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Clear plaque mutation sites linked to the *immI* region of the Salmonella phage P22

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SUMMARY

A complete genetic map has been established for the P22 clear plaque forming mutations *cir4*-1, *cir5*-1 and *cir6*-1. These are located within or closely linked to the *immI* region of P22 and represent a new class of clear plaque forming mutants located outside and rather distant from the *immC* region. They were mapped with respect to the markers *mnt*, *vy* and *ant* of the *immI* region and to genes *16* and *9* which span it. The three *cir* mutations complement each other and – with one possible exception – the *c1*, *c2* and *c3* mutations of the *immC* region. P22 *cir6*-1 – like P22 *cir5*-1 (Harvey *et al.* 1981) – is suppressed by the *ant*$^-$am19 allele, whereas P22 *cir4*-1 is not. The results are discussed in terms of the regulation of early *ant* expression.

1. INTRODUCTION

The temperate Salmonella-bacteriophage P22 harbours two chromosomal regions, the *immC* and the *immI*-region, which both are involved in the regulation of superinfection immunity in lysogenic bacteria. Genes within the *immC* region regulate establishment and maintenance of lysogeny by the c2-repressor (Levine 1957; for review see Susskind & Botstein, 1978), whereas genes of the immI region regulate the expression of the antirepressor gene *ant*, which is an antagonist of the c2-repressor (Levine *et al.* 1975; Botstein *et al.* 1975; Susskind & Botstein, 1978). The expression of *ant* occurs very early after infection (Harvey, Heil & Prell, 1979; Harvey *et al.* 1981) and is negatively regulated by two repressors, the products of genes *cir5* (Harvey *et al.* 1981; *cir* = control of immunity *I* region) and *mnt* (Gough, 1968). A defect in either of these regulatory products shifts the infection response predominantly (with P22 *cir5*$^-$) or completely (with P22 *mnt*$^-$) to lysis resulting in the formation of a more or less clear plaque in contrast to the turbid plaque formed by P22 *wild type*. The mnt-repressor is required to *maint*ain the lysogenic state. It is synthesized by the P22 prophage and prevents the expression of *ant* by attaching to its operator site O_{ANT} thus preventing transcription of gene *ant* and finally the inactivation of the c2-repressor by the antirepressor protein. On the other hand the cir5-repressor is synthesized very early after infection before

mnt-repression is established thus turning off *ant* expression after a short burst of synthesis from 3 to 6 min after phage infection (Harvey *et al.* 1981).

The mutant P22 *cir5*-1 (Harvey *et al.* 1981) is defective in this early ant repression and exhibits only a low frequency of lysogenization as compared with P22 *wild type*. P22 *cir5*-1 was one of several clear plaque isolates carrying a mutation mapping within or closely linked to the *immI* region of P22. In this communication we report the map position of *cir4*-1, *cir5*-1 and *cir6*-1 with respect to other genes within or to both sides of the *immI* region. The three mutants complement (with one exception) each other as well as the *immC* clear plaque mutants P22 *c1*, P22 *c2* and P22 *c3* to turbid plaque formation. Results of other experimental approaches (to be published: different other phenotypes of the *cir* mutants and their suppression, synthesis kinetics of ant protein and of phage DNA in the different P22 *cir*-mutants) suggest the conclusion that not only the *immC* region of P22 but also the chromosomal segment harbouring the *immI* region is involved in processes essential for lytic and lysogenic phage development. The construction of a detailed genetic map as reported in this communication furnishes the basic frame work for further analysis of the gene functions in this chromosomal region of phage P22.

2. MATERIALS AND METHODS

(*a*) *Bacteria*: Strains used are listed in Table 1 (*a*).

(*b*) *Phages*: Strains used are listed in Table 1 (*b*).

(*c*) *Media*: As described by Prell (1973).

(*d*) *Isolation of mutants*: The mutant P22 *cir4*-1, isolated by Dr Bezdeck, was picked after nitrosoguanidine mutagenesis of P22 c^+. Mutant P22 *cir6*-1 (isolated by Dr Hava) arose spontaneously. Both mutations are located outside the *immC* region since after growth of P22 *cir4*-1 and P22 *cir6*-1 in *LT2* (Px1) lysogenic bacteria (Fig. 1; Prell, 1970*a*, *b*) the lysates contain a fraction of less then 10^{-3} turbid plaque formers. On the contrary P22 *c2* which harbours within its *immC* region genetic material homologous to the Px1 prophage (Fig. 1) yields under the same conditions a fraction of $2-4 \times 10^{-2}$ turbid plaque formers by marker rescue from the prophage. This strongly suggests that the mutation in P22 *cir4*-1 and in P22 *cir6*-1 – like that of P22 *cir5*-1 (Harvey *et al.* 1981) – maps outside the *immC* region. Preliminary deletion mapping (Harvey *et al.* 1981) using lysogenic bacteria with partly deleted P22 prophages strongly suggested that the mutations map

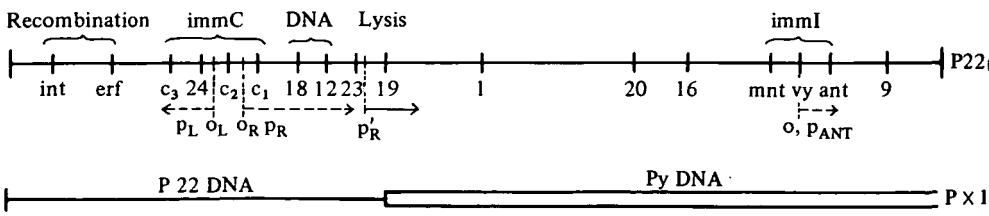

Fig. 1. Map of P22 *wild-type* prophage and of Px1 prophage. Px1 is a hybrid between phages P22 and Py (Prell, 1970). The marker distances shown in the figure correspond approximately to the distances on the genetic map.

