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Chronology of Viral Functions in Bacteriophage α

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Temperature-sensitive mutants of phage α were subjected to short pulses of permissive temperature at various times during the lytic cycle. All the mutants showed an optimal response to the permissive pulse at a specific time after infection. The optimal responses of the mutants belonging to the same complementation group fell close together in the same time interval; the optimal responses of mutants contained in 20 different complementation groups were more or less uniformly scattered throughout the lytic cycle. Temperature sensitivity, therefore, seems to afford, at least in the case of phage α, an independent way of grouping the genes in an ordered sequence with respect to the steps they control.

The successful synthesis, within the infected host cell, of each required macromolecular product at the proper time seems to be the necessary condition for the completion of the assembly process, which ultimately leads to the production of complete and active viral particles.

Until now, only a gross distinction of the viral functions in two classes, early and late, has been proposed (6, 9, 10; H. Echols et al., *personal communication*). Furthermore, the distinction between the two classes is only an operative one (2, 4, 8; C. Levinthal, H. Hosoda, and D. Shub, *personal communication*), in that it stems from a distinction between the group of events taking place after infection of the host cell but before the viral deoxyribonucleic acid (DNA) starts its replication and the group of events subsequent to this stage; it does not imply an understanding of the nature of the change from one series of events to the other. If one wants to inquire more deeply into the nature of such mechanisms, one should, in the first place, try to reach a better understanding of the "fine structure" of the time sequence during which the final products appear and start to operate.

A large number of temperature-sensitive mutants of bacteriophage α were isolated and classified by complementation tests (T. Eremenko-Volpe and F. Graziosi, *in preparation*); we then proceeded to study their response to short pulses of permissive temperature at different times of their life cycle. The idea was that in so doing one might detect a specific time, for each mutant, at which the pulse shows a detectable effect. A similar procedure was followed by Ephrussi and Herold (5), who observed mutants in *Drosophila* showing phenotypic curing produced by temperature pulses given at the proper time during pupal development. More recently, another technique has been used by Edlin (3) with some T4 amber mutants: 5-fluorouracil (5-FU) pulses, each lasting a few minutes, given at different times during the lytic cycle, succeeded in efficient curing only when supplied at proper times; inferences on the time of transcription could thus be drawn.

Phage α is simpler than T4, the molecular weight of α DNA being 3×10^7 (1). The lytic cycle lasts longer (about 50 min at 35 C), and moreover it presents the special property of having all the transcribed codons on the same DNA strand, suggesting a simpler organization of the genetic material (11). For these reasons, phage α appears as a suitable organism for an attempt to establish the grouping of its genes in an ordered sequence on the basis of their temperature sensitivity.

MATERIALS AND METHODS

Phage. All the mutants used were derived from α_{c3}, a clear mutant of the temperate phage α.

Host. Bacillus megaterium (Paris strain) from the collection of the Institute of Microbiology of Rome University was used as the host.

Media. Costantino broth was made by adding to 1 liter of distilled water 5 g of NaCl, 10 g of Costantino peptone, and 10 g of Costantino meat extract; the pH was adjusted to 7.2 (medium C).

Growth medium (medium A). Medium A contained 1 part of Costantino broth, 8 parts of $MgSO_4$ (0.01% solution), and adenosine to a final concentration of 0.5 g/liter.

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TABLE 1. *Mutants used*

Identified complementation groups	Total no. of mutants	*ts* mutants tested
I	2	*ts 180 ts 228*
II	18	*ts 60 ts 202 ts 156*
III	1	*ts 137*
IV	1	*ts 111*
V	2	*ts 102 ts 115*
VI	6	*ts 23 ts 107*
VII	3	*ts 11 ts 142*
VIII	2	*ts 109*
IX	9	*ts 9 ts 110*
X	2	*ts 63*
XI	3	*ts 51 ts 78*
XII	1	*ts 114*
XIII	2	*ts 39 ts 98*
XIV	5	*ts 40 ts 86*
XV	7	*ts 67 ts 84*
XVI	1	*ts 103*
XVII	1	*ts 175*
XVIII	1	*ts 205*
XIX	1	*ts 237*
XX	1	*ts 229*

Resuspension broth (medium B). Medium B consisted of 9 parts of A plus 1 part of $MgSO_4$ (10% solution).

Solid media. Constantino agar of the same composition as Costantino broth plus 15 g/liter of Costantino agar (17.5 ml per petri dish) was used. Soft agar had the same composition as Costantino broth, plus 10 g/liter of Costantino agar.

