MARKER STIMULATION OF CROSSING OVER: A TEST OF THE THEORY YIELDING NEGATIVE RESULTS AND USING *rII* MUTANTS OF PHAGE T4^{1,2}

C. E. FOLSOME

Microbiology Department, University of Hawaii, Honolulu

Received October 12, 1964

CONCLUSIONS based on data collected from the phage T4 *rII* system (BENZER 1957) retain certain assumptions derivative from classical genetic analyses of intergenic recombination (HALDANE 1919) which may not be applicable when extended to intragenic levels. Two of these assumptions are stated and are the subject of experimental test as reported here.

Assumption I: It is generally accepted that r^+ recombinants issuing from intragenic T4 *rII* crosses are identical in genotypic constitution to standard r^+ phage. However, CRICK, BARNETT, BRENNER and WATTS-TOBIN (1961) and JINKS (1961) have clearly established that closely linked intragenic internal suppressors of the original *rII* or *hIII* genotype exist. For example, crosses between close single base pair addition (+) and deletion (−) mutants will generate some “ r^+ ” progeny which prove to contain both parental (+) and (−) loci. Assumption I, that r^+ progeny from *rII* crosses are of identical information sequence to standard type is not necessarily true and is dependent upon the nature of *rII* mutant sites employed in the cross.

Assumption II: The various mutants at a specific *rII* site employed in the cross have no effect upon recombination frequencies. One can conceive (as did HERSHEY 1958) that as more and more *rII* sites are involved in a single mating event the physical presence of an array of heterologous sites will itself lead to altered recombination frequencies. In this fashion HERSHEY suggested the marker stimulation theory to account for the high negative interference (HNI) coupled with apparent additivity of map distances observed by CHASE and DOERMANN (1958) for T4 *rII* multifactor biparental crosses. If HNI were caused by switch areas as proposed by CHASE and DOERMANN one would expect to observe disproportionately larger recombination frequencies between two closely linked markers. Hence the sum of distances between successive two-factor crosses would be expected to exceed the recombination frequency observed for two terminal markers over the span considered.

On the other hand, BOETTCHER (1963) has proposed that internal suppression is sufficient to account for HNI. Assume that vicinal (+) and (−) *rII* mutant parents can generate by recombination an internally suppressed sequence (+ −)

¹ Supported by Research Grant GB-459 of the National Science Foundation and, in part, by the Genetics Department, Melbourne University, Australia.

² Contribution No. 46 from the Pacific Biomedical Research Center, University of Hawaii.

which possesses phenotypically wild-type function to some degree. Then, in a three factor cross of $r_1 r_3 \times r_2$ requiring two recombinational events to produce an r^+ recombinant, HNI is to be expected if r_2 and either r_1 or r_3 are of disparate signs because " r^+ " phenotypes will be produced by *single* recombinational events between (+) and (-) sites as well as by double recombinational events. Since about 0.85 of spontaneous *rII* mutants are acridine dye revertable (BRENNER, BARNETT, CRICK and ORGEL 1961) and thus most likely (+) or (-), one might expect HNI to result frequently from fortuitous combinations of (+) and (-) sites. However, it does seem somewhat odd, on this model, that all three-factor crosses thus far reported demonstrate HNI while none of the expected cases of like signs [say $r_1 r_3 (+) \times r_2 (+)$] which should not produce HNI have yet to be reported.

A test of the applicability of the internal suppression and marker stimulation theories for HNI can be engineered by performing many sets of two- and three-factor *rII* crosses in which the mutant sites can be identified and systematically altered. It has been found that HNI is not correlated with the nature of the point-mutant site. Thus neither mechanism appears capable in simplest form to account for HNI.

EXPERIMENTAL METHODS

Bacteria: (i) *Escherichia coli* B (BENZER) termed B, was used for assay of r and r^+ phage and as host for crosses; (ii) *E. coli* Bb (BENZER), lysis inhibited for *rII* and r^+ , for growth of high titer *rII* stocks; (iii) *E. coli* K-12 (lambda) (BENZER) termed K, for selective assay of r^+ phage; (iv) *E. coli* K-12 (lambda), streptomycin resistant, termed K/S, derived from (iii), was employed in replica plating procedures for multiple *rII* mutant selection (CHASE and DOERMANN 1958); (v) *E. coli* HS/6, streptomycin resistant, termed H/S, was used in conjunction with (iv) for *rr* selection.