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within or closely linked to the *immI* region. P22 *cir4*-1 and P22 *cir6*-1 mutants belong to sets of two or more independently isolated mutants (Hava, Soska & Bezdek, unpublished; Harvey, unpublished) which exhibit a semiclear temperature-sensitive phenotype: plaques are clear at 37 or 40 °C but more or less turbid at 25 °C. Selection of clear plaque-forming mutants released spontaneously from lysogenic *LT2 recA*$^-$ (P22 *sieA*$^-$) bacteria and having their mutations within the *immI* region yielded exclusively *cir6* mutant types. P22 *sieA*$^-$ prophages are unable to exclude homoimmune superinfecting P22 mutants (Rao, 1968; Susskind, Wright & Botstein, 1971; Susskind, Botstein & Wright, 1974). Such lysogens can be used to test for the presence and/or specificity of the *immI* and *immC* regions of a superinfecting phage without interference with superinfection immunity (Gough, 1968; Bezdek & Amati, 1968; Levine *et al.* 1975; Botstein et al. 1975).

3. RESULTS

(i) *Mapping by three- and four-factor crosses of the* cir4-*1*, cir5-*1 and* cir6-*1 mutant sites*

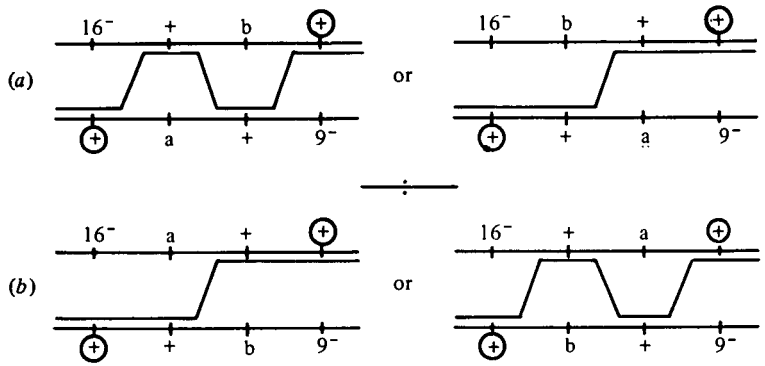
The corresponding crosses were performed using conditional lethal mutations (*amber*, *ts*) of genes *16* and *9* as selective markers. Non-selective markers were *cir4*-1, *cir5*-1, *cir6*-1 and the mutations *mnt*, *vy* and *int*. The map positions of the latter three mutations have been established earlier (for review see Susskind & Botstein, 1978). Each individual non-selective marker of a pair to be mapped was combined with either the *16*$^-$ or with the *9*$^-$ mutation. Reciprocal crosses were performed with such double mutants and *16*$^+$*9*$^+$ recombinants were selected. Among them the frequency of different non-selective marker combinations was scored (for details see Table 2). The frequencies of different recombinant types were expected to be different in reciprocal crosses depending on marker sequence and number of crossovers required for their formation.

In Table 2 four-factor crosses for mapping of *cir5*-1 and *cir6*-1 with respect to *mnt*-ts1, *vy*1, and *cir4*-1 are shown. In all the crosses the frequencies of non-selective + + (turbid) recombinants among the selected *16*$^+$*9*$^+$ progeny phages were scored. In some crosses the frequencies of − − (clear) recombinants were also scored. Harvey *et al.* (1981) established the marker sequence as *16–mnt–*(*vy*/*cir5*-1)*–ant–9*. We find according to the scheme of Table 2 the sequence is *16–vy*1*–cir5*-1*–9* (reciprocal cross A, Table 2). There is a very close linkage between *vy*1 and *cir5*-1, since we find in crosses A (*a*) and (*b*) 16·9 and 13·6 % *16*$^+$*9*$^+$, but only 0·096 and 0·066 % *16*$^+$ *cir5*$^-$ *vy*$^-$ *9*$^+$ and *16*$^+$ *cir5*$^+$ *vy*$^+$ *9*$^+$ recombinants, respectively. The latter two recombinants are formed by one crossover between *vy* and *cir5* and the low frequency suggests the close linkage. Taking into account, that *vy*1 maps to the right of *mnt* (Levine *et al.* 1975; Botstein *et al.* 1975), crosses B, C and D were performed to map *cir6*-1. These crosses establish the sequence *16–mnt–vy–cir5*-1*–cir6*-1*–9*. The position of *cir6*-1 with respect to *9* is based on the finding, that *cir6*-1 maps to the left of *ant* (see below) and *ant* to the left of *9* (Botstein *et al.* 1975). Note that the linkage *vy*1*–cir5*-1 is much closer than *vy–cir6*-1 (comparison of the frequencies of recombinations among unselected markers; Table 2, crosses A and B) confirming the map position of *cir6*-1.

Table 1.