Choice of mutants. The mutants used were chosen taking care that at least one from each different identified complementation group (CG) would be tested; up to three different mutants belonging to the same CG were assayed in some cases. From some 69 classified temperature-sensitive mutants, we selected 32, as listed in Table 1. Criteria of choice were a low level of leakiness and a low frequency of back-mutation. These two criteria often allowed us to reduce the "background noise" to negligible values.

Choice of temperatures. We chose 35.8 C as nonpermissive temperature, and 25 C as the permissive temperature. We operated at temperatures different from these in only a few experiments, as we shall point out later. Two conflicting requirements oriented our choice of the aforesaid temperatures. One was that of choosing a nonpermissive temperature high enough to get as sharp as possible a distinction between nongrowing lytic centers and the pulse-favored growing ones; on the other hand, the replication of α_{c3} wild type is inhibited at 38 C.

Adsorption technique. Cells were taken from a stationary-phase culture and grown overnight with aeration in medium A at 25 C. The rate of growth was followed by measurement of the optical density (OD) of the culture at 560 mμ; 0.5 hr before the culture reached an OD of 2 (corresponding to approximately to 1.5×10^8 cells/ml), it was put at the nonpermissive temperature. As soon as the required OD was reached, 10 ml of the culture was centrifuged and the cells were resuspended in adsorption medium (medium B) to give a fourfold increase in concentration of the cells; these suspensions were placed in a bath at 35.8 C. Phage was then added, and the concentrations were adjusted to give a multiplicity of about 0.1 adsorbed phage per cell; approximately 10% of the phage was adsorbed during the 30 sec allowed for adsorption.

Temperature shifts. At 30 sec after phage was added, the adsorption process was stopped by the addition of antiserum and by a subsequent 5,000-fold dilution in medium C. All these operations were carried out at the nonpermissive temperature in less than 3 min. At the 3rd min after the phage was added, a 1-ml sample of infected bacteria was shifted to the permissive temperature and left there for 5 min. The sample was then returned to the nonpermissive temperature and left there for the rest of the experiment. This process was repeated every 5th min for 12 successive samples. All the samples were then left at the nonpermissive temperature for a further 40 min and then put at 0 C and plated at various dilutions; as a consequence, each sample remained 98 min at the nonpermissive temperature and 5 min at the permissive temperature.

Controls. A first group of controls were plated with part at 25 C and part at 35.8 C, and the titer of the phage suspension used for adsorption, plating efficiency, and frequency of revertants were assayed.

A second group of controls were plated at 25 C at at 4th, the 24th, and the 64th min; these samples were drawn from the same reservoir, kept at 35.8 C, from which the samples used for the shifts were drawn. These controls gave the number of infected cells, making it possible to determine the average burst size and the approximate time of lysis.

A third group of controls were plated with part at 35.8 C and part at 25 C from a tube kept at the nonpermissive temperature. This was done to determine the background due to leakiness.

RESULTS

Figure 1 shows three cases representative of our results. Mutant *ts 180* was the earliest of all the mutants we tested, since the first sample, shifted at the permissive temperature at the beginning of the experiment, already showed some positive effect of the "pulse," and the optimal response was obtained at the 17th min. However, this mutant was not as early as, for instance, the mutant *am 122* of phage T4, tested by Edlin (3) with a different technique. Mutant *ts 111* gave the optimal response at 22.5 min, and mutant *ts 40* gave a still later response at 32 min.

All the results thus obtained are reported in Table 2 and are expressed in graphic form in Fig. 2. One can see that whenever we tested more than one mutant per CG we found the respective peak times not to differ more than 3 min.

In Fig. 3, a histogram of the number of mutants showing the peak within successive time intervals is given. In this figure, we have included in each

