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Constitutive Expression of Bacteriophage P2 Early Genes Resulting from a Tandem Duplication

(lysogeny/gene regulation/genetic recombination/phage P2 map)

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ABSTRACT **A tandem-duplication mutant of bacteriophage P2 was isolated. Physically, its particles are characterized by a higher buoyant density and lower heat stability than the wild type, both consequences of increased DNA content. Genetically, the mutant is easily recognized by its insensitivity to control by the immunity-specific repressor. The duplication covers part of gene B, necessary for phage DNA replication. To explain the immunity-insensitivity of the duplication it is proposed that the promoter, but not the operator site, in the early gene operon is duplicated in this mutant. By crosses with a gene-B mutant, a recombinant carrying a heterozygous duplication was isolated.**

The temperate bacteriophage P2 has two "early" genes, A and B, that are essential for the synthesis of phage DNA (1, 2). These genes are contiguous on the genetic map of P2 (Fig. 1), and are under the control (3, 4) of the immunity repressor specified by gene C. Immunity-insensitive mutants are obtained with relative ease in P2, suggesting that in this phage only one early transcription unit is essential for phage replication rather than two as in phage λ. Those mutants that have been studied are *cis*-dominant (5), and are located on the genetic map immediately to the left of gene B (1). It seems, therefore, that such mutations to immunity-insensitivity define the control segment (the operator-promoter site) of an operon BA (1).

We describe here a new *cis*-dominant immunity-insensitive mutation, *vir37*, which is a small tandem duplication (as shown by electron microscopy, ref. 6) and affects that same segment of the P2 chromosome.

MATERIALS AND METHODS

Temperate bacteriophage P2 (7) and *amber* mutant derivatives were used (1). Host bacteria were *Escherichia coli* strain C and derivatives: C-1a (3), C-85 (3), C-94 (the parent of C-89, ref. 9), C-117 (3), C-1055 (8), C-1080 (a P2-lysogenic derivative of C-85), and C-1757 (10). This last is permissive for *amber* mutants.

Media were LB broth, LB agar, soft agar (11), SSC (0.15 M NaCl–0.015 M Na_3citrate, pH 7.0).

Fresh cultures were grown with aeration in broth with 2.5 mM $CaCl_2$ to a titer of 4 to 7 $\times 10^7$/ml, centrifuged, and suspended in broth with 5 mM $CaCl_2$ at a concentration of 4 to 5 10^8/ml. Phage was added and adsorbed for 10–20 min. Dilutions in growth tubes were made in broth with 2.5 mM $CaCl_2$ (30° or 37°). One-step growth lysis was considered terminated at 65 min (37°) or 3 hr (30°), at which times chloroform or streptomycin was added to inactivate any surviving bacteria. For phage crosses, the bacteria were infected with four to seven particles of each type. To increase the frequency of recombination, the phages to be crossed were first irradiated with a dose of UV light producing one or two inactivation hits (see Table 1).

Phage stocks were prepared on C-1a or C-1757, at 30°, and concentrated with polyethyleneglycol (11).

For equilibrium density gradient centrifugation in CsCl (12), 4-ml samples of 1.42 g/ml density were centrifuged in Spinco rotors SW39 or SW50 for 2 days at 26,000 rpm, at about 9°.

RESULTS

Revertibility and Other Biological Properties of P2 vir37. Wild-type phage P2 forms turbid plaques on indicator bacteria, *E. coli* C. Spontaneous clear-plaque mutants—having lost the power to establish lysogeny—are found with a frequency of about 10^{-5} per particle plated. Some of these mutants are also immunity-insensitive, as shown by their ability to plate well on P2-lysogenic strains. Among 40 or 50 independent mutants of the latter kind, one, P2 *vir37*, was noted which had the unusual property of reverting spontaneously to the turbid phenotype, even after repeated isolations. In all preparations of P2 *vir37*, turbid plaques were found at frequencies of from a few percent down to 10^{-5}. The turbid-plaque type thus obtained was quite stable, and was immunity-sensitive like wild-type P2. Independent revertants are here symbolized as P2 *Rvir37-1*, P2 *Rvir37-2*, etc.

