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Genetic Map of Bacteriophage α

J. KEJZLAROVÀ,[1] P. DONINI,[2] T. EREMENKO-VOLPE, AND F. GRAZIOSI
International Laboratory of Genetics and Biophysics (C.N.R.), Naples, Italy

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Temperature-sensitive mutants of phage α were obtained by means of various mutagens and assigned to 25 complementation groups. Temperature-sensitive mutants belonging to 21 complementation groups and a mutant giving turbid plaques were used to perform two- and three-factor crosses. Seventeen of the cistrons and the turbid mutant were shown to belong to the same linear linkage group, which showed no signs of circularity. The remaining four unlinked cistrons showed peculiarities in their recombination properties. Genes which are known to be expressed earlier appear to be grouped together in a terminal segment of the linkage group.

Bacteriophage α is a temperate bacterial virus active on *Bacillus megaterium* (Paris strain). It contains a double-stranded deoxyribonucleic acid (DNA) molecule (2), the strands of which exhibit a density difference in CsCl gradients (3, 21) and are separable on a methylated albumin-kieselguhr column (4). The DNA of this virus also presents an abnormally high sensitivity to X-irradiation and ^{32}P decay (5, 13, 14). The temperate nature of the phage is peculiar in that lysogenized bacteria undergo spontaneous curing at a high rate (15), the frequency of which can be increased by acridine dye treatment (1).

Bacteriophage α has, therefore, been fairly well characterized physically, chemically, and biologically. To facilitate further work on this phage, it was considered desirable to proceed to a genetic study, which could most easily be accomplished by making use of conditional lethal mutants (16, 17, 18).

The work reported here describes the isolation of temperature-sensitive mutants of phage α with various mutagens and describes a genetic study of the phage carried out with such mutants.

MATERIALS AND METHODS

Phage. A clear mutant of the turbid wild-type phage, α_{c3}, was chosen as the reference type and will be designated α^+, whereas the wild type will be referred to as *αtu*.

Bacterial strain. *B. megaterium* (Paris strain) was used as the host in the crosses and as the plating indicator. This strain originates from the collection of the Institute of Microbiology of the University of Rome.

[1] On leave of absence from the Czechoslovak Academy of Sciences, Institute of Microbiology, Prague.

[2] Present address: University of California, Virus Laboratory, Berkeley, Calif. 94720.

Media. Nutrient broth (NB) 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 *p*H was adjusted to 7.2. Dilute broth (DB) consisted of NB diluted 10-fold; dilute broth plus Mg^{2+} (MB) consisted of DB plus 0.01% $MgSO_4$ (w/v) plus 500 μg of adenosine per ml. Adsorption medium (AM) consisted of DB plus 1% $MgSO_4$ (w/v). Dilution buffer was prepared by adding 4 g of NaCl, 3 g of KH_2PO_4, and 14.7 g of $Na_2HPO_4 \cdot 12H_2O$ to 1 liter of distilled water; after sterilization, 2 ml 10% $MgSO_4 \cdot 7\ H_2O$ was added. M9 medium was made by dissolving 5.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl in 1,000 ml of distilled water.

Solid media. For titration of phage on petri dishes, NB plus 1.5% Costantino agar was used in the bottom layer and NB plus 1% Costantino agar was used for the top layer. In the preparation of phage stocks and titration of bacteria, NB plus 2% Costantino agar was used for the bottom layer and NB plus 1.5% Costantino agar was used for the top layer.

Optical density (OD) measurements. OD measurements were made with a Zeiss PMQ II spectrophotometer at a wavelength of 560 nm.

Preparation of bacteria. When used as indicator, the bacteria were grown in NB with aeration at 35.7 C. When used as hosts for crosses and single-step growth experiments, they were grown in MB. [The adenosine present in MB prevents chain and filament formation (23)]. The bacterial cultures were aerated and grown until the end of the exponential growth period (OD = 2), which corresponds to about 2×10^8 bacteria/ml.

Preparation of phage stocks. Petri dishes with about 5×10^4 phage and 2×10^8 bacteria were incubated overnight at 28 C. Plates were then washed with 1.5 ml of DB for 2 hr at room temperature. The resulting lysate was collected by means of a pipette and freed from bacterial debris by low-speed centrifugation.

Bacteria were prevented from growing in the lysates by the addition of a small crystal of thymol.

