ALLELIC AND NONALLELIC GENES CONTROLLING HOST SPECIFICITY IN A BACTERIOPHAGE *

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BACTERIA infected with any two independently arising *r* (plaque type) mutants of the bacterial virus T2H have been found, as a rule, to yield wild-type and double-mutant individuals among the viral progeny (HERSHEY and ROTMAN 1948). This result has been interpreted in terms of recombination among nonallelic genetic factors, and it is permissible to speak of the test in which bacteria are infected with two different viral mutants as a genetic cross.

Preliminary crosses (HERSHEY and ROTMAN 1948, and unpublished) between pairs of independently arising host-range mutants of the same virus, however, have failed to yield recombinants, even when the two mutants were clearly different. LURIA (personal communication) has had the same experience with host-range mutants of the related phage T2L.

If the results with the two kinds of mutant are to be given a consistent interpretation, it has to be assumed that different host-range mutations, unlike the *r* mutations, tend to occur at a single genetic locus. This assumption is not implausible, inasmuch as the host-range character can be readily combined with the *r* character in the appropriate genetic crosses (HERSHEY and ROTMAN 1949).

Nevertheless, we felt that the genetic interpretation could be greatly strengthened by establishing one authentic example of multiple allelism in the virus. This we have done, by three independent means. The demonstration is all the more convincing because one mutant was found that proved to be carrying a mutation in a second locus controlling host specificity.

METHODS

The technique of crossing viral mutants has been modified slightly since last described (HERSHEY and ROTMAN 1949). The bacteria are now grown in nutrient broth, suspended in buffered saline, admixed with virus, and diluted in broth after allowing five minutes for adsorption to occur. Adsorption in the absence of nutrient is physiologically equivalent to simultaneous adsorption of the added virus particles (BENZER *et al.* 1950), and the effects we observe are understandable on the assumption that this causes a more

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nearly equal intracellular growth of the two parental types of virus in individual bacteria. The yields of recombinants obtained by this method are about 1.3 times larger than those previously reported. The linkage relations remain unchanged.

Serial passage of viral stocks for the analysis of patterns of back-mutation is carried out by growing bacteria in 10 ml of nutrient broth to a titer of about 10^7 per ml, and seeding the culture with about 0.03 ml of the lysate of the preceding day. The cultures are incubated overnight at 37°C, sampled for plating, and then heated for 10 minutes at 60°C before transplantation. Since the rate of population change varies with the conditions of propagation, only parallel series are used for comparing the behavior of different viral lines.

All the viral mutants described below are derived from a line called T2H, otherwise referred to as the wild type. The term " sensitive bacteria " means *Escherichia coli* strain S (for seeding agar plates) or strain H (for all other purposes). " Indicator bacteria " means strain No. 2 B/2H,2K, which is resistant to wild-type T2H. " Mixed indicator " means a mixture of sensitive and indicator bacteria.

Plaques arising from stocks of wild type on single indicator plates contain host-range (*h*) mutants only (LURIA 1945). Pure lines of *h* mutants are isolated by sampling from these plaques with an inoculating needle, replating the diluted sample on sensitive bacteria, and resampling from one of the secondary plaques.

Different *h* mutants may be classified in terms of the ratio of the plaque count on indicator plates to the plaque count on plates seeded with sensitive bacteria. This ratio, called the efficiency of plating (e.o.p.), is a heritable characteristic of the virus (HERSHEY 1946) and varies between 0.01 or less and unity for different mutants.

Plaques arising from mutant stocks on single indicator plates, and plaques arising from either wild or mutant stocks on mixed indicator plates, contain principally phage of the parental type, but sometimes also an appreciable proportion of mutants that originated within the plaque. The e.o.p. of a clone sampled from indicator plates is therefore measured only after replating on sensitive bacteria and sampling from one of the secondary plaques. This procedure gives consistent measures of the e.o.p. of different clones of the same line sampled from indicator plates.

DESCRIPTION OF MUTANTS

Mutants with e.o.p. measuring 0.3 or less form turbid plaques (phenotype h^t) on indicator plates. Mutants with e.o.p. near unity form clear plaques (phenotype h^c) on indicator plates. Mutants with intermediate e.o.p. form plaques of intermediate type. Also on mixed indicator plates, the plaques are more turbid the lower the e.o.p., with the difference that the number of plaques is the same as it is on sensitive bacteria. A differential count of mixtures can therefore be made on mixed indicator plates if the component viral types have sufficiently dissimilar e.o.p. On sensitive bacteria, all the mutants form plaques indistinguishable from those of the wild type.