Turbid-plaque revertants of *vir37* were obtained independently of the host strain (C-1a, C-85, C-1055, or C-1757) used for growing the phage. They were also obtained with a

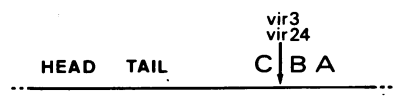

FIG. 1. Genetic map of phage P2 (highly simplified from ref. 1). *Head* and *tail* stand for some 15 genes responsible for phage-particle structure. Gene C specifies the immunity repressor, which is thought to act at the site defined by immunity-insensitive mutants *vir3* and *vir24* (both point mutations or very small chromosomal aberrations). B and A are "early" genes, necessary for phage DNA synthesis.

Abbreviation: SSC, 0.15 M NaCl–0.015 M Na_3 citrate, pH 7.0.

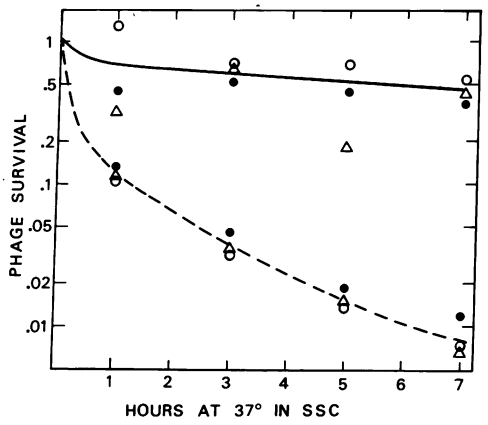

FIG. 2. Inactivation of P2 *vir37* in SSC at 37°. The phages were diluted in ice-cold SSC; 2.5-ml aliquots were placed in a bath at 37° at time 0 and titrated at various times. *Upper set of values:* turbid plaques, i.e., *Rvir37*. *Lower set of values:* clear plaques, i.e., *vir37*. (●) P2 *vir37*(+/+); (○) P2 *vir37*(+/*am*); (△) P2 *vir37*(*am*/*am*). The *continuous line* represents corresponding data obtained with wild-type P2. The initial frequency of turbid plaques in the three preparations was between 0.001 and 0.004, hence the large variability of the turbid-plaque titers.

hybrid of strains C and K12 carrying the *recA13* mutation, which inactivates (29) the main bacterial recombination pathway.

A backcross of one of the turbid-plaque revertants to P2 wild type (with UV irradiation to both phages in order to increase the frequency of recombination) gave less than 10^{-6} immunity-insensitive recombinants in the progeny. There was no support, therefore, for the possibility that the turbid-plaque revertants might result from extragenic suppressor mutations.

UV irradiation of P2 *vir37* produced an appreciable increase in the proportion of turbid plaques (Table 1), even though the survival of the two types was the same. The magnitude of the effect suggests that the transition of *vir37* to the turbid-plaque type is caused by UV-enhanced genetic recombination rather than by reverse mutation.

TABLE 1. *Reversion of P2 vir37 to wild type after UV-light irradiation of the phage*

	UV-light exposure, sec	Inactivation hits	Incidence of turbid plaque revertants: Total plaques scored	Turbid plaques	%
P2 *vir37*	0	0	2047	4	0.2
	200	2.3	1081	13	1.2
	400	4.8	833	24	2.9
	600	7.8	419	30	7.2
P2 *Rvir37-1*	200	2.3			
	400	4.8			
	600	7.9			

Phage suspensions in SSC at about 10^8 particles per ml were exposed on ice, in thin layer, to a 15W "germicidal" lamp (incident energy: 12.5 erg mm^{-2} sec^{-1}). Inactivation hits are calculated from the survival ratio S as x in $S = e^{-x}$. The frequency of turbid plaque-formers before irradiation reflects the history of the particular phage preparation used and is no direct indication of the spontaneous rate of formation of such particles. This rate is at most 0.00002 per progeny particle in one reproductive cycle (unpublished experiments).

TABLE 2. *Buoyant density determinations for various strains of phage P2*

Exp.	Phage 1	Phage 2	Density difference (1 − 2) in g cm^{-3}
a	*Rvir37-1*	REF	0.00003
b	*Rvir37-2*	REF	−0.00004
c	*vir37*	*Rvir37-2*	**0.00261**
d	*vir37*	*lg*	0.00222
e	*lg*	REF	0.00032
Calculated(*d*,*e*)	*vir37*	REF	**0.00252**
f	*vir37*	*lg*	0.00213
Calculated(*f*,*e*)	*vir37*	REF	**0.00245**
g	*vir37*(+/*am*)	*lg*	0.00192
Calculated(*g*,*e*)	*vir37*(+/*am*)	REF	**0.00224**
h	*vir37*(+/*am*)	w.t. 283	0.00140
i	w.t. 283	REF	0.00060
Calculated(*h*,*i*)	*vir37*(+/*am*)	REF	**0.00200**
j	*vir37*(*am*/*am*)	*lg*	0.00182
Calculated(*j*,*e*)	*vir37*(*am*/*am*)	REF	**0.00214**
k	*vir37*(*am*/*am*)	w.t. 283	0.00154
Calculated(*k*,*i*)	*vir37*(*am*/*am*)	REF	**0.00214**