(*a*) Bacteria	
LT2	Prell (1973)
su$^+$*527*	Whitfield *et al.* (1968)
DB5009	Chan & Botstein, (1972)
DB123	Chan & Bothstein, (1972)
(*b*) Phages: mutant strains	
Px1	Prell (1970*a*)
P22 *c*$^+$	Dr M. Demerek
P22 *ant*$^-$am19	Levine *et al.* (1975)
P22 *c1* 7	Levine (1957)
P22 *c2* 5	Levine (1957)
P22 *c3*am08	Dopatka & Prell (1973)
P22 *cir4*-1	This paper
P22 *cir5*-1	Harvey *et al.* (1981)
P22 *cir6*-1	This paper
P22 *int*$^-$N16	Prell (1973)
P22 *mnt*ts	Gough (1968)
P22 *vy*1 (= P22*virA*)	Bronson & Levine (1971)
P22 *9*$^-$amN9	Botstein *et al.* (1972)
P22 *9*$^-$tsU38	Dr J. King
P22 *12*$^-$am80	Kolstadt & Prell (1969)
P22 *16*$^-$am34	Botstein *et al.* (1972)
P22 *16*$^-$ts25 . 2 (= P22tsx)	Bezdek & Soska (1970)
Constructed recombinants:	
P22 *cir4*-1*ant*$^-$am19	This paper
P22 *cir6*-1*ant*$^-$am19	
P22 *9*$^-$amN9*cir4*-1	
P22 *9*$^-$amN9*cir5*-1	
P22 *9*$^-$amN9*cir6*-1	
P22 *9*$^-$amN9*mnt*ts	
P22 *9*$^-$tsU38*cir4*-1	
P22 *9*$^-$tsU38*cir5*-1	
P22 *9*$^-$tsU38*cir6*-1	
P22 *9*$^-$tsU38*int*$^-$N16	
P22 *9*$^-$tsU38*vy*1	
P22 *12*$^-$am80*int*$^-$N16	Prell (1973)
P22 *12*$^-$am80*cir4*-1	This paper
P22 *12*$^-$am80*16*$^-$ts25 . 2*c2*	
P22 *16*$^-$am34*cir4*-1	
P22 *16*$^-$am34*cir5*-1	
P22 *16*$^-$am34*cir6*-1	
P22 *16*$^-$am34*mnt*ts	
P22 *16*$^-$ts25 . 2*cir4*-1	
P22 *16*$^-$ts25 . 2*cir5*-1	
P22 *16*$^-$ts25 . 2*cir6*-1	
P22 *16*$^-$ts25 . 2*vy*1	

Table 2. *Four-factor crosses for mapping of the mutant* mnt*ts*, vy*1*, cir4-*1*, cir5-*1 and* cir6-*1*



		Crosses	Unselected markers: *a*	Unselected markers: *b*	*16⁺9⁺* recombinant plaques scored with unselected markers: Total	a^+b^+ (turbid) (a^-b^-) (clear virulent): no.	frequency (%)	Deduced sequence
A	(*a*)	P22 *16*⁻ts 25·2 *cir5*-1 × P22 *9*⁻tsU38 *vy*1	*vy*1	*cir5*	1055	0 (6)	<0·09 (0·57)	*16–vy*1*–cir5–9*
	(*b*)	P22 *16*⁻ts 25·2 *vy*1 × P22 *9*⁻tsU38 *cir5*-1			1439	7 (1)	0·49 (0·07)	
B	(*a*)	P22 *16*⁻ts 25·2 *cir6*-1 × P22 *9*⁻tsU38 *vy*1	*vy*1	*cir6*	57400* 574	427 (40)	0·74 (7·0)	*16–vy*1*–cir6–9*
	(*b*)	P22 *16*⁻ts 25·2 *vy*1 × P22 *9*⁻tsU38 *cir6*-1			710	30 (4)	4·2 (0·56)	

Table 2. (*cont.*)

		Crosses	Unselected markers *a*	Unselected markers *b*	*16*⁺*9*⁺ recombinant plaques scored with unselected markers: *a*⁺*b*⁺ (turbid) (*a*⁻*b*⁻) (clear virulent) Total	no.	frequency (%)	Deduced sequence
C	(*a*)	P22 *16*⁻am34 *cir6*-1 × P22 *9*⁻amN9 *mnt*ts	*mnt*	*cir6*	2636	35	1·3	*16–mnt–cir6–9*
	(*b*)	P22 *16*⁻am34 *mnt*ts × P22 *9*⁻amN9 *cir6*-1			2032	172	8·5	
D	(*a*)	P22 *16*⁻am34 *cir6*-1 × P22 *9*⁻amN9 *cir5*-1	*cir5*	*cir6*	3015	31	1·0	*16–cir5–cir6–9*
	(*b*)	P22 *16*⁻am34 *cir5*-1 × P22 *9*⁻amN9 *cir6*-1			3113	150	4·8	
E	(*a*)	P22 *16*⁻ts25·2 *cir4*-1 × P22 *9*⁻tsU38 *vy*1	*vy*1	*cir4*	746	81	10·9	*16–vy1–(cir4/9)*
	(*b*)	P22 *16*⁻ts25·2 *vy*1 × P22 *9*⁻tsU38 *cir4*-1			737	375	50·9	
F	(*a*)	P22 *16*⁻am34 *cir4*-1 × P22 *9*⁻amN9 *cir5*-1	*cir5*	*cir4*	5280	437	8·3	*16–cir5–(cir4/9)*
	(*b*)	P22 *16*⁻am34 *cir5*-1 × P22 *9*⁻am34 *cir4*-1			5880	1668	28·4	

G	(*a*)	P22 *16*⁻am34 *cir4*-1 × P22 *9*⁻amN9 *cir6*-1	*cir6*	*cir4*	4163	326	7·8	*16–cir6–(cir4/9)*
	(*b*)	P22 *16*⁻am34 *cir6*-1 × P22 *9*⁻amN9 *cir4*-1			3508	801	22·8	

* To count the *wild type* recombinants the lysate of progeny phages had to be plated in 100 times higher concentration as was required for counting *vy*1 *cir6* recombinants.

Bacteria growing exponentially in a roller at 37 °C and having reached a titre of $4 \cdot 10^8$ ml were infected in m.o.i. 3 with each of the parental phages indicated in the table. The phages were UV-irradiated before infection (dose 330 erg.mm^{-2}) to enhance recombination. After a 10 min adsorption period at 37 °C the mixture was diluted 1/50 in nutrient broth and incubated for 55 min at 37 °C. Then two drops of chloroform were added to aid lysis and incubation was continued for 30 min. The crosses between the P22 *ts* mutants were performed at 37 °C, since at 25 °C the frequency of recombination is rather low. According to the average burst size and the frequency of recombination obtained there is good complementation at 37 °C between the *ts* mutants. The yield of parental 16^- and 9^- (*amber* or *ts*) genotypes in all crosses was similar and varied at most by a factor of four. 16^+9^+ recombinants were selected by preadsorbing the progeny of the cross on logarithmically growing LT2 bacteria (4×10^8/ml) at 37 °C (in the crosses with *amber* mutants) or at 39·5 °C (in the crosses with *ts* mutants). The complexes formed were plated on overnight grown *LT2* indicator bacteria and incubated at 37 and 40 °C respectively. The table shows the numbers of 16^+ and 9^+ recombinants scored for wild-type alleles among the unselected markers. Depending on the sequence of the non-selective markers one or three crossovers are required for the formation of wild type ($16^+9^+a^+b^+$) or the reciprocal recombinant type ($16^+9^+a^-b^-$). Whenever possible other combinations of unselective markers were also counted. Their numbers were consistent with the number of crossovers required for their formation. In crosses A and B with low numbers of wild-type recombinants the number of reciprocal recombinant types are also shown in parentheses in the table.