An additional point of difference among the *h* mutants is revealed by heating stocks diluted in phosphate buffer of pH 7.0 for 10 minutes at 64°C, and measuring the proportion surviving. (Identical results are obtained when undiluted broth stocks are heated.) This test shows that the *h* mutants are less resistant to heat than the wild type, and that the h^c mutants are less resistant than the h^t. Five independently arising mutants of the phenotype h^c proved to be equally heat-sensitive. Several *r* mutants, however, were as resistant as the wild type.

When large amounts of wild-type stocks are plated out on indicator bacteria, the numbers of plaques of h^t and h^c mutants are about equal, in the proportion of about 10^{-6} to the titer of the stock. The proportion of h^c mutants in the stock is therefore about 10^{-6}, and the proportion of h^t mutants is at most 10^{-4}. The proportion of h^c mutants in initially pure clones of h^t is less than 10^{-5}. These circumstances make it unlikely that an h^c mutant isolated from a stock of wild type arose by a second mutation from the h^t virus present, whereas an h^c mutant coming from a stock of h^t clearly did so. Both classes of mutant are used in the following experiments. The origins and properties of several lines are summarized in table 1.

TABLE 1

Origin and properties of several h mutants.

Line	Origin	e.o.p.	Percent survival at 64°C, 10 min.
$h^c1 \ldots h^c5$	Wild type	1.0	1.4–5.3
h^c6	h^t1	1.0	1.1–2.0
h^c7	h^t5	1.0	3.0–5.7
h^t1	Wild type	0.1	13
h^t2	Wild type	0.3	Not tested
h^t5	Wild type	0.01	24–30
$h^t1\ h^t5$	cross	0.5	9.5–25
Wild type	....	$< 10^{-5}$	40–48

$h^c1 \ldots h^c5$ are five independently arising mutants of identical phenotype.

The column headed e.o.p. gives the ratio of the titer on indicator bacteria to the titer on sensitive bacteria.

The data for survival at 64° for 10 minutes show the range of results obtained with two or more different stocks of each line, including stocks propagated in glucose-ammonia medium as well as stocks propagated in nutrient broth.

ADSORPTION OF *h* MUTANTS

LURIA (1945) showed that a host range mutant of T2 was adsorbed at measurable rate to its indicator strain of bacteria, whereas the wild type was not, and suggested that this is the primary difference between the two viruses. He also found that the rate of adsorption of the mutant to indicator bacteria was considerably slower than to sensitive bacteria, and wished to explain the low and variable efficiency of plating of the mutant on this basis.

We have measured the adsorption of several *h* mutants to bacteria with the expectation that the rates would parallel the e.o.p., and that either could be taken as an expression of different degrees of adaptation of virus to host.

In a general way this expectation proves correct, but the effects of mutation do not appear to be entirely explainable on this simple basis.

For the measurement of slow rates of adsorption, we have used the following method. Phage and bacteria are mixed, held for a measured time at 37°C, and the unadsorbed phage is inactivated by the addition of antiphage immune serum. The suspension of cells is then titrated before the end of the latent period of phage growth to determine the number of cells yielding phage. Provided the proportion of cells infected is kept small, their number gives an estimate of the number of phage particles adsorbed. The results given by this method agree with those obtained in the usual way, by titrating unadsorbed phage after centrifugation, when the rates can be measured by both methods: namely, in the adsorption of wild type or *h* mutants to sensitive bacteria, and in the adsorption of h^c to indicator bacteria.

As far as could be determined, a solution containing 0.15 M NaCl and 10^{-4} M $CaCl_2$ supplies all the requirements for adsorption to bacteria of T2H and its host range mutants. The calcium is needed only for the adsorption to indicator bacteria; the adsorption of *h* mutants to sensitive bacteria is practically unaffected by calcium. The rate of adsorption is not affected by added nutrient broth (or L-tryptophan) provided the broth suspension of bacteria is kept at low temperature until the moment of adding phage. Incubation at 37°C of washed bacteria with broth (or bacteriological peptone, or L-tryptophan) for a few minutes produces a powerful soluble inhibitor of adsorption that may be indol, since indol also has an immediate inhibiting effect. The sensitivity to indol is a characteristic of T2H not shared by all its relatives. The *h* mutants are somewhat less sensitive than wild type to the inhibitor derived from peptone when tested by adsorption to sensitive bacteria. They are equally sensitive among themselves when tested by adsorption to either sensitive or indicator bacteria. For this reason, the inhibitor cannot be a primary factor in determining the e.o.p. of the *h* mutants.