am, *amB116*. *REF*, a mutant, P2 *amK12 tsD4 vir3*, used here as a density standard. P2 *lg* is another mutant (27). Note that w.t. 283, a wild-type stock of P2, does differ from the standard used. In experiments *d*, *f*, *g*, and *j*, CsCl was dissolved in 0.015 M Na_3 citrate; in the others, it was dissolved in SSC. The results would suggest that *vir37*(+/*am*) and *vir37*(*am*/*am*) are not quite as dense as *vir37*(+/+): this might be a consequence of their origin in recombination with P2 *amB116*, whose density has not been measured.

Like other immunity-insensitive mutations (5), *vir37* was *cis*-dominant. Whereas wild-type P2 and P2 *vir37*, simultaneously infecting a nonlysogenic host (C-1a), multiplied equally well, very few wild-type P2 particles appeared in the progeny under the same conditions when the host (C-117) was lysogenic for P2, hence immune. This evidence is against the idea that P2 *vir37* might make an excess of some product that counteracts immunity to superinfection in the lysogenic cell, but is consistent with the notion of *vir37* being a *cis*-dominant mutation of the "operator-constitutive" type. P2 *vir37* is insensitive even to the higher levels of immunity typical of doubly lysogenic bacteria, as exemplified by strain C-94.

Physical Properties of P2 vir37 Particles. Preparations of P2 *vir37* suspended in SSC were less stable to heat than similar preparations of the wild-type or of the turbid-plaque revertants. For example, after 30 min at 45° in SSC, P2, P2 *Rvir37-1*, and P2 *Rvir37-2* gave survivals of about 10%, whereas only between 0.01 and 0.1% of P2 *vir37* gave plaques after such treatment. A similar difference in particle stability could be established by exposure at 37° (Fig. 2). This observation suggested the possibility that *vir37* carries extra DNA, which is then lost in the process of reversion to the turbid-

plaque type. An inverse correlation between amount of DNA in the virus particle and its stability to heat in the presence of chelating agents (in this case the citrate of SSC) is well documented for various bacteriophages (e.g., ref. 14).

The possibility was confirmed by buoyant density determinations in CsCl gradients: P2 *vir37* was more dense by 0.0025 density units (average of three estimates; Table 2, lines *c*, *d*, and *f*) than the two turbid-plaque revertants tested, P2 *Rvir37-1* and P2 *Rvir37-2*.* From the observed density difference, by the criteria of ref. 15, one can calculate that P2 *vir37* contains a 2.8% excess over the normal P2 DNA content. This estimate is based on the known DNA and protein contents of normal P2 (11), which, for a DNA density of 1.71 and a protein density of 1.30, lead to a nominal density of normal P2 of 1.434 g cm^{-3}.

Electron microscopic studies with the heteroduplex method (6) not only showed that *vir37* phage DNA contained a 2.8% DNA addition, as compared with a wild-type P2, but also that the extra DNA resulted from a tandem duplication of a preexisting DNA segment. This conclusion is consistent with a recombinational origin of the *vir37* revertants (Fig. 3).

Location of vir37 on the Genetic Map of Phage P2. In previous work (16), two deletions of phage P2, *vir22* and *del1*, were localized by electron microscopic methods on the P2 chromosome at 72.0 to 77.1% and at 92.1 to 99.7%, respectively, from the left end, left and right being defined on the basis of partial-denaturation map studies (17). The duplicated segment in P2 *vir37* could be located (6) between 77.2 and 80.0% from the left end. The segment is then immediately to the right of the *vir22* deletion. Genetic crosses (G. Bertani, unpublished) indicated (*a*) that the *vir22* deletion covers mutations *vir3* and *vir24* (these three mutations all confer upon P2 the immunity-insensitive phenotype), and (*b*) that *del1* is linked to mutations in gene A.

