coincidences, calculated basing on the frequency of *ts* and *ac* single HETs.

‡ In brackets : ratio of *r*9-*ac* to *ts*-*r*9 interval.

TABLE 7

Constitution and number of revertant HETs from shift mutants

PARENTS	<u>1</u>	<u>r</u>	<u>3</u>
	<u>2</u>	<u>r</u>	<u>4</u>
ts HETS	<u>1</u>		<u>3</u>
	<u>2</u>		
	<u>1</u>		
	<u>2</u>		<u>4</u>
ac HETS	<u>1</u>		<u>3</u>
			<u>4</u>
			<u>3</u>
	<u>2</u>		<u>4</u>

Selection and mutants	Alleles present			
	1/2,3	1/2,4	1,3/4	2,3/4
<i>r</i> 3, <i>r</i> 9 B ₁	17	15	10	12
B ₂	7	5	2	4

See legend to Table 6.

The two parents and the four possible single HET genotypes are represented.

possible single HETs are shown. Within the two classes of *ts* and *ac* HETs, the two possible genotypes with respect to the other (non-HET) marker are equally frequent. Since the frequencies of either class of HETs (*ts* and *ac*) seem to be related to the distance of either marker from the *r* site (Table 6), one may conclude that the occurrence of any different allele in the revertant HET structures is essentially random. A consequence of this assumption could be that some HETs may be too "short" to cover either one of the outside markers (10 to 13 map units from the *r* site). However, since the overall frequency of HETs cannot exceed 50%, one should conclude that most revertant HETs are unusually "long."

The structure of newly arisen revertant HETs may be defined formally as one which carries (a) at least the *r*⁺ information at the *r* shift-site, and (b) the information of two alleles (from different parents) at one side and of a single allele at the other side, for the outside markers at random. Whether the *r* site is actually HET or not (carrying possibly the original *r* allele) cannot be answered directly with these experiments, since revertant plaques (and therefore the *r*⁺ allele) were always selected through K.

Backcrosses: According to the working hypothesis, one would expect that most revertants arising through unequal crossing over from a sign mutant would carry the *r*⁺ information as a result of a second shift in the reading frame close enough to the former. In principle, both mutations (and particularly the original one) could be separately recovered by backcrossing a revertant with a wild type, as was in fact done by CRICK *et al.* (1961) in the left segment of the *rIIB* cistron. In this case the second shift might occur as far apart as about 0.5 map unit from the original one. This segment, however, was previously shown to be nonessential to the function (CRICK *et al.* 1961; CHAMPE and BENZER 1962).

In our case, a preliminary attempt to recover the original mutation from revertants was unsuccessful. Two procedures were followed: (1) Six independent revertants (3 from *r*3 and 3 and *r*9) were backcrossed to wild-type T4 and 10,000 plaques in each progeny scored. A total of 41 *rII* mutants were isolated and none failed to recombine with the original mutant by mixed infection on B as shown by plating infective centres on K. (2) A sample of *r*3 and one of *r*9 containing about 1000 *r*⁺ revertants were cycled four times on K to get rid of the *rII* phages. The final *r*⁺ stocks which had arisen from random samples of different revertants were backcrossed to wild-type T4 grown on K. Here again none of the rare *rII* mutants isolated in the crossed progeny (12 on 20,000 plaques scored) failed to recombine with the original mutant.

Therefore, in the present case the second shift should be required to occur in most cases closer than .02 units to the original one.

DISCUSSION

The following remarks will especially consider results with c 1 revertants (selected as *r*⁺ in clones of size 1). These were shown to represent a fairly pure sample of new revertants. Moreover, though little is known about the maturation mechanism, they are likely to include a large fraction of *primary* revertants rather than the products of their replication. The latter statement is only based on the assumption that *r*⁺ structures, since they are formed in the DNA replicating pool, may be matured and then recovered as plaque forming units in clones of size 1, or may be replicated and then recovered after maturation in clones of size ≥ 1 .