Phage: (i) T4 B r^+ standard type, was obtained from DR. S. BENZER; (ii) T4 B *rII* mutants of spontaneous origin (SF-) or induced by ultraviolet irradiation of extracellular r^+ standard phage (UF-) were from the *rII* set described and partially mapped by FOLSOME (1962a, b).

Media: (i) Broth, used for all dilutions, growth tubes, phage stock preparations, and growth of plating bacteria contained, per liter, 8g Bacto peptone, 1g Bacto yeast extract, and 5g NaCl; (ii) Agar plates and soft agar were prepared according to CHASE and DOERMANN (1958).

Analogues and mutagens: 2-aminopurine, 5-bromodeoxyuridine, and proflavin were obtained from California Foundation for Biochemical Research, Los Angeles.

Incubation: All plates, growth tubes, and plating bacteria were incubated at 37°C.

Preparation of *rr* phage stocks: Replica plating procedures of CHASE and DOERMANN (1958) were followed. Five different *rr* stocks were isolated.

Standard crosses: KCN at final concentration of $2 \times 10^{-3}M$ was added to exponential phase B at 10^8 per ml. After 2.5 min one ml B was pipetted to each adsorption tube containing appropriate input phage and incubated 10 min without aeration. One aliquot of the adsorption tube was chloroformed and assayed on B for unadsorbed phage and a second was diluted 100-fold into warm broth tubes which were aerated 90 min by gently shaking. Growth tubes were chloroformed and assayed on B and on K. In general the criteria set for crosses by CHASE and DOERMANN (1958) were followed: minority/majority input parent ratios ranging from 0.8 to 1.0 and multiplicities of each phage from 4 to 7 were required before further analysis of cross results was undertaken. KCN concentration of $2 \times 10^{-5}M$ in the growth tube appeared to have no effect upon recombination frequencies.

Calculations: (i) Recombination frequencies are expressed as twice the frequency of progeny plating on K. (ii) Efficiency of plating (e.o.p.) corrections for K and B phage titers were found

unnecessary and were not made; e.o.p.'s of K relative to B varied from 0.9 to 1.1. (iii) Interference was calculated as the ratio of observed $r_1 r_3 \times r_2$ recombination frequency to the product of $r_1 \times r_2$ and $r_2 \times r_3$ recombination frequencies. (iv) Corrections for *rr* phage stock single *r* content and for back mutation have been estimated by experiment and in all cases reported contribute less than 0.05 of the progeny phage plating on K. In general these corrections were derived as those of CHASE and DOERMANN (1958) and are not included in these data as their effect is negligible.

Terminology: (i) For simplicity, mutational sites were assigned to an alphabetical series as depicted in Table 1. Where more than one mutant at a site is considered, numerical prefixes identify each mutant. Thus, a cross of $1b1d \times 2c$ would represent a three-factor cross in which two recombinational events must occur to produce a standard-type r^+ phage (one event in region *b-c*, a second in region *c-d*). (ii) All mutants at all sites were tested for reversion induction by (1) low pH (FRESE 1963), (2) base analogues 2-amino-purine and 5-bromodeoxyuridine (FRESE 1959), (3) proflavin (ORGEL and BRENNER 1961). In addition an approximation of the leakiness (partial wild-type function) of each mutant was obtained by measuring the transmission coefficient (BENZER 1957). Table 2 lists origins and provisional mutant properties.