Table 2 shows the rates of adsorption of several phages to bacterial suspensions prepared from cultures of various kinds. In each case the bacteria were washed and resuspended in a saline phosphate buffer containing 10^{-3} M

TABLE 2

Rates of adsorption of h mutants to bacteria.

Bacteria	Phage				
	Wild	h^c1	h^c2	h^t1	h^t5
Sensitive					
2 hour broth	3×10^{-9}	....	3×10^{-9}	3×10^{-9}	3×10^{-9}
Indicator					
2 hour broth	$< 2 \times 10^{-14}$	5×10^{-10}	6×10^{-10}	6×10^{-14}	1×10^{-12}
day old broth	$< 2 \times 10^{-14}$	....	5×10^{-10}	7×10^{-13}	1×10^{-12}
synthetic medium	1×10^{-13}	....	1×10^{-9}	6×10^{-11}	1×10^{-11}

The rates given are per bacterium per ml per minute at 37°C.

$MgSO_4$, 10^{-4} M $CaCl_2$, and 10 mg of gelatin per liter, in which the phages are stable over long periods. Most of these rates have been verified several times using various concentrations of bacteria and phage. That the slow rates are not artifacts due to the rapid adsorption of phage to a small fraction of sensitive bacteria in the population is shown both by the measurements with wild type, and by the fact that the number of bacteria infected is proportional to the amount of phage added over the ten-fold range subject to test. In particular, measurements of bacterial survival show that at least 80 percent or so of the cells in a culture grown in a glucose-ammonia medium adsorb the mutants h^t1 and h^t5 at the rates shown in the table.

The main features of the data in table 2 are the following:

All the *h* mutants, as well as the wild type, are adsorbed at equal rates to sensitive bacteria.

All the *h* mutants, but not the wild type, are adsorbed at measurable rates to indicator bacteria, and the mutants h^c are adsorbed much faster than the mutants h^t.

The mutant h^t5 is adsorbed to indicator bacteria from broth cultures at a faster rate than the mutant h^t1. This is contrary to the expectation based on measurements of e.o.p.

The relative rates of adsorption of the different *h* mutants to indicator bacteria varies with the age of the culture from which the bacterial suspension is prepared. The rate increases with age for the mutant h^t1, and remains constant for the mutants h^c2 and h^t5. Both types of behavior are contrary to general experience; in fact, the rate of adsorption of these same mutants to sensitive bacteria from old cultures is considerably less than to sensitive bacteria from young cultures.

The adsorption of all the mutants, and of wild type as well, is very much faster to indicator bacteria grown in a synthetic medium than to indicator bacteria grown in broth. The rates in this instance parallel the efficiencies of plating, which are, on synthetic medium agar: 0.07 for wild type, 0.6 for h^t5, and 1.0 for h^t1 and h^c2.

The distinction between indicator and sensitive bacteria is evidently a relative one in the case of our indicator strain growing in synthetic medium, and the question arises whether the broth cultures and the synthetic medium cultures have the same genetic composition. We do not believe this question can be answered except to say that both kinds of population appear to be homogeneous, and to transform in either direction during a single culture cycle whether started from a massive inoculum or from a single colony. In any case, our data show that the relative susceptibility to different viral mutants is subject to non-heritable as well as heritable modifications in the bacteria.

The very low e.o.p. of the mutant h^t5 on nutrient agar containing peptone is left unexplained except to say that it must depend on conditions in the agar medium which we are unable to reproduce in adsorption measurements. It is perhaps significant that this mutant alone gives a higher plaque count when plated with sensitive bacteria on synthetic medium agar than when plated on agar containing peptone.

Rates of adsorption evidently provide a quantitative measure of mutational effects that is considerably more sensitive than the measurement of e.o.p. The class h^c, for instance, should include all the mutants with rates of adsorption in excess of a certain value, and there is no reason to anticipate that any two of them would be identical. The failure to distinguish h^c1 from h^c2 (table 2) by this method thus adds materially to the evidence that these arose by independent occurrences of the same mutation.