Considering the genetic map of P2 (Fig. 1) it is then likely that the essential, early genes B and A are located between deletions *vir22* and *del1*, in the sequence *vir22 vir37 B A del1*. To test this suggestion, crosses were made to construct double mutants P2 *vir37 amB* and P2 *vir37 amA*. The latter type was obtained without complications. An attempt to isolate P2 *vir37 amB* failed, although several recombinants of an unexpected type were found in such a cross. These recombinants, symbolized here as P2 *vir37*(+/*amB116*), gave clear plaques (like *vir37*) and behaved like leaky *am* mutants (unlike the *amB116* parent, which definitely does not grow on a nonpermissive host). In buoyant density they resembled *vir37* (Table 2, lines *g* and *h*). They segregated turbid-plaque types (i.e., they reverted, like *vir37*): of these, some were frank am^+, and others (like *amB116*) nonleaky *am*. Phages from either turbid-plaque, segregant type were (like P2 *Rvir37*) more heat stable then the clear-plaque, leaky *am* parent (Fig. 2). These findings suggest that at least part of cistron *B*, including *amB116*, is included in the chromosome segment that is duplicated in *vir37*, and that the unexpected recombinant type described above is heterozygous for *amB116*. Its structure and recombinational origin are interpreted in Fig. 3. Since the ratio of *am* to am^+ haplo-segregants is different from one (Table 3), *amB116* is very probably located off center within the duplicated piece, barring polar effects in recombination.

From a backcross of P2 *vir37*(+/*amB116*) to P2 *amB116* a new recombinant type was isolated. This, symbolized as P2

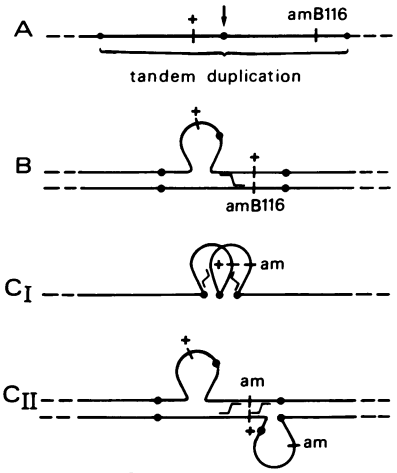

FIG. 3. (*A*) Proposed structure for P2 *vir37*(+/*amB116*). The direction is the same as that of the standard P2 map. The swellings along the chromosome define the end points of the duplicated segment. The *arrow* indicates the discontinuity ("novel joint") produced by the duplication in the normal base sequence. (*B*) The most likely recombination event to have produced P2 *vir37*(+/*amB116*) in the cross P2 *vir37* × P2 *amB116*. (*C*) Possible simple recombination events giving origin to various genetic types from P2 *vir37*(+/*amB116*). In either case, a single crossover as indicated on the right would produce a wild-type "revertant," whereas a single crossover as indicated on the left would produce an *am* "revertant." The complementary products of recombination would be, in C_I, a small ring fragment, and in C_{II}, a triplication-carrying chromosome. Other structures, for example P2 *vir37*(+/+), identical to the original duplication mutant, will originate from double crossovers. C_I could occur in single infection even in the absence of multiplication. C_{II} requires an interaction between two chromosomes, for example two daughter copies of the infecting chromosome. No implication is made concerning the molecular mechanism of recombination.

TABLE 3. *Immunity-sensitive segregants produced by the heterozygous, duplication-carrying phage P2 vir37*(+/*amB116*)

Type of segregant	Incidence
Turbid-plaque, nonleaky *am*	34
Turbid-plaque, frank am^+	10

Each of 44 (clear) plaques formed by P2 *vir37*(+/*amB116*) on the permissive indicator was picked and streaked out for plaque reisolation. One (clear) plaque from each streak was collected in 1 ml of SSC and incubated at 37° overnight. This enriches for the more stable, haplo-segregant types (see Fig. 2). From each tube a sample was then plated, always on the permissive indicator, and incubated. The turbid plaque nearest to the center of the plate was picked and tested for the *amber* phenotype by spotting it on permissive and nonpermissive indicators.

* The wild-type phage clone within which *vir37* originated is no longer available, so that a direct density comparison between P2 *vir37* and its parent is not possible. Furthermore, minor but significant density differences exist between various "wild-type" P2 preparations available in our laboratory; these have not been fully investigated.