Choice of temperature. For liquid media, the precise nonpermissive temperature to be used was chosen by performing one-step growth experiments at different temperatures to determine the highest temperature that does not significantly affect the growth of α^+ (Fig. 1). We chose 35.7 C as the nonpermissive temperature and allowed phage growth to proceed for 90 min. The permissive temperature chosen was 25 C, since at 30 C the growth of some of our *ts* mutants was significantly affected. A typical one-step growth curve of α^+ and a *ts* mutant at 25 C is shown in Fig. 2. The latent period takes about 70 min, the eclipse lasts for 50 min, and, after 190 min, lysis is complete.

For solid media, preliminary experiments showed that the most convenient temperatures (permissive and nonpermissive) were 28 and 37.7 C, respectively. The latter temperature is also quite critical since, on solid media, growth of α^+ begins to be inhibited at 38.2 C.

Mutagenization with hydroxylamine (HA). The phage was treated at 37 C with 1 M hydroxylamine in 0.2 M phosphate buffer at *p*H 6 for 25 min, which resulted in a survival of 1%. The action of HA was arrested by diluting the treated suspension 100-fold into 0.1 M tris(hydroxymethyl)aminomethane buffer at *p*H 8.5 and plating immediately.

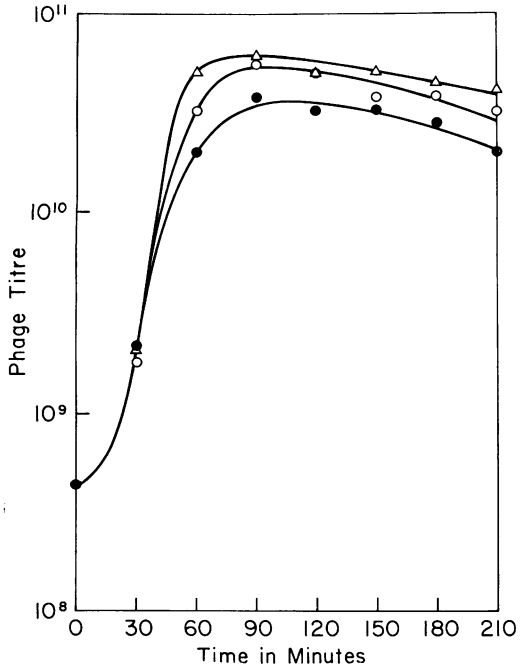

FIG. 1. *One-step growth of α^+ at different temperatures in the range of 35 to 36 C. Bacteria were infected and then kept at different temperatures as indicated. At different time intervals after infection, samples were taken and immediately plated. Time 0 is the time of dilution of the adsorption mixture. Symbols: △, 35.4 C; ○, 35.7 C; ●, 36.0 C.*

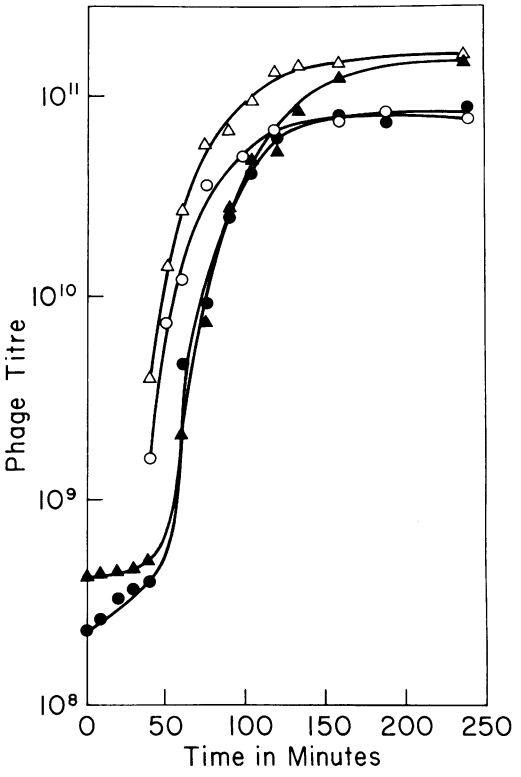

FIG. 2. *One-step growth of α^+ and ts 190 at 25 C. Bacteria were infected as described in Materials and Methods. At different time intervals after infection, a sample was taken and divided into two parts; one part was directly plated and to the other 0.4 ml of chloroform was added to bring about the premature lysis of bacteria. The samples were kept for 15 min at room temperature and then plated. Time 0 is the time of dilution of the adsorption mixture. Symbols: ▲, α^+ (△ treated with chloroform); ●, ts 190 (○ treated with chloroform).*

Mutagenization with nitrous acid. Phage α^+ was suspended in 0.1 M citrate buffer at a *p*H of 5.2 and at a temperature of 25 C. The phage was then treated with 0.5 M KNO_2 for 70 min, thereby obtaining a survival of about 10^{-4}. The reaction was stopped by diluting 100-fold into NB.