RESULTS

Sequence determination: Table 1 provides a genetic map of the *B* cistron as determined by two-factor crosses. Note that the map regions are not additive, thus corroborating the findings of EDGAR, FEYNMAN, KLEIN, LIELAUSIS, and STEINBERG (1962). Pairwise summation over all regions is 3.88 map units, while the distance between terminal markers *1a* and *1h* is 3.08 units. In all crosses involving

TABLE 1
Genetic map of loci a-h of rII cistron B

a-h map distance by pairwise summation of smallest distances equals 3.88 units; *a-h* map distance by cross of $a \times h$ equals 3.08 units. All map distances are averaged from five or more crosses.

TABLE 2

Identification of mutant type by reversion-induction profile

Mutant	Number and origin	Response to				Spontaneous $\times 10^{-6}$	Transmission coefficients	Type
		pH 4.2	BD	AP	Proflavin			
1a	UF-65	<.01	0	tiny	0	0.7	L	tv
2a	SF-312	<.01	+	0	0	0.3	L	t
3a	SF-698	0.03	+	+	0	1.3	L	t
4a	SF-650	<.01	+	$\pm$	0	0.5	L	t
1b	SF-663	<.01	0	0	+	0.5	N	$\pm$
1c	r-117	<.01	0	0	+	0.5	N	$\pm$
2c	UF-98	0.3	0	0	+	0.7	N	$\pm$
3c	SF-645	0.8	0	0	+	2.3	N	$\pm$
4c	UF-58	0.9	0	0	+	1.3	N	$\pm$
1d	UF-84	<.01	0	0	+	0.8	N	$\pm$
1e	UF-170	<.01	0	+	0	1.5	L	t
1f	UF-33	0.16	0	0	+	2.9	N	$\pm$
2f	UF-133	<.01	0	0	+	1.4	N	$\pm$
1g	UF-1	<.01	tiny	0	0	0.02	L	tv
2g	UF-72	<.01	0	0	+	0.05	N	$\pm$
3g	UF-173	<.01	0	0	+	0.001	N	$\pm$
4g	UF-27	<.01	0	0	0	0.9	L	tv
5g	UF-69	<.01	0	0	+	0.007	N	$\pm$
6g	UF-88	<.01	0	0	0	0.004	L	tv
1h	UF-163	5.3	0	0	0	7.0	L	tv

pH 4.2: reversion-induction indicated when slope of *revertants/time of treatment* is greater than 1 (FREESE 1963). BD, AP, and proflavin reversion-induction: see text.

Transmission coefficients less than 10^{-3} were obtained for nonleaky (N) mutants. Higher transmission coefficients were obtained for leaky (L) mutants.

Type: transition (t), transversion (tv), or single-base addition or deletion ($\pm$).

alternate mutants occupying the same site, recombinants in essentially equal frequencies were observed.

Site differentiation: Table 2 summarizes the reversion profile of each mutant at all sites and includes estimates of leakiness based upon transmission coefficients. Each mutant can be assigned a class based upon its reversion-induction profile. Analogue revertable mutants neither proflavin nor low pH revertable which show elevated transmission coefficients will be termed *transition* (t) mutants (FREESE 1963). A second distinct class is that in which members are proflavin revertable, but nonleaky and not analogue or low pH revertable: these will be termed *addition* (+) or *deletion* (−) mutants (as by CRICK *et al.*, 1961). A third class which is neither analogue nor proflavin revertable but which may be low pH revertable and may be leaky will be termed *transversion* (tv) mutants (as by FREESE 1963). Only mutants which had finite spontaneous reversion indices were employed for these studies.