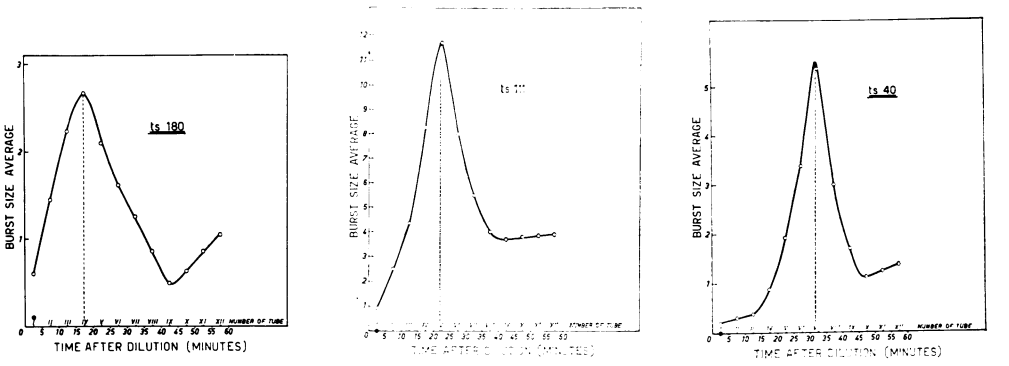

FIG. 1. *Permissive pulse experiments with mutants ts 18,0 ts 111, and ts 40. Abscissa: arabic numbers = time (in minutes) after dilution; roman numbers = progressive numbers of shifted samples;* ● *= value of backgronud. Ordinate: burst size average.*

TABLE 2. *Peak times of the various mutants*

CG	Mutant	Peak time (min)	CG	Mutant	Peak time (min)
I	*ts 180*	17	X	*ts 63*	28.5
I	*ts 228*	19.5	XI	*ts 51*	28.5
VI	*ts 107*	18	XI	*ts 78*	29
VI	*ts 23*	19	XII	*ts 114*	29.5
III	*ts 137*	22	XV	*ts 84*	28.5
IV	*ts 111*	22.5	XV	*ts 67*	31
V	*ts 102*	23.5	XVIII	*ts 205*	30
V	*ts 115*	24	XIII	*ts 39*	29.5
VII	*ts 11*	25	XIII	*ts 98*	31.5
VII	*ts 142*	25.5	XIV	*ts 86*	29.5
VIII	*ts 109*	26	XIV	*ts 40*	32
IX	*ts 110*	25.5	XVI	*ts 103*	33.5
IX	*ts 9*	27	XX	*ts 229*	36
II	*ts 202*	27	XIX	*ts 237*	45–50
II	*ts 60*	27.5			
II	*ts 156*	29.5	VI	*ts 44*	1st mutation 19
XVII	*ts 175*	28	II	*ts 44*	2nd mutation 30

time interval all the available *ts* mutants on the basis of the observation made above, that different mutants of the same CG give a positive response at the same time. As one can see, the overall distribution shows a crowding at the late times. It should be noted here that no special procedure was adopted to isolate early mutants.

The results obtained with mutant *ts 44*, which, on the basis of genetic evidence, is thought to be a double *ts* mutant in CG II and CG VI, confirm the validity of our experimental procedure. In this case, the normal pulse technique would not be expected to yield any result. The sample getting the pulse of permissive temperature at the right time, to pass over the first mutational defect, would be blocked by the second, and vice versa; in fact, in the pulse experiment no peak was observed with *ts 44*. On the other hand, by shifting the samples to a permissive temperature, one after the other at 5-min intervals, and then leaving each sample at the permissive temperature throughout the rest of the experiment, one should be able to detect the time at which the product of the gene containing the first mutated site enters into action. In a similar way, by doing the reciprocal experiment, carrying out infection and the initial operations at the permissive temperature and shifting the samples to the nonpermissive temperature one after the other at 5-min intervals, one should be able to detect the time when the product of the gene containing the second mutated site goes into action.

Such experiments were carried out and gave the foreseen results (Fig. 4). In this way, we could ascertain that *ts 44* behaved as expected for a double mutant, the two mutations being located