Mutagenization with ultraviolet light. Ten-milliliter samples of phage α^+ in standard saline were placed in glass petri dishes having a diameter of 10 cm. Irradiation was obtained from a BUG ultraviolet germicide lamp at a distance such that it gave a dose of 10.5 erg per mm^2 per sec. Doses of 90 and 140 sec were given, which resulted in survivals of 10^{-4} and 2×10^{-5}, respectively.

Mutagenization with ethyl methanesulfonate. The phage suspended in M9 was treated with 2% ethyl methanesulfonate for either 100 or 120 min, after which the action of the mutagen was arrested by

diluting the suspension 100-fold into 6% $Na_2S_2O_3$ in standard saline.

Mutagenization with nitrosoguanidine. A one-step experiment was performed with α^+ at 25 C (*see below*). Nitrosoguanidine was added at either 3.5 or 10 μg/ml to the bacterial suspension 8 min before the addition of the phage and to the dilution tube in which the lysate was produced.

Selection of mutants. Samples of mutagenized phage expected to contain 100 to 150 plaque-forming units were either plated directly or, more often, the plating was preceded by preadsorption. When the latter technique was used, host bacteria in DB were infected at a low multiplicity by the mutagenized phage and preadsorption was allowed to proceed for 10 min at 37 C. To eliminate nonadsorbed phage, the infected bacteria were spun down and washed twice in fresh DB and then plated.

The plates, obtained by either of the above methods, were incubated for about 7 hr at 25 C until the plaques first appeared and then were shifted to 37.7 C for another 5 to 6 hr. All of the plaques which had remained small were streaked by means of sterile toothpicks onto two plates which had already been seeded with indicator bacteria. One plate was incubated at 25 C and the other at 37.7 C. Lysates were subsequently made from single plaques of all strains which had grown poorly or not at all at the restrictive temperature and were plated at 37.7 C to obtain confirmation of the temperature-sensitive nature of the mutants.

One-step experiments. Bacteria were grown to a concentration of about 2×10^8 cells/ml, spun down, concentrated fourfold in AM, and placed in a bath at 35.7 C. Phage was added (at a multiplicity of about 10 phage per cell), and, after 6 min, adsorption was stopped by the addition of α antiserum which was allowed to act for 3 min and killed 99.9% of the free phage; the adsorption mixture was then diluted 10^4-fold into DB. One sample was kept in a bath at 35.7 C for 90 min and the other was kept in a bath at 25 C for 3 hr. Within 10 min after dilution of the adsorption mixture, a sample was taken to assay for infective centers; the plates were incubated at 28 and 37.7 C. Just before adding the serum, a sample of the adsorption mixture was diluted 10-fold and spun down, and an assay of the unadsorbed phage was made from the supernatant. The lysates were assayed for phage yields as follows. The lysate obtained at 35.7 C was plated at 28 C, and the lysate obtained at 25 C was plated at 28 and 37.7 C. The ratio between the yield at 35.7 C and at 25 C is an indication of the degree of "leakiness" of the mutants. The ratio between plaques obtained at 37.7 C and at 28 C from the lysate kept at 25 C served as a measure of the frequency of revertants.

Spot-test complementation on plates. This technique usually gives complications caused by intracistronic complementation when it is used with temperature-sensitive mutants. With phage α, we have found that there is a critical phage concentration at which these complications are minimized. The procedure finally adopted was to prepare phage suspensions at a concentration of 10^7 to 10^8/ml and streak them onto plates covered with bacteria containing 1.5% soft agar by means of fine paint brushes.

Relative-yield test. The procedure was analogous to the one used for determining the burst size of the single mutants; the cells were infected with two mutants at a multiplicity of five each. Complementation was expressed as the ratio of the yield of the lysate obtained at the nonpermissive to that obtained at the permissive temperature (both plated at the permissive temperature). As this ratio for α^+ varied from experiment to experiment in the range of 0.2 to 0.8, a control with α^+ was run together with the complementation tests, and all values obtained were corrected by assuming a theoretical ratio of 1 for α^+.

Two-factor cross. The procedure was the same as for complementation by means of the relative-yield test, with the difference that the infected cells were incubated only at the permissive temperature, whereas platings were made at both temperatures. To calculate the number of recombinants, the number of plaques obtained at the nonpermissive temperature was multiplied by two. Frequency of recombination was then expressed as the per cent of plaques obtained at the permissive temperature. The observed recombination frequencies were multiplied by a factor of 1.25 because of the lower efficiency of plating of α^+ at the higher temperature.