A very large fraction (40%) of the c 1 revertants from *rII* shift mutations were found to be HETs. This figure might be an underestimate, since a fraction of HETs could be lost due to the technique of analysis (see MATERIALS and METHODS) and another fraction could be too "short" to cover the distance between the *r* mutant site and either one of the outside markers (see RESULTS). On the other hand, the figures of 40% could include a fraction of c 1 HETs unrelated to reversion. This fraction should not exceed the average (4%) expected in the mass lysate, since the fraction of HETs reaches equilibrium very early in the latent period (HERSHEY and CHASE 1951; SECHAUD *et al.*, unpublished). However, even if the fraction of HETs (13%) found amongst c 1 revertants from substitution mutants is taken as an estimate of the no-correlation value (between reversion and heterozygosity), this background remains significantly lower than the corresponding figure in the case of shift mutants. Alternatively, it is compatible with the model of LERMAN that some substitution mutation could also be reverted—with lower efficiency—by rare double unequal crossing over involving two opposite shifts at both sides of it. Some evidence favouring this explanation was given for the mutant *r*105.

It seems safe to rule out the objection that these HETs might only be artifacts produced by some mixing or rescue or recombination with *r* phages occurring on the plates. These simple explanations, as a matter of fact, lead to the further as-

sumption that the probability for these phenomena would be dependent on the mutation type and its reversion rate, rather than on the number of plated *r* phages. It seems more simple to believe that a strong correlation exists between spontaneous reversion of a shift site and heterozygosity of a nearby region. Since close to half of the primary revertants are genetically HETs, nearly all of them may be assumed to carry information of biparental origin (having similar structures, either heterozygous or homozygous for the considered markers). This is true, at least, for two spontaneous *r* mutants, one of them having a very high reversion rate (about 10^{-4}).

This correlation by itself establishes neither a cause-effect relationship, nor its direction. Further experiments with more mutants are required to establish the generality of the phenomenon described here. In this regard it is relevant that two more *rII* mutants (of the set isolated by CRICK *et al.* 1961) have shown again a high frequency of HETs (for the same outside markers used throughout the present work) amongst newly arisen revertants (STRIGINI unpublished). In any event, it seems worthwhile to consider some implications of the proposed model with respect to genetic recombination.

Two assumptions will be explicitly stated here for the discussion to follow: (1) the *r* site is included (possibly at one end) in the HET region; (2) the HET region as an average does not extend much more than about 10 map units. The first point is not easily susceptible to direct experimental verification for technical reasons. However, this was found to be the case for most *rII* forward mutations induced by proflavine (DRAKE 1964). The second point is weakly supported by data reported in Table 6 (last column) and more strongly by similar data (STRIGINI unpublished) which include more *rII* mutants such that the ratio of *r-ac* to *ts-r* intervals ranges now from 0.7 to 1.8. The inverse relationship thus found between the length of the *r-x* interval and the frequency of *x* HETs (where *x* symbolizes either *ts* or *ac*) strongly supports the second point, and also suggests that the *r* site is at one end of the HET region.

A HET genome may be formally defined as one carrying both the parental alleles for a given marked region. The nature of its physical linkage to the adjacent (non-HET) regions of the polynucleotide sequence is a matter of hypothesis. Different models may define different classes of HET structures, which seem to derive one from the other (EDGAR 1961; SECHAUD *et al.* unpublished; WOMACK 1964) and finally segregate pure recombinants. Accumulated evidence, however, favours the notion that recombinants in phage crosses arise from HET structures. Different models were presented by LEVINthal (1954) and STREISINGER (unpublished); direct evidence was provided by EDGAR (1961), SECHAUD *et al.* (unpublished), DOERMANN and BOEHNER (1963) and WOMACK (1963) for T4, and by KELLENBERGER *et al.* (1962) for λ bacteriophage.

The model of LERMAN (1963) described in the introduction predicts that reversion of shift mutations is produced by unequal crossing over. Data presented here may mean that reversion (in the case of shift mutations) always takes place in a HET structure, or involves the production of a HET structure.

Crossing over is probably a multi-step process, involving pairing, breakage, and

reunion of at least two polynucleotide strands from two different parental DNA molecules. Since r^+ revertants are originally HETs in a nearby region, this would imply that reversion results from an (unequal) crossing over, or from some preliminary step leading to crossing over. In other words, the shift required by the model to get reversion could occur at, or before, the stage of reunion. If, as seems simplest, it is assumed to occur at the reunion, then the r^+ strand (carrying both the original sign mutation and a second one of opposite sign) should also carry, in about 50% of the cases, a recombinant set of outside markers. Heterozygosity could then result from a redundancy in the genetic information (either terminal or not) through the presence of at least a second strand carrying the r allele, and accordingly—in most cases—a parental set of outside markers. Since revertant plaques have always been selected on K, the r^+ strand was strongly selected, and the majority of segregants (according to this hypothesis) were expected to be recombinant for the outside markers. As a matter of fact, HETs are close to 50% amongst c 1 revertants, but most of them segregate a *majority* of parents with respect to the outside markers: as a whole, c 1 HETs only segregate about 20% of recombinants, as will be reported in detail elsewhere.