Identification of recombinant genotypes:

Crosses A, B, E: according to plaque morphology: c^+ (wild type): turbid; *vy*: semiturbid; *cir4*-1, *cir5*-1 or *cir6*-1: each nearly clear. Identification of *cir4*-1 *vy*, *cir5*-1 *vy*1, *cir6*-1 *vy*: more or less clear plaques were picked on *LT2* indicator plates and replicated on plates seeded with *LT2* (P22 *sieA*⁻*int*⁻N16), where plaques containing the *vy*1 allele replicate and form a semi-clear lysis area.

Crosses F and G: according to plaque morphology: c^+: turbid; *cir4*-1, *cir5*-1 *cir6*-1, *cir4*-1 *cir5*-1 or *cir4*-1 *cir6*-1: nearly clear, no discrimination between the particular clear phenotypes attempted.

Crosses D: according to plaque morphology: c^+: turbid; *cir5*-1: clear with a small sparse centre of bacterial growth; *cir6*-1 and *cir6*-1 *cir5*-1: both clear, no discrimination attempted.

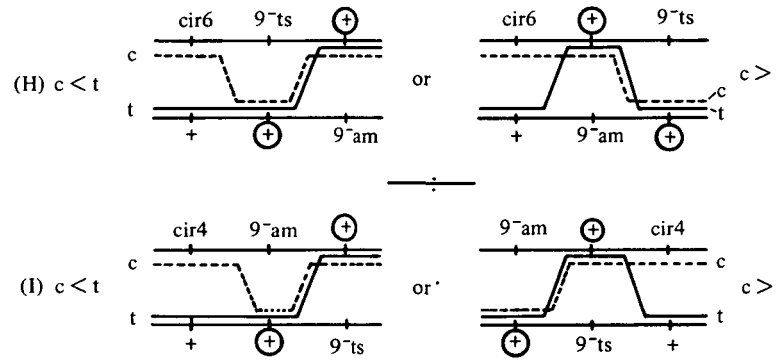
Crosses C: according to plaque morphology: c^+ turbid; *mnt*: bulls-eye-like plaque; *cir6*-1: clear.

(ii) *The map position of* cir4-*1 as established by three- and four-factor crosses*

Crosses E, F and G in Table 2 place *cir4*-1 to the right of *vy*1, *cir5*-1 and *cir6*-1, but its position with respect to gene *9* cannot be derived from these data. To map *cir4*-1 with respect to gene *9*, two three-factor crosses were performed. In the first cross (Table 3, cross H) the sequence of *9*⁻am and *9*⁻ts with respect to *cir6*-1 was established and in the second cross (Table 3, cross I) the position of *cir 4*-1 with

Table 3. *Three-factor crosses for mapping of the mutant sites* 9⁻ *tsU38*, 9⁻*amN9 and* cir4-*1*

The crosses were performed as described in Table 1. Among the *9*⁺ recombinants selected at 40 °C the number of clear and turbid plaques was estimated. According to the scheme on top of the table the ratio of clear to turbid plaques, which depends on the number of crossovers formed, allows us to deduce the marker sequence.



	Crosses	Unselected markers		*9*⁺ recombinant plaques scored with unselected markers: Total no.	Clear	Turbid	Deduced sequence
H	P22 *9*⁻tsU38 *cir6*-1 × P22 *9*⁻amN9	*cir6*	*Wild type*	824	661	163	*cir6*–*9*⁻am–*9*⁻ts
I	P22 *9*⁻amN9 *cir4*-1 × P22 *9*⁻tsU38	*cir4*	*Wild type*	2405	1760	645	*9*⁻am–*9*⁻ts–*cir4*

respect to both markers in gene *9*. In both crosses the one *9*⁻ parental phage had an *amber* (or a *ts*) mutation whereas the other parent of the genotype *cir6*-1 (or *cir4*-1) carried in addition a *9*ts (or *9* amber) allele (see diagrams in Table 3). *9*⁺ wild-type recombinants were selected and among them the ratio of turbid (wild type) and clear (*cir6*-1 or *cir4*-1) plaques was estimated. This ratio reflects the number of crossovers required according to the marker sequence. Cross H in Table 3 establishes the marker sequence as *cir6*-1–*9*⁻am–*9*⁻ts. Based on this sequence, cross I shows, that *cir4*-1 maps at the other side of gene *9* and hence the sequence

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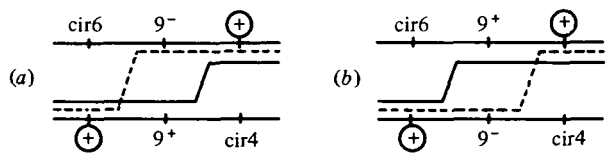
reads as *cir6*-1–*9*$^-$am–*9*$^-$ts–*cir4*-1. This marker sequence is confirmed by the reciprocal three factor crosses K in Table 4: among the turbid plaque forming recombinants scored, the percentage ratio of (non-selected) *9*$^-$amN9 and *9*$^+$ genotypes obtained in both crosses was reciprocal (16:84 and 67:33), suggesting that *cir6*-1 and *cir4*-1 span gene *9*, and *cir6*-1 is more closely linked to the *9*$^-$amN9 site than *cir4*-1.

