Spontaneous λ o_R Mutations Suppress Inhibition of Bacteriophage Growth by Nonimmune Exclusion Phenotype of Defective λ Prophage

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Survivor clones with defects in gene functions that participate in the replicative killing of thermally induced *Escherichia coli* constructs with integrated λ *N* through *P* or *c*III through *P* gene fragments were selected at a frequency of about 10^{-6}. Among the population of survivors, clones were identified that exhibited normal λ immunity at 30°C, as shown by their ability to prevent the plating of λ wild type and to support the plating of a nearly identical heteroimmune bacteriophage λ*imm*^{434}. However, when placed at 42°C to inactivate the *c*Its857 repressor, these survivor isolates excluded the plating of both λ wild-type and λ*imm*^{434} phages, a phenotype designated nonimmune exclusion (Nie). Spontaneous mutants of λ wild type were isolated that overcame the Nie phenotype and would plaque at 42°C on cell lawns of these isolates. The acquired λ*se* mutations suppressed nonimmune exclusion, prevented lysogenization by interrupting repressor expression from p_{RM}, and made the phage insensitive to replicative inhibition. The *se* mutations were genetically mapped and sequenced within the rightward λ operator site.

The induction of λ prophage replication by thermal derepression of an N⁻ *c*Its857 prophage residing within an *Escherichia coli* chromosome results in extensive cell killing (replicative killing [5]). The viability of cells carrying an integrated, nonexcisable λ DNA fragment encoding the capacity to support initiation of λ replication depends upon the constant repression of λ genes *O* and *P* carried on the fragment. The products of these genes participate in the initiation of replication from the λ fragment. Cells that can be killed through the derepression of lambda replication when shifted to 42°C (at which temperature the *c*Its857-encoded repressor is denatured) are considered herein to have a replicative killing-competent (RK⁺) phenotype. Replicative killing-defective (RK⁻) cells, which are able to grow up as colonies at 42°C on agar plates, arise in RK⁺ cell cultures at frequencies of 10^{-5} or less.

The powerful forward selection for RK⁻ cells has been used to select spontaneous mutations within the rightward λ promoter p_R, distal genes *O* and *P* (1, 6, 7, 17), and the λ origin-replicator (19) and for determining the genotoxicity of environmental substances by RK mutatest (10, 11). The latter system replaced the intact N⁻ prophage with an integrated λ fragment comprising genes N through P, deleting prophage genes *int* through *kil*, the open reading frames between *ninC* and gene *Q* (unpublished data), and the lysis and other late gene functions from *Q* through the *b* region. The expression of *kil*, the lysis functions, and the suggested overproduction of *ninC* and *ninG ninH* (13) are lethal to the cell (2, 11).

E. coli constructs with integrated N⁺ or N⁻ and *cro*⁺ or *cro*⁻ λ fragments comprising genes N through P or *c*III through P were used to obtain spontaneous RK⁻ survivor cells with mutations in replicative killing functions. One class of survivor clones occurred frequently in each of the six selector strains and had an identifiable trait we termed nonimmune exclusion. This thermally inducible phenotype

is manifested as an ability of the survivor clones acquiring it to inhibit the plating of λ and λ*imm*^{434} bacteriophages. The nonimmune exclusion (Nie) phenotype is illustrated herein but will be fully described elsewhere. It is not identical to the Hyp phenotype (6) nor to the *rex* (25) or *sie* (23) exclusion system, although it may represent a composite of more than one such system.

In this report we describe the properties of spontaneous *se* (for suppress nonimmune exclusion) mutations arising within the o_R region of λ wild-type (wt) phage. The λ*se* phage are selected for their ability to form plaques at 42°C on lawns of RK⁻ cells displaying the Nie phenotype.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The origin and properties of *E. coli* strains TC600, 594, W3350, and SA431 were previously reported (9). The last strain carries a deletion that includes the prophage genes to the right of *P* (i.e., *Q* through *Jb*) and contiguous *E. coli* genes (including the *bio* operon through *chlA*). We prepared the lysogenic derivatives to be used. Starting with SA431, we constructed six *sup*⁰ RK⁺ selector strains carrying at *att*λ the integrated λ fragments N *c*I857 P (Y832) or N *c*I857 *cro27* P (Y834) (leftward endpoint marker *bio*10, at about base pair [bp] 34,249 [3]), and *c*III N *c*I857 P (Y836) or *c*III N *c*I857 *cro27* P (Y838) (leftward endpoint marker *bio*275, about bp 33,303), each including a *c*I *ind*⁻ mutation. The Nam7,53 derivatives of Y832 and Y834 were also prepared (R. Tuer and S. Hayes, unpublished data). The construction techniques used were partially described by Hayes et al. (11), and specific details are available from the authors. The phage used are listed in Table 1.