Biparental three-factor crosses: Two-factor map distances presented in Table 1

TABLE 3

Three-factor crosses of type $r_1r_3 \times r_2$

Cross	Mutational type	r_1-2	r_2-3	r_1-3	O	C	O/C	Predictions	
								Internal suppression	Marker stimulation
$2a1e \times 1d$	$t \ t \times \pm$	1.63	0.14	2.00	60.2	2.28	26.4	0	L
$\times 1b$	$t \ t \times \pm$	0.71	1.93	2.00	91.8	18.39	5.0	0	L
$\times 1c$	$t \ t \times \pm$	1.33	0.87	2.00	77.7	11.57	6.7	0	L
$\times 2c$	$t \ t \times \pm$	1.33	0.87	2.00	69.9	11.57	6.1	0	L
$\times 3c$	$t \ t \times \pm$	1.33	0.87	2.00	82.5	11.57	7.1	0	L
$\times 4c$	$t \ t \times \pm$	1.33	0.87	2.00	87.4	11.57	7.5	0	L
$1a1f \times 1e$	$tv \pm \times t$	2.00	0.22	2.22	98.0	4.40	22.2	0	I
$\times 1d$	$tv \pm \times \pm$	1.63	0.54	2.22	219.0	8.80	24.9	+ or 0	H
$\times 1b$	$tv \pm \times \pm$	0.71	2.81	2.22	188.0	17.54	10.7	+ or 0	H
$\times 1c$	$tv \pm \times \pm$	1.33	1.09	2.22	70.5	17.16	4.1	+ or 0	H
$\times 2c$	$tv \pm \times \pm$	1.33	1.09	2.22	57.2	17.16	3.3	+ or 0	H
$\times 3c$	$tv \pm \times \pm$	1.33	1.09	2.22	68.9	17.16	4.0	+ or 0	H
$\times 4c$	$tv \pm \times \pm$	1.33	1.09	2.22	63.1	17.16	3.7	+ or 0	H
$1c1e \times 1d$	$\pm \ t \times t$	0.93	0.14	0.87	57.5	1.30	44.2	0	L
$1e1g \times 1f$	$t \ tv \times \pm$	0.22	0.39	0.61	17.5	0.86	20.3	0	I
$\times 2f$	$t \ tv \times \pm$	0.22	0.39	0.61	9.7	0.86	11.2	0	I
$1f1h \times 1g$	$\pm \ tv \times tv$	0.39	0.37	0.85	19.1	1.44	13.2	0	I
$\times 2g$	$\pm \ tv \times \pm$	0.39	0.37	0.85	26.9	1.44	18.7	+ or 0	H
$\times 3g$	$\pm \ tv \times \pm$	0.39	0.37	0.85	22.5	1.44	15.6	+ or 0	H
$\times 4g$	$\pm \ tv \times tv$	0.39	0.37	0.85	30.9	1.44	21.4	0	I
$\times 5g$	$\pm \ tv \times \pm$	0.39	0.37	0.85	17.7	1.44	12.3	+ or 0	H
$\times 6g$	$\pm \ tv \times tv$	0.39	0.37	0.85	32.3	1.44	22.4	0	I

r_1-2 , r_2-3 , r_1-3 : two-factor recombination frequencies $\times 10^{-2}$, averaged over a minimum of six crosses. O: observed frequency of recombinants from three-factor crosses $\times 10^{-5}$; C: calculated frequency of recombinants $\times 10^{-5}$ ($r_1-2 \times r_2-3$); O/C: interference value.

Predictions: *Internal suppression* not expected to generate HNI (0), could generate HNI if signs dissimilar (+ or 0). *Marker stimulation*, high (H), intermediate (I), or low (L) effect on HNI dependent on the contribution expected from all three marker-types used in the cross.

were used to calculate expected frequencies of double recombinants for the appropriate three-factor crosses. Where more than one mutant was available for each site, all mutants of that site were crossed. No significant differences in recombination frequencies for different mutants of the same site were observed for two-factor crosses. Table 3 presents results of three-factor crosses of the type $r_1r_3 \times r_2$ with predictions for HNI as based upon the internal suppression model and the marker stimulation model.

According to the internal suppression model, crosses of the rr type $t-t$ or $t-tv$ to either t , tv , or $\pm$ single-site mutants are not expected to yield HNI. Crosses of this type do demonstrate HNI with an average interference value of 16.5, ranging from 44.2 to 5.0 for 13 sets of crosses. Hence these results are inconsistent with this model.

However, internal suppression *could* occur between the r_2 and the r_1 or r_3 loci if the appropriate mutants are of opposite sign. Nine crosses of the type $t \pm$

(or $tv \pm$) $\times \pm$ are presented in Table 3. The average interference value is 10.8, ranging from 3.3 to 24.9. These results are also inconsistent with the internal suppression model.