The location of *cir4*-1 outside the *imm1* region to the right of gene *9* was an unexpected result, since so far only the *a1* (antigen conversion) gene has been shown to map (Gough & Scott, 1972) between gene *9* and the right arm end of the P22

Table 4. *Three factor crosses to map* cir6-*1 and* cir4-*1 with respect to gene* 9

The crosses were performed as described in Table 2. Turbid recombinant plaques appearing on su$^+$ indicator plates were picked in parallel on plates seeded with su$^+$ and with *LT2* bacteria to test for *9* amber of wild type.



	Crosses	No. of turbid 9$^+$ and 9$^-$ recombinants scored	9$^+$ Plaques	9$^+$ %	9$^-$ Plaques	9$^-$ %	Deduced sequence
K (a)	P22 *cir6*-1 *9*$^-$amN9 × P22 *cir4*-1	172	144	84	28	16	*cir6–9–cir4*
K (b)	P22 *cir4*-1 *9*$^-$amN9 × P22 *cir6*-1	153	51	33	102	67	*cir6–9–cir4*

prophage at *att*. However, during vegetative replication P22 forms a circle by joining the right and left arms of the prophage map. Therefore we asked if *cir4*-1 is located even further from *9*, namely to the right of gene *int*, which is the last gene so far known on the left arm of the prophage map. In order to map *cir4*-1 with respect to *int* the reciprocal four-factor crosses L in Table 5 were performed using as selective markers *9*$^-$tsU38 and *12*$^-$am80. Among the *9*$^+$*12*$^+$ recombinants two kinds of analysis were performed considering frequencies of recombinants for the non-selective markers. The first analysis involves scoring the frequency of recombination expected for the formation of turbid plaques (irrespective to being *int*$^+$ or *int*$^-$) by one crossover (evaluation like a three-factor cross; stippled lines in the scheme on top of Table 5). Depending on the location of *cir4*-1, this could occur either by general (low-frequency) recombination or by int-promoted, site specific (high-frequency; cross hatched area) recombination. According to this evaluation the marker sequence is *9–cir4*-1–*int–12* since in cross L (*b*) the frequency of turbid recombinants is much higher than in (*a*).

The second analysis was performed like that of a four-factor cross by looking

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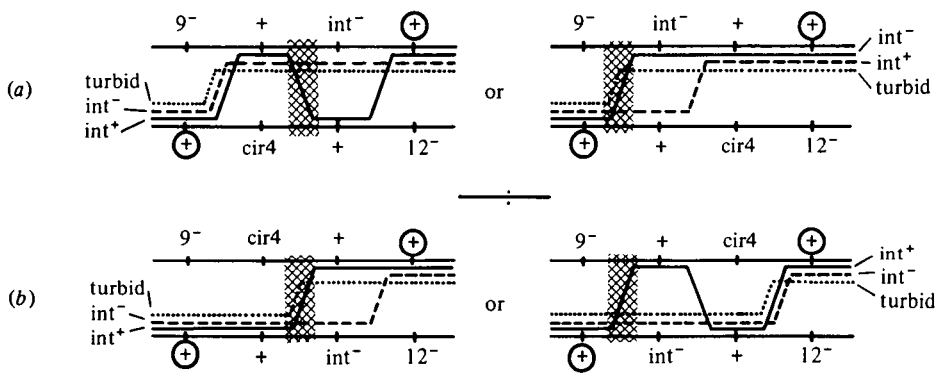
Table 5. *Four factor crosses to map* cir4-*1 with respect to genes* 9 *and* int

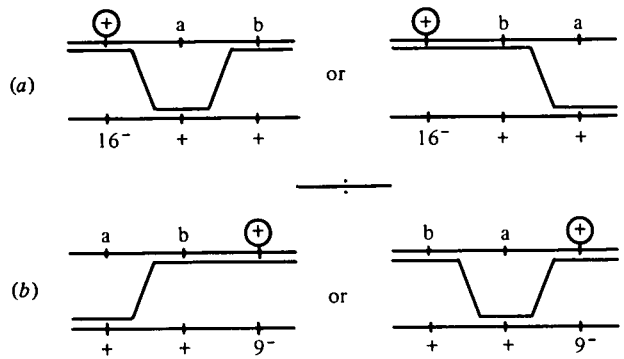
The cross was performed as described in Table 1 except that the UV-irradiation was omitted. In the scheme on top of the table the crossed area indicates the region of site specific, *int*-promoted recombination. The evaluation of the reciprocal crosses (*a*) and (*b*) according to the formation of turbid *int*± recombinants is shown by the stippled lines; the evaluation according to the ratios of *int*$^+$/*int*$^-$ turbid recombinants is shown with straight and with dashed lines. For details see text.



Crosses		Unselected markers		9^+12^+ recombinant plaques scored with unselected markers: Total no.	*cir4*$^+$*int*± (turbid) no.	*cir4*$^+$*int*± (turbid) frequency %	Deduced sequence
L (*a*) P22 *9*$^-$tsU38 *int*$^-$N16 × P22 *12*$^-$am80 *cir4*-1		*cir4*	*int*	274	51 (*int*+/*int*$^-$ = 5/122)	18·6	*9–cir4–int–12*
L (*b*) P22 *9*$^-$tsU38 *cir4*-1 × P22 *12*$^-$am80 *int*$^-$N16		*cir4*	*int*	360	314 (*int*$^+$/*int*$^-$ = 43/86)	87·2	*9–cir4–int–12*

Table 6. *Three factor crosses to map* cir6-*1 with respect to* ant

The crosses were performed as described in Table 2. The frequency of *9*$^+$*ant*$^+$*cir4*-1 or *16*$^+$*ant*$^+$*cir4*-1 and *9*$^+$*ant*$^+$*cir6*-1 or *16*$^+$*ant*$^+$*cir6*-1 recombinants was estimated by preadsorption (39·5 °C) and plating on *LT2* (Px1) bacteria (incubation at 40 °C). P22 *ant*$^+$*cir4*-1 and *ant*$^+$*cir6*-1 form nearly clear plaques, *ant*$^+$*cir*$^+$ recombinants turbid plaques. The frequency of clear plaques depends on the sequence of *ant* and *cir* and the number of crossovers required for their formation (see scheme on top of the table).