Three-factor cross. The procedure used was the same as that for two-factor-cross experiments. The cells were infected simultaneously with a double and a single mutant. Double mutants were isolated from descendants of the pertinent two-factor crosses and checked by the routine test used for single mutants. Usually sets of three crosses were carried out in which the same three markers were distributed on the two infecting phage in the three possible combinations.

Complete analysis of cross progeny. From the yield of a cross plated at the permissive temperature, plaques (600 to 1,000) were picked with a needle and short streaks were made on a plate already seeded with indicator bacteria. These master plates were always preincubated at 37 C for about 2 hr until incipient turbidity caused by growing bacteria appeared. After streaking, the plates were incubated overnight at the permissive temperature. The next day they were used for spot-test complementation with all of the parent types by means of the replica plating technique. By this method, it was possible to recognize all possible classes of recombinant and parental phage.

RESULTS

Temperature-sensitive mutants. As a result of treatment of α^+ with the various mutagens used, 174 temperature-sensitive mutants were obtained. Of these, 84 were obtained with HA, 14 with ethyl methanesulfonate, 65 with nitrosoguanidine, 19 with nitrous acid, and 2 with ultraviolet light. The mutants obtained by HA treatment were designated by a number, and those obtained with the other mutagens were designated by a

number preceded by a capital letter (E for ethyl methanesulfonate, G for nitrosoguanidine, N for nitrous acid, U for ultraviolet light).

Most of the mutants exhibited a normal or near-normal plaque morphology; the most frequent aberration observed was a slight reduction in the size of the plaques. Other changes in plaque morphology associated with some temperature-sensitive mutants were separable from the temperature-sensitive mutation by means of back crosses with α^+.

Complementation groups. Spot-test complementation was carried out with the 174 mutants in all pairwise combinations, most of the tests being performed twice. In most instances, clear-cut results were obtained, but some dubious cases had to be checked by the more sensitive burst-size test in liquid medium. As a result of this work, the mutants were finally assigned to 25 complementation groups. Nine mutants gave the abnormal result of failing to complement with two or more complementation groups; by crossing these mutants with α^+, it was possible to isolate recombinants with the expected property of complementing with all groups except one. This class of mutants was therefore interpreted as consisting of multiple mutants; eight of them yielded two different mutants each, whereas one gave three. The mutants stemming from the same multiple mutant were distinguished by adding the letters A, B, or C to their numerical designation. The total number of mutants used in the complementation work thereby increased to 184.

Table 1 gives a list of the 25 complementation groups, the total number of mutants which was assigned to each group, and the number of mutants produced in each cistron by the different mutagens. This classification differs slightly from the one already presented by Gaeta et al. (20) on the basis of earlier complementation work performed on 69 of the mutants obtained with HA. One class, XX, has been deleted since later results showed that the previous assignment of a mutant to this class was the result of highly efficient intracistronic complementation. Furthermore, six new classes have been created, XXI to XXVI, to accommodate new mutants which could not be assigned to existing complementation groups.

From Table 1, one can conclude that the various cistrons vary greatly in their susceptibility to mutagenic treatment. In general, when a particular mutagen produces several mutants in a certain cistron, mutants in that same cistron will be found by means of the other mutagens used by us. One exception to this rule is the case of cistron II in which HA has yielded 20 mutants, whereas none has been found with any of the other mutagens.

TABLE 1. *Classification of temperature-sensitive mutants of phage α by complementation group and mutagen employed*[a]

Cistron	HA	EMS	NG	NA	UV	Total
I	3	2	8	3		16
II	20					20
III	1					1
IV	1		2			3
V	2		1		1	4
VI	8	1	4	4		17
VII	4	2	9	1		16
VIII	2		2			4
IX	10	1	7	2	1	21
X	2		3			5
XI	4					4
XII	2	1	4	3		10
XIII	2		2			4
XIV	5	5	5	3		18
XV	8		2	1		11
XVI	1					1
XVII	3					3
XVIII	1					1
XIX	1	2	4			7
XXI	1		5			6
XXII	1					1
XXIII	1					1
XXIV	1					1
XXV			2	1		3
XXVI			5	1		6
Total	84	14	65	19	2	184

[a] Abbreviations: HA, hydroxylamine; EMS, ethyl methanesulfonate; NG, nitrosoguanidine; NA, nitrous acid; UV, ultraviolet light.