Marker stimulation of crossing over as a cause of HNI requires that the physical presence of additional loci, probably by a disturbance of base pairing between heterologous strands, itself induces further switches in the immediate area. The simplest test of this model is to examine crosses of the type $r_1 r_3 \times r_2$ where r_2 parents are either of $\pm$ type, or tv type, or t type. One might expect that the presence of $\pm r_2$ loci would disturb pairing considerably more than tv or t type r_2 loci. Furthermore, tv loci might be expected to disturb pairing more than t loci and less than $\pm$ loci. Consistent with these expectations, predictions for the effect of marker stimulation are presented in Table 3 in which H, I, and L correspond respectively to higher, intermediate, and lower degrees of HNI. It can be seen from the table that these expectations do not correspond to observed patterns of interference. Seventeen interference values ranging from 3.3 to 26.4 (average 11.0) were predicted to be *high*. Two interference values predicted *low* are 22.2 and 44.2. Three values predicted *intermediate* are 13.2, 21.4, and 22.4. Note also that within any group of $r_1 r_3 \times r_2$ crosses in which one common $r_1 r_3$ parent is crossed to r_2 parents of varying mutational type that no consistent pattern emerges. Thus, marker stimulation does not appear to be sufficient to explain HNI.

"Test crosses" of r^+ progeny: To explain HNI by internal suppression one would expect from the $1f1h \times 3g$ cross of Table 3, for example, that the ratios of internally suppressed to r^+ progeny would be $15.6 - 1.6$ to 1.6 (observed interference minus interference without HNI to interference expected without HNI [CHASE and DOERMANN 1958]). Thus, of every nine progeny r^+ sampled, one might be standard r^+ and the remaining eight might be $(+ -)$ mutants with r^+ phenotype but internally suppressed $(+ -)$ genotype.

r^+ plaques growing on K from $r_1 r_3 \times r_2$ crosses involving sites f , g , and h were picked and stocks which did not contain rII phage (due to recombinant heterozygotes [EDGAR 1958]) were grown on Bb. Progeny r^+ phage were test-crossed to T4 r^+ standard type. Twenty plates containing 500 phage per plate were prepared for each progeny r^+ cross and the frequency of r types was determined. The results are presented in Table 4.

TABLE 4

Testcrosses of r^+ recombinants from three-factor rII crosses by T4 r^+ standard type

Source of r^+ recombinant progeny	Frequency* of r mutants in testcross No.					
	1	2	3	4	5	6
$1f1h \times 1g (\pm tv \times tv)$	0	3	40	10	0	1
$1f1h \times 3g (\pm tv \times \pm)$	0	6	5	0	6	43
$1g \times 1h (tv \times tv)$	2	20	18	15	0	1
$1f \times 1g (\pm \times tv)$	13	8	5	6	1	0
standard r^+ control	4	8	0	0	0	0

* r phage observed per 10⁴ plaques scanned (20 plates of 500).

Since there is no difference in progeny r phage frequencies for $\pm\text{-tv} \times \text{tv}$, or $\times \pm$ crosses, and since the increase above r frequencies observed from r^+ standard phage treated identically is not significant, it is concluded that r^+ progeny phage derived from $r_1r_3 \times r_2$ crosses are identical in genotype to standard r^+ phage.

DISCUSSION

These data clearly show internal suppression to be an insufficient mechanism to account for all instances of HNI. In fact, of 22 sets of three-factor crosses nine instances of mutant type arrangements were conceived in which internal suppression *could* have occurred, but no evidence exists to show that it did. Furthermore, 13 sets of three-factor crosses arranged by mutant-type selection so that internal suppression could not occur demonstrated HNI in the same pattern and of the same magnitude. I conclude that HNI in biparental three-factor crosses is not necessarily related to the phenomenon of internal suppression. However, it is possible to conceive of cases in which internal suppression could act in conjunction with events leading to HNI. In this eventuality higher than normally observed interference values would be expected.