	Crosses	Unselected markers *a*	Unselected markers *b*	*16*$^+$ or *9*$^+$ recombinant plaques scored with unselected markers $a^+b^-(= ant^+\ cir^-)$ Total no.	no.	(%)	Deduced sequence
M (*a*)	P22 *cir4*-1 *ant*$^-$am19 × P22 *16*$^-$ts25 . 2	*ant*	*cir4*	801	199	24·8	*16–ant–*(*cir4*/*9*)
M (*b*)	P22 *cir4*-1 *ant*$^-$am19 × P22 *9*$^-$tsU38	*ant*	*cir4*	982	629	64·1	*16–ant–*(*cir4*/*9*)
N (*a*)	P22 *cir6*-1 *ant*$^-$am19 × P22 *16*$^-$ts25 . 2	*ant*	*cir6*	1272	417	32·8	*16–cir6–ant–9*
N (*b*)	P22 *cir6*-1 *ant*$^-$am19 × P22 *9*$^-$tsU38	*ant*	*cir6*	1548	160	10·3	*16–cir6–ant–9*

at the ratio of *int*$^+$/*int*$^-$ recombinants among the turbid plaques. If the map order is *9–cir4*-1–*int–12* in cross (*a*) (scheme on top of Table 5) three crossovers are required (two by general and one by site-specific recombination; straight line) for formation of an *int*$^+$, and one crossover (general recombination; dashed line) for an *int*$^-$ recombinant. This would lead to a very low ratio of int$^+$/int$^-$ recombinants. For the same map order in the reciprocal cross (*b*), a higher *int*$^+$/*int*$^-$ ratio is expected, since both recombinants require only one crossover (for *int*$^+$ by site specific recombination; for *int*$^-$ by general recombination between the rather distant genes *int* and *12*). The ratio *int*$^+$/*int*$^-$ = 5/122 in cross (*a*) and 43/86 in cross (*b*) supports the map order proposed above. In summary: the results presented suggest the following marker sequence:

16–mnt–vy-1–*cir5*-1–*cir6*-1–*9*$^-$amN9–*9*$^-$tsU38–*cir4*-1–*int–12*.

(iii) *Map position of ant with respect to the* cir *mutant sites*

Double mutants *cir4*-1 *ant*$^-$am19 and *cir6*-1 *ant*$^-$am19 were constructed in order to map *ant* with respect to *cir4*-1 and *cir6*-1. The crosses P22 *16*$^-$ts25 . 2 *ant*$^-$am19 × P22 *9*$^-$tsU38 *cir4*-1 and P22 *16*$^-$ts25 . 2 *ant*$^-$am19 × P22 *9*$^-$tsU38 *cir6*-1 were performed and *16*$^+$*9*$^+$ recombinants selected. These were scored for the unselected marker combination *cir*$^-$*ant*$^-$am19.

P22 *cir4*-1 *ant*$^-$am19 forms clear plaques on *LT2* but (like P22 *ant*$^-$) no plaques on *LT2*(Px1) indicator bacteria, since, in this lysogen, the c2-repressor of P22-specificity (Prell, 1970*a*,*b*) prevents replication of a superinfecting P22 *ant*$^-$ phage (Prell, 1977). Clear plaque formation is observed with *su*$^+$(Px1) indicator bacteria because active ant product is synthesized by suppression of the *ant*$^-$am19 mutation thereby inactivating the c2-repressor in the lysogenic bacteria. Hence this test verifies the presence of the *ant*$^-$am19 mutation.

When performing the cross P22 *16*$^-$ts25 . 2 *ant*$^-$am19 × P22 *9*$^-$tsU38 *cir4*-1 we were surprised to find only 0·2 % *cir4*-1 *ant*$^-$am19 genotypes among the *16*$^+$*9*$^+$ recombinants. This was much less than expected supposing that *cir4*-1 maps like *cir6*-1 to the left of gene *9*. Later on, when the correct map position of *cir4*-1 was established to the right of *9* (see paragraph 3(ii)), this result was easily explained. The possibility that the constructed P22 *cir4*-1 *ant*$^-$am19 mutant forms clear plaques only because it harbours an additional mutation responsible for the clear plaque phenotype was excluded since, in a backcross with P22 *cir4*-1, P22 *cir4*-1 *ant*$^-$am19 gave the same frequency of turbid plaques as a control infection with P22 *cir4*-1 alone (about 10^{-4}).

In contrast to P22 *cir4*-1 *ant*$^-$am19, P22 *cir6*-1 *ant*$^-$am19 forms turbid plaques on *LT2* indicator bacteria of somewhat different morphology than P22 *wild type*. Also P22 *cir6*-1 *ant*$^-$am19 does not form plaques on *LT2* (Px1) but will form turbid plaques on *su*$^+$ (Px1). After backcrossing of P22 *cir6*-1 *ant*$^-$am19 with P22 *c*$^+$, clear plaque forming P22 *cir6*-1 segregants are formed with a frequency of about 10^{-2}.

To map the *cir* mutations with respect to *ant*, three-factor crosses were performed (Table 6). Each of the double recombinants P22 *cir4*-1 *ant*$^-$am19 and P22 *cir6*-1 *ant*$^-$am19 was crossed with either P22 *16*$^-$ts25 . 1 or P22 *9*tsU38. Among the selected *16*$^+$ and *9*$^+$ progeny phages the frequency of segregated *ant*$^+$*cir*$^-$ was

estimated. The latter depends on the number of crossovers required (one or two; scheme of Table 6). Crosses M in Table 6 suggest the marker sequence *16–ant–(cir4/9)* which together with the results of cross I (Table 3; marker sequence *9*$^-$am–*9*$^-$ts–*cir4*) and crosses K (Table 4 marker sequence *cir6–9–cir4*) is actually *16–ant–9–cir4*. The segregation frequencies obtained in crosses N in Table 6 suggest that the sequence is *16–cir6–ant–9*.