THE PATTERN OF BACK-MUTATION

If the mutant h^c6, arising from h^t1 by a second mutational step, carried alterations at two independently mutating loci, it should be incapable of reverting to the wild genotype in a single step. During serial passage accompanied by loss of the h character, one would expect, among other possibilities, to observe reversion to the phenotype h^t1. If, on the other hand, h^c6 arose by a second mutation in a single mutational locus, so that the second mutation erased the first, reversion to wild type in a single step would be possible, and there should be no preferential selection of the phenotype h^t1 on serial passage. Table 3 shows the patterns of reversion observed when different single-plaque stocks of h^c6 are subjected to serial passage. The behavior of the single-step mutants h^c2 and h^t1 is shown for comparison. The table shows that h^c6 can return to wild type (or to a phenotype resembling wild) in a single mutative step and, more important, that the pattern of reversion of h^c6 is identical to that of the single-step mutant h^c2. We conclude that the muta-

TABLE 3

Serial passage of the mutants h^t1, h^c2, and h^c6.

Series	Percent of turbid plaques on mixed indicator, and analysis of population, after passage number				
	2	4	6	8	10
h^t1 (1)	0	0	1	5(a)	60
h^t1 (2)	0	0	0	5(a)	50
h^t1 (3)	0	0	1	20(a)	50
h^c2 (1)	1	50(b)			
h^c2 (2)	0	50(b)			
h^c2 (3)	0	70(c)			
h^c6 (1)	0	70(c)			
h^c6 (2)	0	50(b)			
h^c6 (3)	0	50(b)			

Each series was started from a different plaque of the specified type. After the indicated number of serial passages, pure lines of phage were isolated from turbid plaques on mixed indicator, and also from turbid plaques, if any, on single indicator, and identified by measuring the e.o.p. The following types of result were obtained in the above and similar experiments:

(a) Mixture of h^t1 and wild type.

(b) Mixture of h^c, h^t, and wild type. The proportion of h^t was usually less than the proportion of wild type, and never exceeded the proportion oi h^c before wild type arose. In several instances it was established that the e.o.p. of the isolated h^t (0.2–0.3) was significantly different from that of h^t1.

(c) Mixture of h^c and wild type only. This result was observed in two out of six series starting from h^c2, and three out of seven series starting from h^c6.

tion giving rise to h^c6 has obliterated the previous mutation carried by h^t1, and therefore that these are alternative modifications of a single mutational locus.

In view of the rather complicated pattern of reversion just described, it was decided to repeat the experiment with a two-step mutant derived from a different h^t stock. For this purpose the mutant h^t5, and h^c7 derived from it, were isolated. It was found, quite unexpectedly, that h^c7 reverted during serial passage to h^t5, long before any wild type appeared in the stocks. This behavior points clearly to the conclusion that the two mutations have both left their marks in the stock h^c7, and therefore that at least one of them has occurred in a mutational locus distinct from the site of the mutation h^t1.

It is of some interest that these experiments were done before any crosses had been made with the stocks h^t5 and h^c7, so that the existence of a second locus governing host specificity was discovered by the means just described.

It has happened before (HERSHEY 1946) that the analysis of patterns of back mutation has furnished information about the genetic structure of phage that was later confirmed by other methods. In view of these successes, it is probably unnecessary to enumerate here the several situations in which mutational analysis might suggest erroneous conclusions.

INTERCROSSES AMONG *h* MUTANTS

The results of the following crosses are expressed qualitatively, owing to difficulties in the quantitative recognition of the several viral types. When a recombinant was found, it was always present in the proportion of several percent. The absence of a given recombinant was established by sampling a number of the plaques that resembled it, and identifying the phage in the samples by measuring the e.o.p. Recombinants not found would have been detected by this method if present in a proportion exceeding 0.1 percent.

As previously mentioned, the five indistinguishable mutants of phenotype h^c failed to yield wild type in intercrosses, and the same was true of the crosses $h^c2 \times h^t1$ and $h^c2 \times h^t2$. (The mutants h^t3 and h^t4 could not be distinguished, in terms of e.o.p., from h^t2, and were not used in these experiments.) Evidently all these mutants came from mutations at the same combinative locus, which we shall call locus 1. Our further interest centers on the relation of the mutants h^c6, h^c7, and h^t5 to this locus.