The more general theory of marker stimulation of recombination advanced by HERSHEY (1958) is not definitely ruled out by the experiments reported here. If one assumes a progression of pairing difficulty ranging in order from $\pm$, tv , and t mutants and if one assumes that the greater the pairing heterogeneity the more marked the stimulation of recombination frequencies, then this form of the marker stimulation theory has been ruled out by the data presented.

Other more recent experiments clearly demonstrate that HNI is a variable dependent upon heterozygote frequency (FOLSOME and MONK 1965). When corrections are computed for the role of heterozygotes, HNI does not appear to exist.

The outstanding technical assistance of Miss M. LINCOLNE and Dr. M. MONK is gratefully acknowledged.

SUMMARY

An array of eight rII sites of cistron B of phage T4 have been mapped by two-factor crosses. Twenty-one mutants occupying these eight sites have been individualized by their reversion-induction profile and assigned to single base-pair addition or deletion, transition or transversion classes. Biparental three-factor crosses involving all mutants have been performed. High negative interference was observed regardless of the mutational type of each mutant. It is concluded that the general theory of marker stimulation of crossing over and its special case of formation of "recombinants" by internal suppression is insufficient as a mechanism to account for high negative interference.

LITERATURE CITED

- BENZER, S., 1957 The elementary units of heredity. pp. 70-93. *The Chemical Basis of Heredity*. Edited by W. D. McELROY and B. GLASS. Johns Hopkins University Press, Baltimore. —

- 1961 On the topography of the genetic fine structure. *Proc. Natl. Acad. Sci. U. S.* **47**: 403-415.
- BOETTCHER, B., 1963 A suggested explanation of some cases of high negative interference and certain mapping anomalies in phage T4B. *J. Theoret. Biol.* **5**: 108-113.
- BRENNER, S., L. BARNETT, F. H. C. CRICK, and A. ORGEL, 1961 The theory of mutagenesis. *J. Mol. Biol.* **3**: 121-124.
- CHASE, M., and A. H. DOERMANN, 1958 High negative interference over short segments of the genetic structure of bacteriophage T4. *Genetics* **43**: 332-353.
- CRICK, F. H. C., L. BARNETT, S. BRENNER, and R. J. WATTS-TOBIN, 1961 General nature of the genetic code for proteins. *Nature* **192**: 1227.
- EDGAR, R. S., 1958 Phenotype properties of heterozygotes in the bacteriophage T4D. *Genetics* **43**: 235-248.
- EDGAR, R. S., R. P. FEYNMAN, S. KLEIN, I. LIELAUSIS, and C. M. STEINBERG, 1962 Mapping experiments with *r* mutants of bacteriophage T4D. *Genetics* **47**: 179-186.
- FOLSOME, C. E., 1962a Specificity of induction of T4 rII mutants by ultraviolet irradiation of extracellular phages. *Genetics* **47**: 611-622. — 1962b Topographical effects of ultraviolet irradiation upon recombination in phage T4. *Z. Vererb.* **93**: 404-416.
- FOLSOME, C. E., and M. MONK, 1965 Genetic interference: the role of heterozygosity in phage T4. (Manuscript in preparation.)
- FREESE, E., 1959 The specific mutagenic effect of base analogues in phage T4. *J. Mol. Biol.* **1**: 87-105. — 1963 Molecular mechanism of mutations. pp. 207-269. Academic Press, New York. *Molecular Genetics*, Part I. Edited by J. H. TAYLOR.
- HALDANE, J. B. S., 1919 The combination of linkage values, and the calculation of distances between the loci of linked factors. *J. Genet.* **8**: 299-309.
- HERSHEY, A. D., 1958 The production of recombinants in phage crosses. Cold Spring Harbor Symp. Quant. Biol. **23**: 19-46.
- JINKS, J. L., 1961 Internal suppressors of the *hII* and *tu* 45 mutants of bacteriophage T4. *Heredity* **16**: 241-254.
- ORGEL, A., and S. BRENNER, 1961 Mutagenesis of bacteriophage T4 by acridines. *J. Mol. Biol.* **3**: 762-768.