In summary, all the mapping data of the last three paragraphs establish the following map order:

16–mnt–vy–cir5–cir6 ant–9–cir4–int–12.

(iv) *Complementation tests*

The mutants P22 *cir4*-1 and P22 *cir6*-1 were recognized by their clear plaque morphology. This phenotype reflects a reduced ability to lysogenize the infected bacterium. Accordingly only a few surviving lysogenic bacteria can be isolated from the clear plaque area. After infection at high m.o.i. in liquid medium, bacteria lysogenic with P22 *cir4*-1 or P22 *cir6*-1 are easily isolated, although with much lower frequency than after infection with P22 *wild type*. The exact frequency of lysogenization after infection in m.o.i. 6 with the P22 *cir*$^-$ mutants was estimated by scoring the number of lytic responses and, in an aliquot of the infection mixture, the number of surviving colony formers after a challenge with P22 *c2 int*$^-$ at high m.o.i. For details see description of Table 7. The frequency of lysogenization was used as a criterion for complementation among the mutants P22 *cir4*-1, *cir5*-1 and *cir6*-1 and the P22 mutants *c1*, *c2* and *c3* (Table 7). Complementation of P22 *cir4*-1

Table 7. *Complementation between the P22 mutants* cir4-*1*, cir5-*1*, cir6-*1*, c1, c2 *and* c3

LT2 log bacteria were prepared as described in Table 2. They were infected in a total m.o.i. of six with either one type of phage or the pair of phages (each m.o.i. of three) to be tested for complementation. After 10 min adsorption period at 37 °C one aliquot was withdrawn, diluted and plated with *LT2* indicator bacteria in soft agar for the estimation of lytic infections. To score lysogenic infections the other aliquot was treated for a total period of 70 min with anti-P22 serum to inactivate phages produced by lytic responses. The suspension was then diluted and plated on soft agar plates containing 8×10^8 P22 *c2* 5 *int*$^-$N16 phages in the soft agar layer. This challenge with superinfecting P22 *c2 int*$^-$ survive only immune and/or excluding lysogenic bacteria together with a very low fraction of phage resistant or 'infection refractory' bacteria. This fraction ranges between 0·2 and 0·4 % of the bacteria as estimated from an infection with P22 *c2* 5 and was neglected in the calculation. The frequency of lysogenization was calculated as the fraction in percent of surviving colonies among the total amount of lytic infections and surviving colonies recovered.

	c$^+$	c1	c2	c3	cir4	cir5	cir6
c$^+$	50·9	29·1	13·8	17·1	38·8	55·8	39·4
	c1	0·39	18·2	10·5	19·8	27·2	30·2
		c2	0·21	9·7	16·0	14·7	13·5
			c3	0·75	12·5	29·4	19·7
				cir4	0·54	32·9	27·9
					cir5	2·4	22·1
						cir6	12·4

and of P22 *cir5*-1 by all mutants and by each other is clearly to be seen. The outcome of the complementation tests of P22 *cir6*-1 with some of the clear plaque mutants is less clear. P22 *cir6*-1 exhibits in single infection with 12·4 %, already a rather high frequency of lysogenization, whereas P22 *cir4*-1 and P22 *cir5*-1 yield frequencies of only 0·54 and 2·4 %. The 'complementation level' among all P22 clear plaque mutants tested ranges between 10 and 30 percent (Table 7). P22 *cir6*-1, with a frequency of lysogenization of 12·4 % is already within this range

Table 8. *Complementation of P22* cir4-*1 and of P22* cir6-*1 by P22* $12^{-}16^{-}$ c2 *for increased frequency of lysogenization*

The complementation tests were performed as described in Tables 8. The P22 *cir4*-1 or P22 *cir6*-1 prophages in lysogenic bacteria were identified in a separate experiment, in which colony formers were plated on agar plates without superinfection with P22 *c2 int*$^{-}$. Colonies were streaked twice for purification and the P22 immune among them were tested for the type of phage production on LT2 indicator plates under non-permissive conditions.

Infecting phages	Frequency of lysogenization (%)
P22 c^{+}	48·0
P22 *cir4*-1	1·4
P22 *cir6*-1	8·0
P22 *12*$^{-}$am80 *16*$^{-}$ts25·2 *c2*	<0·8
P22 c^{+} + P22 *12*$^{-}$am80 *16*$^{-}$ts25·2 *c2*	25·0
P22 *cir4*-1 + P22 *12*$^{-}$am80 *16*$^{-}$ts25·2 *c2*	10·0*
P22 *cir6*-1 + P22 *12*$^{-}$am80 *16*$^{-}$ts25·2 *c2*	16·3†

* 50 % lysogenic for P22 *cir4*-1 (the remainder were wild-type recombinants).
† 85 % lysogenic for P22 *cir6*-1 (the remainder were wild-type recombinants).

making assessment of complementation in some cases weak or even questionable. Complementation of P22 *cir6*-1 with P22 *c1* and P22 *cir4*-1 (30·2 and 27·9 %) seems beyond question while complementation with P22 *cir5*-1 and P22 *c3* (22·1 and 19·7 %) is somewhat weak. However, even the lysogenization frequency of 13·2 % for the mixed infection P22 *cir6*-1 × P22 *c2* does not exclude complementation, since P22 *c2* also yields rather low values with P22 *cir4*-1 and P22 *cir5*-1 (16·0 and 14·7 %). Apparently, this type of complementation test is unfit for testing complementation between P22 *cir6*-1 and P22 *c2*.