Mutants used in recombination experiments. One or more representatives from each of the complementation groups described in Table 1 were subjected to the routine tests described above. Table 2 furnishes the data obtained from such single-burst experiments with all of the mutants that were finally considered suitable for recombination experiments. Most of the figures given have been obtained from a single experiment, whereas a few are the result of two tests.

With most of the mutants, repeated tests gave between 50 and 75% adsorption in 6 min, with the exception of mutants 60, 156, and others belonging to the same cistron which reproducibly gave values ranging between 10 and 40%. This result indicates that gene II is involved in the formation of normal tail structures; when mutants from this gene were used in crosses with mutants from other genes, the initial input was adjusted in such a way as to obtain equal adsorption of the two infecting phages.

Several mutants give very low burst sizes at 25 C; this is interpreted as an indication that these mutants are mildly temperature-sensitive

TABLE 2. *Characteristics of ts mutants of phage α used in genetic crosses*

Cistron	Phage	Multiplicity of Infection	Adsorption	Burst size avg at 25 C	Leakiness	Frequency of revertants
			%		%	
	α^+	9.6	65.4	225	100.0	
I	180	6.8	64.8	158	3.6	1.6×10^{-5}
	228	8.2	40.0	584	2.5	5.6×10^{-5}
II	60	2.5	19.3	80	28.3	3.2×10^{-5}
	156	6.7	39.6	86	28.0	2.2×10^{-5}
IV	111	12.0	57.3	160	17.0	1.0×10^{-5}
V	102	11.6	70.4	120	11.0	1.7×10^{-4}
VI	23	7.8	64.4	227	0.8	1.0×10^{-4}
VII	11	9.0	62.5	161	0.7	5.5×10^{-5}
VIII	109	7.4	58.1	19	12.0	1.5×10^{-5}
IX	7	10.0	87.7	147	0.2	1.1×10^{-5}
	N9	8.0	64.0	48	2.9	8.9×10^{-6}
X	63	8.5	49.1	88	5.9	3.2×10^{-4}
XI	51	10.1	66.0	128	3.3	3.3×10^{-5}
XII	114	10.0	67.0	269	0.1	1.4×10^{-6}
XIII	39	10.1	78.7	53	0.3	1.0×10^{-4}
XIV	40	9.3	64.0	51	9.5	1.0×10^{-4}
	86	8.4	50.6	160	1.0	1.7×10^{-5}
XV	75	4.1	44.6	38	13.1	9.2×10^{-5}
	G4	19.0	76.0	64	14.0	4.0×10^{-5}
XVII	175	8.7	58.6	53	2.1	1.7×10^{-4}
	229	9.3	34.4	155	1.0	$<10^{-6}$
XVIII	205	11.1	59.5	102	0.8	1.7×10^{-6}
XIX	237	6.0	48.4	610	8.0	1.0×10^{-4}
XXI	190	7.5	35.3	340	0.3	2.4×10^{-7}
XXII	255	7.8	76.0	156	17.9	9.5×10^{-5}
XXV	N1	6.2	43.2	19	1.8	3.6×10^{-6}
XXVI	N2	8.3	54.9	142	0.7	2.2×10^{-6}

even at this low temperature. That some of the mutants give much higher burst sizes than wild type is not so easy to understand, except for the case of mutant 237 for which there are preliminary results indicating that it is involved in cell lysis. If such is the case, some residual temperature sensitivity at the permissive temperature will delay lysis, resulting in a larger burst.

Some of the mutants appear to be extremely leaky in liquid medium, which reduces the sensitivity of complementation tests involving them but does not affect their usefulness in genetic crosses.

A low frequency of revertants was the main criterion used in the final selection of mutants for genetic work. The upper limit established was 4×10^{-4}, since it seemed desirable to be able to measure accurately recombination frequencies of the order of 1%.

Four cistrons which appeared in Table 1 do not appear in Table 2. These are cistrons that contained only one mutant which failed to meet either the low reversion rate requirement, or which gave burst sizes so low at the permissive temperature that lysates of titers sufficiently high to obtain the multiplicities required by the cross experiments could not be produced.

Recombination. Two-factor crosses were performed with the mutants listed in Table 2, which were used in almost all pairwise combinations. The results of these crosses are summarized in Table 3. It can be seen that, for most cistrons, linkage relationship can be established with other cistrons, whereas five cistrons appear to be unlinked to all others. The cistrons showing linkage to others can be arranged in a linear order which is univocal over most of the linkage group, whereas there is some uncertainty with respect to the order in two regions of the genome. In Table 3, the mutants of these two regions are disposed according to what we considered was the most probable order indicated by the data. From the last column of the table, one can also see that the mutants from the five cistrons not showing linkage furnish frequencies of recombination in crosses with all others that are either lower (cistrons II, IV, XXI) or higher (cistrons XVII and XXV) than the frequencies obtained from crosses between mutants from linked but distant cistrons (maximum recombination frequencies).