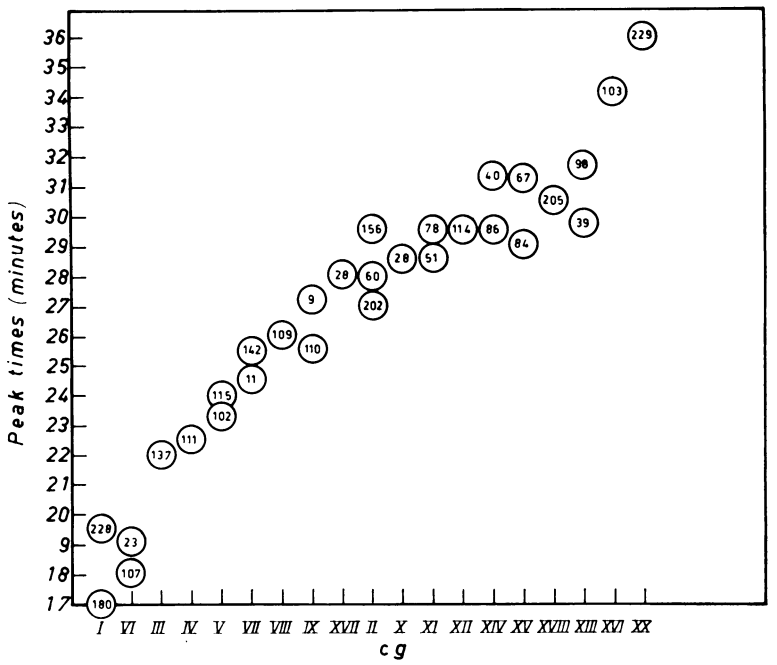

FIG. 2. *Sequence of peak times during the lytic cycle. Abscissa: Complementation groups in chronological order (see Table 1). Ordinate: Time of the peaks.*

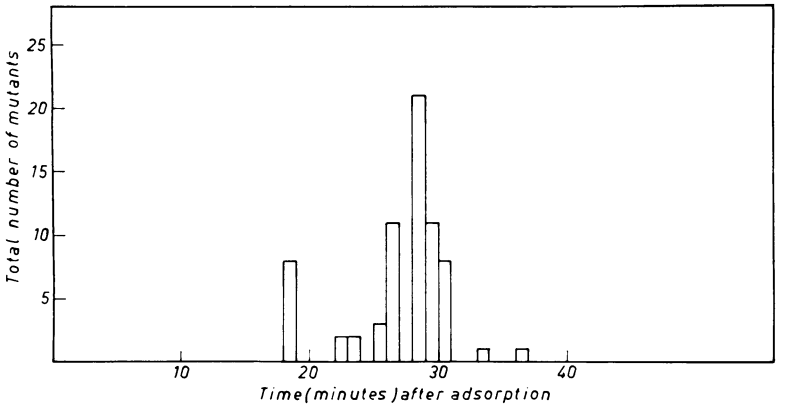

FIG. 3. *Histogram where all the available ts mutants have been assigned to the time interval of the ts mutants assayed with the pulse technique and belonging to the same complementation group.*

in different complementation groups. This result was confirmed by complementation tests, indicating that *ts 44* contains two mutations located in CG II and CG VI.

We also carried out another kind of experiment which can be considered the inverse of the pulse experiment described above: a 5-min pulse of nonpermissive temperature was given to the various samples at 5-min intervals. With 12 mutants tested in this way, two groups could be distinguished. One of them (*ts 102*, *ts 229*, *ts 175*, and *ts 39*) gave an ill-defined negative peak, in correspondence to the positive peak of the inverse experiment; a second group (*ts 180*, *ts 114*, and *ts 23*) showed a continuous down-sloping curve, representing a gradually decreasing average burst size. Such a result may mean that the gene products of these *ts* mutants are irreversibly damaged by the high temperature to which they were submitted during the nonpermissive pulse.

A peculiar mutant is *ts 237*, which gave by far the latest response (Fig. 5), at about the 48th

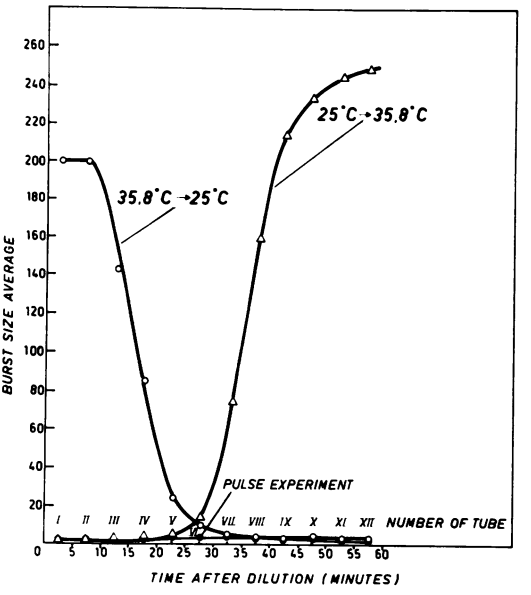

FIG. 4. *Response of mutant ts 44 to pulses of permissive temperature and shifts from permissive to nonpermissive temperature and vice versa. Abscissa: arabic numbers = time after dilution; roman numbers = progressive number of shifted samples; ○ = samples shifted from nonpermissive to permissive temperature (and left at this temperature until the end of the experiment); △ = samples shifted from permissive to nonpermissive temperature (and left at this temperature until the end of the experiment); ● = samples receiving a 5-min pulse of permissive temperature. Ordinate: burst size average.*