This might suggest that the shift postulated by the model occurs prior to the reunion, and it produces a functional r^+ sequence which still segregates *preferentially* in connection with a parental set of outside markers. One might speculate whether this phenomenon (formally equivalent to high negative interference) is not due to some mechanism requiring an even number of exchanges (as the rescue of a fragment) or alternatively a limited copy-choice mechanism as suggested, for instance, by BERNSTEIN (1962) and MESELSON (1964). Thus, at the present moment, one cannot decide whether the HETs resulting from reversion of sign mutants have a peculiar structure and meaning with respect to the mechanism of standard recombination, or the latter also includes a preliminary step involving HET structures which segregate in a similar way, such as the NRH (nonrecombinant HETs) detected by EDGAR 1961.

It has been shown that spontaneous mutation is correlated to genetic exchange in *Saccharomyces*, since the mutation rate is higher during meiosis than in mitosis by a factor of 10 (MAGNI and VON BORSTEL 1962) and spontaneous reversion at a given locus in diploids is strongly correlated to recombination of outside markers (MAGNI 1964). Moreover, acridine was found to be effectively mutagenic in *Saccharomyces* only during meiosis (MAGNI, VON BORSTEL, and SORA 1964); in bacteriophage T4 it was shown to be relatively insensitive to block of DNA synthesis by FUDR and, moreover, to induce r mutants mostly as r/r^+ HETs (DRAKE 1964). All these findings are readily explained as a result of unequal crossing over. However, the mechanism of genetic exchange is poorly understood, and even less so are the conditions which may produce unequal exchange, and possibly switch the crossing over from equal to unequal. As a matter of fact, ultraviolet-stimulated recombination fails to increase the mutagenic action of acridine (DRAKE 1964). The postulated reciprocal products of unequal crossing over have not been found in T4, since mutants induced by acridine occur in pure clones (DRAKE 1964). An inhibitory effect of acridine on recombination and

transformation was reported by LERMAN (1964) and this could be separated from the mutagenic effect. In this context, it may be worth mentioning that revertants induced by proflavine with *r*9 included only about 6 to 10% HETs for outside markers in a cross-reversion system, in spite of the fact that most of the revertants occurred in clones of size 1. A more precise knowledge of the sequence of physical events involved in recombination (and of the physiological implications in any particular system) would be needed to predict the effect of acridine in any case according to LERMAN's hypothesis.

A strong prediction, which may be derived from the model of LERMAN, was verified by CRICK *et al.* (1961), showing that most revertants from sign mutants still carry the original shift mutation plus a second one of opposite sign. It is not too surprising that this could not be achieved in the present work, since shift mutations used were not selected with respect to their location, and this may lie in an indispensable DNA region. In this case the interval available for the second shift to restore effectively the function of the gene product may be very short, in contrast to the situation of the left segment of the *rIIB* cistron (CRICK *et al.* 1961). Moreover, the recombination frequency is not meaningful as an estimate of the physical length of this interval. It has been possible, however, to show that some of the revertants have acquired new properties with respect to the *rII* function (STRIGINI unpublished). This strongly suggests that such an interval out of register is still present, and may be detected with appropriate techniques.

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SUMMARY

The correlation between spontaneous reversion and genetic recombination was studied in crosses with *rII* mutants of bacteriophage T4, carrying the same *r* mutation to be studied and differing for two outside markers on both sides of the *rII* region. Two spontaneous mutants, one highly revertible spontaneously and the other with proflavine, and two 2-aminopurine induced mutants, both revertible with 5-bromodeoxyuridine, were studied. In the cross-progeny the revertants selected as *r*⁺ in clones of size 1 were shown to be mostly newly arisen revertants. About 40% of these were found to be heterozygous for either one of the outside markers (HETs), in the case of spontaneous *rII* mutants. Large clones of revertants, derived from *r*⁺ already present among the infecting parental phages, included only 5 to 6 % of HETs. In the case of substitution mutants, 13% of the new revertants were HETs. It is inferred that spontaneous reversion may generally occur through unequal crossing over, involving heterozygosity of a nearby region. Some implications of this model are discussed with respect to genetic recombination.

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