A further check of the linkage and the order determined by the two-factor crosses was carried out by a series of three-factor crosses which covered the whole linkage group. The results of these crosses (Table 4) give complete confirmation of the order of markers previously established, with the exception of the order of mutants 39 and 114 with respect to their neighbors. To clarify this point, the cross 114 × 63.39 was repeated and a complete analysis of the progeny was carried out as described above. The order which resulted from the frequencies of the various classes of recombinants is 63, 39, 114. A control of this technique was made by also performing two such crosses with mutants from areas of the genome where the order had been firmly established by two-factor crosses.

Negative interference is present in our system, as can be seen from Table 4, which compares the observed and expected frequencies for double recombinants in the three-factor crosses.

As mentioned above, our reference type, designated α^+ in the present work, is really a clear mutant of the wild type which is a temperate phage giving turbid plaques. A region of the genome of our reference type, which can be called the *c* region and which could comprise several cistrons, is therefore genetically silent in our system, and this could be the cause of an apparent absence of linkage relationships between markers to either side. Thus, by using a marker in the *c* region, one could hope to connect one of the "unlinked" genes to the main linkage group.

TABLE 3. *Recombination data from two-factor crosses*[a]

ts	V[b]	I		VI	XXVI	VII	XIX	XVIII	IX		XXII	XI	VIII	X	XIII	XII	XIV		XV		XXV	XVII		IV	XXI	II		MRF[c]
	102	228	180	23	N2	11	237	205	7	N9	255	51	109	63	39	114	86	40	75	G4	N1	175	229	111	190	60	156	
102		10.8[2]	15.9	18.8[2]	20.9	20.8	18.5[2]	17.9	20.8	19.9	25.4	24.9	21.5	24.4	22.6	21.1	26.9	32.1	24.4	26.6	21.8	22.4	29.0	18.1	9.8	8.4	14.6	23.0
228			8.3[2]	12.5	18.9	13.0	19.5	17.8	14.1[2]	17.6	15.0	16.5	24.5	16.4	26.8	17.5	24.6	26.3	28.4		20.6	19.4	19.3		12.5			19.9
180				16.5[2]	16.4	17.1[2]	11.5[2]	17.5	24.3	17.6	18.4	20.5[2]	19.3	18.4[2]	23.4	21.5	26.6	20.1	21.5	20.8	25.8	23.4	21.6	15.0	6.8	8.5[2]	7.0	20.8
23					5.9[2]	11.0[2]	14.4[2]	21.6	25.1	16.4	25.0	23.5	18.0	22.9	27.0	22.9	24.0	22.6	31.9	18.4	26.3	27.0[2]	31.0[2]	7.6	5.9[2]	12.4[4]	9.5	23.1
N2						6.1[2]	13.9	15.0	11.5	14.9	21.8	17.4	24.0	13.5	21.0	19.9	21.9	20.1	17.0	24.8	21.0[2]	18.4	23.8	19.9	5.8			19.8
11							6.0[2]	16.3[2]	15.9[2]	16.6	26.3	17.0[2]	17.5	23.3	29.8	21.1	21.6	15.9	25.3	25.5	26.3	22.0	25.9	6.4	9.3	15.1	12.3	21.7
237								14.2[2]	14.4	13.8	14.1	15.8	15.4	20.8	23.9	19.5	17.3	20.0	23.9		18.0	16.1[2]	22.0	14.5	8.9	19.0	16.3	19.5
205									10.0[2]	7.5	12.0	13.4[2]	15.9[2]	19.0	26.5	17.9	19.5	28.5	29.3	20.9	40.9	31.8	29.4	14.9	4.1	16.4	16.1	21.5
7										4.8[2]	10.1[2]	10.3[2]	17.4[2]	21.0	24.6	16.0	24.3	25.0	31.8	26.0	19.0	35.0	40.0	10.3	8.3[2]	17.4[3]	10.1	22.0
N9											6.5[2]	6.1	11.0	13.5	14.4	15.6	15.6	17.1	18.0		24.1	16.1	25.0	5.3	5.8	13.9	10.8	16.4
255												3.9[2]	15.3[2]	17.8	25.8	19.6	23.4	23.6	28.5		27.3	28.1	15.3[2]		6.9	8.1		22.7
51													15.3[2]	23.1[2]	23.1[2]	23.6[2]	24.8	16.9[2]	23.3	25.0	25.4	29.6	35.8	6.6	6.8	19.6	14.5	21.2
109														9.0[2]	17.9	9.6[2]	21.3	17.8[2]	24.4	15.6	24.5	36.4	30.6	5.5	10.8	15.8	15.3	19.4
63															10.8[3]	5.6[2]	15.4	16.6	21.5	11.6	25.4	37.5	36.1	12.5	13.3	14.0[2]	14.5	19.1
39																5.3[2]	20.4[2]	20.5[2]	17.8	13.5	19.4	39.4	29.9	11.9	18.3	16.6[3]	11.1	24.1
114																	13.4[2]	14.9[2]	20.9	15.1	25.0	27.3	32.5	8.3	13.1	12.8	14.1	19.3
86																		2.9[2]	10.5	12.5	21.4	21.6[2]	25.4	8.8	16.0	10.3	9.4	22.5
40																			7.9[3]	12.1	30.5	30.4[2]	37.1	10.4	15.5	14.0	13.3	22.0
75																				5.8[2]	26.9	33.5[2]	32.1	17.5	16.6	13.5	15.0	25.2
G4																					35.4							20.3
N1																						21.8	23.6					25.3
175																							3.9[4]	17.1	9.6	5.5	10.8	27.1
229																								32.4	18.3		11.5	28.5
111																										21.8	18.5	11.3
190																										14.9	12.3	10.2
60																											0.0015	13.9
156																												12.7