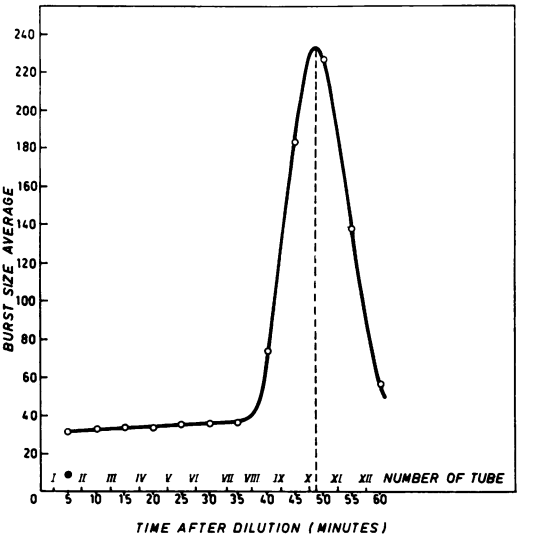

FIG. 5. *Permissive pulse experiments with mutant ts 237. Abscissa: arabic numbers = time after dilution; roman numbers = progressive numbers of shifted samples; ● = value of background. Ordinate: burst size average.*

min. This mutant has not been included in the chronological sequence reported in Fig. 2 because it delays the lysis of the infected cells both at 25 and at 35.8 C. All other mutations gave the same duration of the lytic cycle as the wild type; we checked on this point in detail, since otherwise all the peak times would have to be normalized before being compared and the meaning of the time sequence would have been less direct.

Preliminary results of other experiments made with *ts 237* suggest that it represents a mutation in a gene controlling the lysis of the bacterial cell; indeed, we have observed that slight changes of the nonpermissive temperature affect the length of the lytic cycle and that the average burst size, by far the highest of all the mutants here described, increases with the length of the cycle.

DISCUSSION

Repeated experiments with the same mutant invariably yielded results differing not more than 1.5 min, with the exception only of *ts 237*, which, as we have said, is in many ways a special case. This good reproducibility of the time of the peak holds also when the value of the nonpermissive temperature was slightly changed: in this case, only the ratio peak height-background is affected. The peak height and width did not change when the duration of the permissive pulses was reduced to 1 min, the pulses being administered at the same time intervals of 5 min. The shape of the peaks shown by the late mutants is probably affected by the inactivation of lytic centers due to lysis. In fact, the lytic cycle in this system lasts about 50 min at 35 C, but some lysis begins around 30 min after adsorption.

Whenever we tested two or more mutants belonging to the same CG, we found the peak times (and peak shapes and heights) to be very similar, the times differing by not more than 3 min. As for the overall distribution of the times of the peaks over the lytic cycle, there is a clustering in the late part of the chronological distribution, with a more or less uniform frequency of occurrence between the 23rd and 31st min (if one orders the mutants according to the criterion followed in Fig. 2).

The lower frequency of genes acting best at the beginning of the lytic cycle could be a consequence of the fact that the late genes are more likely to give rise to temperature-sensitive mutants or of the fact that no selective technique was applied for the isolation of early mutants. On the other hand, we have no grounds to assume

that our phage has early and late functions as defined in T4.

We would like to stress the observation that all the mutants tested in the course of our experiments respond best to a pulse of permissive temperature at a certain time during intracellular growth. This seems to indicate that the step affected by the nonpermissive condition is the utilization of a gene product present at a specific time. Such an interpretation is in agreement with all the evidence accumulated in the course of permissive pulse experiments as well as with the results obtained in the nonpermissive pulse experiments and in the treatment used for mutant *ts 44*.

A temperature-sensitive mutant of phage α would, therefore, be defined as one giving rise to an enzyme or a structural protein which is thermally unstable, or which cannot be successfully used, when the temperature exceeds certain rather precisely defined limits. This is what one would expect from proteins containing amino acid replacements in critical positions.

One can speculate about the meaning of the order of appearance of the final macromolecular products: in phage α, one would expect that many of the genes are arranged in a very large unit of transcription, since it has been proved that the transcribed codons of this phage are located in the same DNA strand (the H strand; 11). Genetic work is in progress to establish the topographical order of the genes of phage α and to look for a correlation between chronology and genetic topography (P. Donini, J. Kejzlarova, and F. Graziosi, *in preparation*).

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