TABLE 4. *Limited complementation in mixed infection of P2 amB116 mutant phage with P2 vir37(+/amB116)*

	A	B	C	D	E
Phage I	*am*	*am*	*am*	*am*	*vir37-(am/am)*
MOI	6.0	6.0	4.8	6.0	5.3
Phage II	None	*vir37-(+/+)*	*vir37-(+/am)*	*vir37-(am/am)*	+
MOI		0.38	0.40	0.15	0.35
Average burst size	0	192	1.4	0	154
Fraction of *vir* plaques in yield		0.26	0.23		0.78

Bacteria of nonpermissive strain C-1a were simultaneously infected with phages I and II at 37°. They were diluted at 10 min, and assayed for phage production at 60 min. Indicator: permissive strain C-1757. *Average burst size* is the phage yield divided by the number of bacteria expected to be infected with phage II. Zero, however, means phage yield not significantly different from level of unadsorbed phage. The latter was of the order of 0.3% of the input phage. *MOI*, multiplicity of infection.

vir37(amB116/amB116), resembled the P2 *vir37* parent in all properties [clear plaques, immunity-insensitivity, revertibility, heat stability (Fig. 2), density (Table 2, lines *j* and *k*)], except that it had nonleaky *amber* phenotype, and all turbid-plaque segregants tested were also nonleaky *am*.

The Apparent Leakiness of the am Phenotype of P2 vir37(+/amB116). If the entire gene B were duplicated in the heterozygous phage, the latter ought to have a frank am^+ phenotype, since the mutation *amB116* is well complemented by the wild-type gene in mixed infection (ref. 18, and Table 4, column *E*). We assume, therefore, that only part of gene B is included in the duplicated segment of *vir37*, and that the incomplete, hence inactive, copy is the one carrying the am^+ allele in the heterozygous phage isolated to date (Fig. 3).

Phage P2 *vir37(+/amB116)* is nevertheless quite unlike P2 *amB116* or P2 *vir37(amB116/amB116)*, in that it gave plaques on nonpermissive indicators with a 10–20% efficiency. Of such plaques, a few, both clear and turbid, and accounting for an efficiency of plating of about 1%, were of normal size, whereas the others were small to extremely small. Some of the larger plaques might well have been produced by preexisting recombinant particles. The nature of the others was less obvious. Their smallness might have resulted from small initial burst size, or from delayed lysis. The second alternative was clearly supported by three lines of evidence. (*a*) Plating on permissive indicator phage preadsorbed to nonpermissive bacteria produced roughly the same number of small plaques as plating on nonpermissive indicator. (*b*) Using in the above test, the streptomycin-sensitive, nonpermissive host strain C-1a, and the permissive, streptomycin-resistant indicator C-1757, and spraying streptomycin on the plates after 2 hr of incubation at 37° eliminated the small but not the large plaques. (*c*) In single-burst experiments with nonpermissive bacteria infected with the heterozygous phage at low multiplicity and plated (with streptomycin) well after the usual lysis time on the permissive indicator, more bursts could be demonstrated with still longer incubation times. It would seem, therefore, that the heterozygous particles, when infecting a nonpermissive cell, may remain more or less inactive for a relatively long period of time until a recombination event creates a whole, am^+ B gene, and thus permits the advance of the infectious process. Recombination might be helped by the fact that the *amB116* mutation does permit a small amount of synthesis of phage DNA under nonpermissive conditions (2). Among the single bursts analyzed, some contained frank am^+ recombinants, of either turbid or clear plaque type, that is, wild type or *vir37(+/+)*, in addition to the parental type. About as many bursts were found, however, to contain only particles of the parental type: here, any am^+ recombinants formed were either very few, and were missed in the analysis of the burst, or did not appear as mature phage particles, or were otherwise inviable. The possible presence of permissive mutant bacteria in the culture of the nonpermissive strain to explain these results was excluded by parallel experiments with P2 *vir37(am/am)*.

A definite amount of gene complementation was obtainable with P2 *vir37(+/am)* (Table 4), but it was not more than expected from its capacity to form a few normal sized plaques on nonpermissive hosts.