[a] The mutants have been divided into two groups. The larger of these, corresponding to the data on the left-hand side of the table, contains the mutants showing linkage relationships and are arranged according to the most probable genetic order. The remaining mutants are those which appear to be unlinked to any other. Mutants in brackets belong to the same cistron. The exponents indicate the number of times a particular cross experiment was reported.

[b] Roman numerals refer to cistrons.

[c] MRF, maximum recombination frequency. In the case of the mutants showing no linkage to others, the MRF was obtained by averaging the results of all crosses with other mutants; in the case of the mutants on the main linkage group, the average was taken of all values issuing from crosses between a certain mutant and all mutants more than 30 recombination units away.

TABLE 4. *Results and analysis of three-factor crosses*[a]

ts markers			Recombination frequency			Analysis of B × AC	
A	B	C	A × BC	B × AC	C × AB	Expected	Observed/expected
102	228	23	6.0	3.3	10.1	1.4	2.4
102	180	23	5.6	2.1	8.1	2.6	0.8
23	11	237	3.0	1.5	4.0	0.7	2.1
11	237	205	6.0	3.3	8.9	0.9	3.7
11	237	7	5.0	2.5	9.5	0.9	2.8
11	205	7	7.8	3.0	4.8	1.6	1.9
237	205	7	5.8	1.5	2.5	1.4	1.1
205	7	51	2.4	1.6	9.0	1.0	1.6
205	255	51	11.1	1.6	2.3	0.5	3.2
7	255	51	8.6	0.6	1.4	0.4	1.5
255	51	109	2.0	1.4	14.9	0.6	2.3
51	109	63	11.0	3.4	6.3	1.4	2.4
109	63	114	10.6	2.5	3.6	0.5	5.0
109	39	114	7.0	1.3	1.0	0.5	2.0
63	39	114	5.3	3.3	1.3	0.3	4.7
39	114	40	2.4	1.0	17.9	0.8	1.3
39	40	75	19.7	4.0	6.5	1.6	2.5

[a] *ts* markers are listed in columns A, B, C in the sequence established in two-factor crosses. The appropriate recombination frequencies were calculated in a set of three three-factor crosses, involving always the same markers but with different combinations of double mutants (A × BC, B × AC, C × AB). The expected frequencies of recombination for C × AB were calculated on the basis of the two-factor crosses.

TABLE 5. *Recombination frequencies as a percentage from the crosses α tu ts⁺ × α tu⁺ ts*[a]

ts Mutants	Frequency of recombination	*ts* Mutants	Frequency of recombination
102	8.8	39	31.1
228	16.6	114	30.0
180	20.1[3]	86	25.0
23	25.7[2]	40	21.3
N2	31.7	75	35.4
11	24.1		
237	24.7	N1	2.1
205	36.3	175	22.8[2]
7	26.0	229	16.3[3]
255	20.3	111	16.5
51	30.2	190	15.2
109	34.1	60	15.1
63	34.1	156	10.4

[a] *ts* Mutants are listed in the genetic order found on the basis of two- and three-factor crosses up to *ts* 75 (the remainder of the mutants are unmapped). The exponent-like numbers indicate the number of repetitions of a particular cross experiment.