According to the patterns of reversion previously described, the two-step mutant h^c6 is a single-locus mutant identical, so far as can be determined, with the single-step mutant h^c2. The results of the following crosses support this conclusion.

$$h^c6 \times \text{wild type} \longrightarrow \text{no } h^t$$
$$h^c6 \times h^c2 \longrightarrow \text{no wild type}$$

By contrast, the two-step mutant h^c7 behaves like a double mutant, in confirmation of the conclusions reached by analysis of pattern of back-mutation.

$$h^c7 \times \text{wild type} \longrightarrow h^t5 \text{ (e.o.p. 0.01)}$$
$$h^c7 \times h^c2 \longrightarrow \text{no } h^t \text{ or wild type}$$

The last cross shows that the h^c mutation in h^c7 is allelic with h^c2. The following crosses show that h^t5 is not allelic with h^c2 or with h^t1.

$h^t5 \times h^c2 \longrightarrow$ wild type
$h^t5 \times h^t1 \longrightarrow$ wild type and $h^t1\ h^t5$ (e.o.p. 0.5)
$h^t1\ h^t5 \times$ wild type $\longrightarrow h^t1$ (e.o.p. 0.1) and h^t5 (e.o.p. 0.01)

The second (h^c) mutation in h^c7 lies, therefore, in locus 1, and the mutation h^t5 identifies a second combinative and mutative locus.

LINKAGE TESTS

If the conclusions reached above are correct, any phenotype resulting from a mutation in locus 1 ought to recombine with the same frequency as h^c2 in crosses with selected markers, whereas the mutant h^t5 should behave differently. The markers chosen for this test were *r*1, *r*7, *r*13, and *m* (HERSHEY and ROTMAN 1949). The results are shown in table 4. It may be seen that locus 1 of the mutations h^c2, h^c6, and h^t1 is closely linked to *r*13, whereas the locus of the mutation h^t5 is not. The position of this locus is otherwise uncertain, and the methods employed here are unsuited to determine it.

TABLE 4

Linkage relations among h mutants.

	h^c2	h^c6	h^t1	h^t5
*r*1	16–20	15	17	$\sim$ 13
*r*7	8–10	9	9	$\sim$ 14
*r*13	1–2	1	2	$\lesssim$ 10
m	18–20	20	18	$\sim$ 10

The numbers indicate percentage yields of wild type in the respective crosses. Yields of recombinants in crosses involving h^t are approximate, because of the difficulty of recognizing the plaques on mixed indicator. The minimal yield of wild type in $h^t5 \times r13$ was established by sampling and confirming 26 typical plaques from two plates showing a total of 231 plaques.

DISCUSSION

It has been shown that a number of host-range mutations of phage T2H can be assigned to one or the other of two loci, identifiable by any one of three independent tests. A number of alternative mutations can occur at one of these loci. The unit character of this locus is established by the failure of successive mutations to accumulate in it, by the inability of its alternative mutations to combine in intercrosses, and by its unique and invariant map position in several mutational states. The second locus, at which only one mutation has been found, is distinguishable from the first by the same three tests. The mutual consistency of these tests gives strong support to the notion of localized hereditary determinants in the phage, and shows further that at least two of the mutations that can occur in a single combinative structure occur at a single mutational site within that structure.

At one of the loci controlling host specificity, a number of mutations can

occur leading sometimes to different visible effects, and sometimes to what appear to be recurrences of the same mutation. This is in contrast to mutations affecting the *r* character, which tend to occur each time at a different locus, as if the number of loci were very great, and their rates of mutation very similar. Neither situation, of course, is without precedent in other organisms.

SUMMARY

Host range mutants of the bacteriophage T2H differ among themselves, and from the wild type, with respect to rate of adsorption to their selective host. These changes occur without affecting the rate of adsorption to the common host. Accompanying these changes, and characteristic of them, are changes resulting in decreased stability of the virus at high temperatures.

Several of the host-range mutants (class 1) fail to give rise to wild type when intercrossed. One host-range mutant (h^t5) yields wild type when crossed with examples of class 1.

A mutant arising in two successive steps, both of class 1, is able to lose its mutant character in a single mutative step. A mutant arising in two mutative steps, the first of which is h^t5, the second of class 1, can lose its mutant character only by two mutative steps, the first of which can yield h^t5 again.

The mutational locus in three mutants of class 1 occupies a single map position with respect to a set of four selected markers. The mutational locus in h^t5 occupies a different position.

We conclude that the inheritance of host specificity in T2H is controlled by genes occupying at least two different loci, and that at least one of these genes is capable of alternative mutations.

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