An alternative possibility for explaining the leaky phenotype of *vir37(+/am)* would be an occasional "hopping" of the RNA polymerase from the incomplete to the complete B gene to yield normal, amB^+ messenger RNA. This possibility was made unlikely by the observation that *vir37(+/am)* is just as leaky when plated on a nonpermissive lysogenic indicator: under these conditions the ordinary promoter controlling the B gene ought to be nonfunctional because of repression.

DISCUSSION

We have described a small tandem duplication of the P2 chromosome, which covers a well-defined gene marker. The duplication has been characterized by both genetic (this article) and electron microscopic methods (6). Other tandem duplications have been demonstrated or hypothesized in bacteriophages (19–24). The case reported here is unique in that it can be easily studied by both genetic and electron microscopic methods. Our duplication is much more stable than those described in T4, and this might reflect the fact that the recombination frequency in vegetative P2 is very low (13), and at least partly independent of the main bacterial recombination pathway.

If reversion of the tandem duplication occurs primarily by mechanism C_{II} (Fig. 3), one would expect triplications to occur with the same frequency as revertants. Triplications ought to be easily recognizable by a relatively greater heat instability. Attempts to isolate triplications in this way have failed (even when *vir37* had been coupled to a compensating deletion). This failure would suggest that reversion occurs preferentially by the "self-recombination" mechanism C_I (Fig. 3).

The material described is promising for studies of genetic recombination. For example, a good fraction of the single bursts resulting from single infection of nonpermissive bacteria with the heterozygous duplication phage, yielded only two types of phages, the am^+ recombinant and the parental type, in roughly equal numbers. This result reminds one of formally similar results obtained by mixed infection with other material (28).

The special interest of the duplication here described is that it makes the essential early genes, B and A, of phage P2 unaffected by the immunity repressor. The mechanism of this constitutive expression may be visualized in two ways.

We shall first accept the assumption that the two early genes B and A are under the control of an operator, defined by the immunity-insensitive mutations *vir3* and *vir24* (1).

Second, we distinguish in the control of a transcription unit or operon, the operator function (recognition of repressor) and the promoter function (recognition of RNA polymerase in the process of initiation of mRNA synthesis). These functions obviously correspond to short base-pair sequences, or recognition sites, in the DNA. Neglecting intermediate cases, three possibilities are easily conceived: (*a*) promoter and operator are overlapping and correspond to one DNA site. (*b*) Promoter and operator are contiguous nonoverlapping sites, in the order promoter, operator, structural genes. This is the case in the lactose operon (25), where, however, there might be a minor promoter–operator overlap. In this case, the repressor, in its bound state, can be visualized as a road-block to the attachment or to the progress of RNA polymerase. (*c*) Promoter and operator are contiguous nonoverlapping sites in the order operator, promoter, structural genes. In this case, to be effective in hindering initiation of mRNA synthesis, the bound repressor has to be "protruding" over the promoter; alternatively, promoter and operator may overlap to some extent.

Third, we note that P2 deletions *vir22* (16) and *vir79* (D. K. Chattoraj and G. Bertani, unpublished work), which are located on the P2 map to the left of the duplication, are very near to it, but do not overlap it. Both these deletions cover the sites of the operator mutations *vir3* and *vir24* (G. Bertani, to be published). All these mutations show the same immunity-insensitive phenotype. One has to conclude that the operator has been deleted in *vir22* and in *vir79*. As a consequence, the operator must be outside of the tandem duplication here described. As for the promoter in those deletion mutants: (*1*) it might have also been deleted, in which case their constitutivity in respect to the early cistrons must result from a fusion of the BA operon with another early, nonrepressed operon (e.g., that to which gene *int* presumably belongs, ref. 26) or (*2*) it is still intact, in which case it must find itself to the right of the deletions (since these end very near each other on the right, but have quite different endpoints on the left).

Considering now the structure of the tandem duplication, one can see that its constitutive phenotype may be (*A*) the result of a new repressor-independent promoter [which must then include the "novel joint" of the duplication (Hershey), i.e., the point where the two copies of the duplicated segment are joined together]. Alternatively, (*B*), the normal promoter could be included in its entirety in the duplicated piece: the right-hand copy of the promoter would thus be removed from the operator and would therefore be unaffected by the binding of the repressor.

We cannot distinguish between hypotheses (*A*) and (*B*). The latter, which fixes a sequence for operator and promoter (it implies cases *2* and *c* above), is more attractive, however, since it also explains the quasi-coincidence of the left end of the duplication and the right ends of two deletions, all giving the same immunity-insensitive phenotype.

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