A series of crosses of this type was undertaken, and the marker for the *c* region used was simply α wild type, which we have called *αtu* in the present work. Table 5 shows the result of a series of experiments in which *αtu* is crossed to the *ts* mutants used in the previous crosses and in which clear plaques growing at the nonpermissive temperature were scored for. A perusal of this table shows that the marker *tu* appears to be linked to one of the terminal genes (V) of the linkage group and also to one of the "unlinked" genes (XXV).

A three-factor cross of the type described in the previous section, involving the three pertinent markers, confirmed the linkage and established the following order: N1–*tu*–102.

The results described above allowed us to construct the genetic map presented in Fig. 3, in which the distances between markers are those obtained from the relevant two-factor crosses. The markers seem to be spread quite evenly over the map, and the sum of the smallest adjacent intervals gives a total map length of 175 recombination units. No indication of linkage between the terminal markers has been observed.

DISCUSSION

The DNA of phage α has a molecular weight of 3×10^7 daltons (2) and can therefore be expected to have 50 or 60 genes if all of the DNA has a genetic function. Twenty five cistrons have been discovered in the present study; 21 of them have been characterized genetically; 18 cistrons and a turbid marker appear to be in a single linkage group, whereas four cistrons seem to be unlinked to any other cistron. Possibly the markers linking these genes to the rest of the genome still remain to be found, since Edgar and Lielausis (17) have shown that when a random isolation procedure such as the one adopted in the present work is used to isolate temperature-sensitive mutants of phage T4, one selects strongly against the class of mutants called "early."

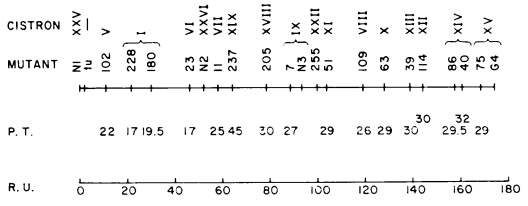

FIG. 3. *Genetic map of phage α. The map shows the distances in recombination units between adjacent cistrons. The mutants under brackets are from the same cistron. PT, time of optimal response to pulses of permissive temperature (peak time) from Gaeta et al. (20). RU, recombination units.*

Mutants in the four unlinked cistrons differ from all others in their recombination properties, in that the maximum recombination frequencies which are obtained when any of these mutants are used as one of the parental phage in crosses with all other mutants are either significantly higher (cistron XVII) or lower (cistrons II, IV, XXI) than average. Several kinds of explanations of this effect are possible, one of them being that the markers themselves affect recombination. It is possible that at the permissive temperature gene products are synthesized from the mutant genes which allow normal burst sizes to be produced but which still determine an effect on recombination which is dominant or partially dominant. It has recently been shown that early mutants of phage T4 often show altered recombination properties when used in crosses in conditions of incomplete permissiveness (6, 7).

Crosses involving the marker *tu* also show alterations in the recombination mechanisms, as evidenced by a higher than average maximum recombination frequency. Either the function or functions that were lost when the clear reference type was selected are involved in recombination or the high recombination frequencies observed are due to selection against the *tu* allele.

A conclusion of the present work is that the vegetative genetic map of phage α, as far as it has been characterized up to now, appears not to be circular. Thus, at least for the present, α behaves like other phages of similar molecular weight, notably the *E. coli* phages T5 (19) and λ (9) and the larger *B. subtilis* phage SP82 (22). Further work will be carried out to ascertain whether the map of phage α in the lysogenic cell is permuted circularly, as one would expect from the work on λ (8, 10, 11, 12) and on P22 (24).

In a recent study, Gaeta et al. (20) have shown that *ts* mutants of phage α show an optimal response to a short pulse of permissive temperature given at a time characteristic for the various genes. In Fig. 3, the times of optimal response to the pulses ("peak times") are reported for the mutants that have been characterized by these authors and which have been found in the present work to be in the main linkage group. On the left-hand side of the map there seems to be a group of genes that express their function at earlier times, whereas all of the genes on the right-hand side seem to express their function later. These two groups are separated by a gene (XXI), which responds very late and which appears to be involved in cell lysis (Graziosi, *unpublished data*). That the genes on the left-hand side are indeed "early" is in agreement with the finding that the *c* region is located genetically in this part of the chromosome.

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