Recently Harvey *et al.* (1981) showed that P22 *cir5*-1 is defective in early turn off of ant synthesis and that it can be complemented by a co-infecting P22 cir^{+} phage. This was taken as evidence for the synthesis of the so-called *cir5* repressor and its action in *trans* on *ant* regulation. The same conclusion might also be drawn from Table 7, namely that gene *cir5* synthesizes a product active in *trans*. It is not clear from Table 6 if either P22 *cir4*-1 or P22 *cir6*-1 synthesize products or if they

suffer from a non-complementable *cis*-dominant defect and only the co-infecting mutant is complemented to yield an increased frequency of lysogenization. To test this hypothesis the experiments in Table 8 were performed. The results show that P22 *cir4*-1 is efficiently complemented for lysogeny by the highly defective mutant P22 *12⁻16⁻c2*, which itself cannot lysogenize. For P22 *cir6*-1 the same conclusion seems to hold true but the high lysogenization frequency of P22 *cir6*-1 in single infection makes this conclusion less stringent.

4. DISCUSSION

A couple of clear and semiclear mutants of phage P22 were isolated. Their sites of mutation were found to map within or closely linked to the *immI* region of P22 (Harvey *et al.* 1981; this communication). Except for mutants P22 *mnt* (Gough, 1968) and P22 *vy* (Bronson & Levine, 1971), all other clear plaque P22 isolates have been found to carry mutations within the *immC* region (for review: Susskind & Botstein, 1978). The new mutants were named P22 *cir* (for: control of immunity *I* region), since preliminary experiments suggested that all of them had their mutations within the *immI* region (Harvey *et al.* 1981).

To map the *cir*-mutants, reference markers within or closely linked to the *immI* region were used. The map established by three- and four-factor crosses shows that the mutant sites *cir5*-1 and *cir6*-1 map between gene *ant* and the operator site *vy* which regulates the expression of *ant*. The mutation *cir4*-1 on the other hand maps outside the *immI* region to the right of gene *9*. The complete map appears as:

16–mnt–vy–cir5–cir6–ant–9–cir4–int–12.

We have shown by complementation tests that the clear plaque mutants P22 *cir4*-1, P22 *cir5*-1 and P22 *cir6*-1 complement each other, albeit complementation between P22 *cir6*-1 and P22 *cir5*-1 is weaker than between P22 *cir6*-1 and P22 *cir4*-1. Each mutation might thus belong to a unique gene, each synthesizing its own polypeptide. This is without doubt the case with mutations *cir5*-1 and *cir4*-1 since at least two genes, *ant* and *9*, map between them. On the other hand, mutant site *cir6*-1 might map either within gene *cir5*, thus allowing for intragenic complementation with P22 *cir5*-1 or belong to a separate gene *cir6*. As long as neither possibility has been ruled out we shall refer to complementation groups *cir5* and *cir6*.

Complementation groups *cir5* and *cir6* seem to be involved in the expression of gene *ant*, since an *ant⁻* mutation suppresses the clear plaque phenotypes as well as the low frequency of lysogenization of both P22 *cir5*-1 and P22 *cir6*-1 (Harvey *et al.* 1981; Prell, to be published). This conclusion is supported by the map position of both mutations, namely between the promotor/operator for *ant* expression, *vy* and gene *ant*. On the contrary, complementation group *cir4* seems not to be involved in *ant* expression since the double mutant P22 *cir4*-1 *ant⁻*am19 forms clear plaques. Accordingly we found the *cir4*-1 site mapping outside the *immI* region to the right of genes *ant* and *9*. Thus the genetic map as established in this communication for the P22 clear plaque mutants *cir4*-1, *cir5*-1 and *cir6*-1 is consistent with their relationship to the expression of gene *ant*. Next we will discuss what is known so far about the functions of the three genes.

Gene *cir5* was shown to specify a repressor for early shut-off of ant synthesis (Harvey *et al.* 1981). A second repressor of ant synthesis is the mnt-protein (Gough, 1968; Levine *et al.* 1975; Botstein *et al.* 1975), which is required for the maintenance of lysogeny. Harvey *et al.* (1981) have shown that mnt-repression is epistatic to cir5-repression. The authors demonstrated that P22 *virB cir5* phages do not express *ant* when multiplying in P22 *sieA*$^-$ lysogens, very likely because the mnt-repressor of the lysogenic cell prevents *ant* transcription on the superinfecting phage DNA.

Recently Susskind (1980) described a conditional lethal *amber* mutant P22 *arc*$^-$ which corresponds in most of its phenotypes to our P22 *cir5*-1 mutant: Both mutations map between *vy* and *ant*, they exhibit an unregulated overproduction of antirepressor and the wild type alleles of the *arc* and *cir5* genes allow for the synthesis of a *trans*-acting protein, most likely a repressor. The *cir5* and *arc* mutants exhibit some minor differences in other phenotypes which we believe depend on the procedure employed for isolation and the type of mutant recovered (*cir5*-1, *missence*, clear plaque, viable in *su*$^+$ and *su*$^-$; *arc*, *amber*, conditional lethal in *su*$^-$). Thus we like to suggest that both, *cir5* and *arc* mutations belong to the same gene.

The particular function of complementation group *cir6* and how it acts in the regulation of ant expression is still unknown. However, since complementation group *cir6* maps between genes *cir5* (= *arc*) and *ant*, the transcript originating at p_{ant} (= *vy*) and terminating at the end of gene *ant* (Susskind & Youderian, 1982) should also include the cir6 message. Furthermore, since the *cir5* (= arc) protein seems to regulate its own synthesis as well as that of antirepressor (Youderian, Chadwick & Susskind 1982) complemenation group *cir6* is also expected to be included in this self-regulatory circuit.

For gene *cir4* no specific function has been identified yet. Its map position between genes *9* and *int* may correspond to a gene coding for the early protein EaB (Youderian & Susskind, 1980). This protein was identified on SDS polyacrylamide gels without knowing the gene coding for it. Since gene *cir4* maps to the right of gene *9*, i.e. outside of the immI region and no relationship to *ant* expression has been found (see above) the designation 'cir' (control of immunity *I* region) might be unjustified. However, as long as the real cir4 function is unknown, we prefer to keep this designation.

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