Isolation of λ *se* mutations. Dilutions of a λ wt lysate (5 × 10⁹/ml) were mixed with 0.2 ml of overnight 30°C TB (tryptone broth: 10 g of tryptone [Difco Laboratories], 5 g of NaCl per liter) cultures of RK⁻ survivor clones plus 3 ml of TB top agar (0.65%), overlaid on TB agar (1.1%) plates and incubated at 42°C. (The RK⁻ clones used were designated Ilr100a, Ilr101b, Ilr109b, Ilr109e, Ilr141e, Ilr145c, and

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TABLE 1. Bacteriophages

Bacteriophage	Source
λ wt.	W. Szybalski
λvir (λv2v1v3)	W. Szybalski
λcI72	W. Szybalski
λcI26	W. Szybalski
λcI47	W. Szybalski
λcII68	W. Szybalski
λcIII67	W. Szybalski
λcy42	D. Wulff
λbio3h-1Δnin5	W. Szybalski
λbio30-7Δnin5	W. Szybalski
λbiot124Δnin5	W. Szybalski
λcIΔKH54pf	F. Blattner
λspi173Δnin5	G. Gussin
λspi275Δnin5	G. Gussin
λspi113Δnin5	G. Gussin
λspi274Δnin5	G. Gussin
λspi156Δnin5	G. Smith
λcI90yc17	W. Szybalski
λprm116	G. Gussin
λcI857prm116	G. Gussin
λcI857(18,12)^{P22} [λhy106]	S. Hilliker
λimm^{434}T	W. Szybalski
λimm^{434}cI$^-$	W. Szybalski
λimm^{434}cI$^-$ds	D. Wulff
λimm^{434}cII68	λcII68 × λimm^{434}T
λimm^{434}cIII67	λcIII67 × λimm^{434}T

Ilr145d, and each exhibited the Nic phenotype at 42°C.) Scattered small plaques appeared on the plates at an efficiency of plating (EOP) of about 10^{-6}. These phage were assumed to carry a spontaneous defect (se mutation) which enabled λ wt to escape nonimmune exclusion. Representative, well-isolated plaques were picked from each plate, added to phage buffer (0.01 M Tris [pH 7.6], 0.1 M NaCl), chloroformed, and streaked onto the Ilr$^-$ (see Results) culture cells from which they were isolated. The λse lysates were prepared on strain W3350 from the individual plaques.

cis-trans effect of *se* mutations. Selected RK$^-$ isolates exhibiting the nonimmune exclusion phenotype at 42°C were grown to stationary phase, pelleted, suspended in TM buffer (0.01 M Tris [pH 7.6], 0.01 M MgCl$_2$) at 10^8 cells per ml, starved (suspended cells were held in TM at room temperature for 1 h), mixed with λ wt plus the λse phage (each at a multiplicity of infection [MOI] of 3), and adsorbed for 10 min at 30°C. After adsorption, the mixture was adjusted to 0.01 M disodium EDTA (pH 8.0), held at 30°C for 6 min, and diluted into 2 ml of TM buffer. The cells were pelleted, washed once in TM buffer, suspended into 10 ml of prewarmed TB at 42°C, incubated for 70 min at 42°C, chloroformed, and repelleted to clarify the lysate. Aliquots of the lysate were plated at 42°C on TC600 for the determination of total yield and plaque type and on their corresponding Ilr$^-$ host cells to verify the λse phage burst.

Lysogenization efficiency. Overnight cultures of *E. coli* 594 were pelleted, suspended in TM buffer at 2 × 10^8 to 4 × 10^8 cells per ml, starved, mixed with λ or λse phage, adjusted to 0.01 M Ca-Mg, and incubated (as below). Subsequently, the cells of the infected cultures were pelleted, suspended, diluted, and spread onto TB agar plates, and the resultant clones from the 30°C incubation were replicated to plates seeded with 10^9 λcI72, and replicate plates were incubated at 30°C. Clones arising were picked and cross-streaked with λcI$^-$ and λvir. Cross-streaked clones that were resistant to

lysis by λcI$^-$ and were lysed by λvir were considered lysogenic for λ. In incubation method 1, cells were mixed with phage at an MOI of 20, adsorbed 15 min at 39°C, diluted into 20 ml of TB, and incubated overnight at 30°C. In method 2, cells were mixed with phage at an MOI of 5, held at 4°C for 15 min, diluted 10 times into prewarmed (30°C) TB, and incubated 20 min with shaking at 30°C. Method 3 was the same as method 2, except that incubation at 30°C was for 30 min.

Phage complementation. TB agar plates were overlaid with a mixture containing an overnight culture of *E. coli* W3350 (0.25 ml), λse plus λimm^λ or λimm^{434} phage (each diluted to give 2 × 10^3 to 3 × 10^3 plaques per plate), and 2.5 ml of TB top agar. Parallel plates were incubated at 30 and 39°C. Control plates of each phage were similarly prepared.

Deletion analysis. Phage stocks ($\sim10^{10}$ phage per ml) were diluted 100-fold into 0.01 M disodium EDTA, (pH 8.5), incubated for 30 min at 37°C, diluted 10-fold into TM buffer to stop the reaction, diluted, and titered on lawns of strain W3350 at 30°C (16).

Deletion mapping. Fresh overnight cells from a TB culture of strain W3350 were pelleted, suspended in TM buffer at 4 × 10^8 cells per ml, and starved. The cells (8 × 10^7) were mixed with λse and the λΔ marker phage (each at an MOI of 5) and adsorbed for 15 min at 39°C. The mixture was diluted into 20 ml of prewarmed (39°C) TB, shaken for 90 min at 39°C, chloroformed, and plated on W3350, with incubation at 42°C.

Replicative inhibition. Sensitivity to replicative inhibition was measured by coinfection of a λ lysogen with homoimmune and heteroimmune phages with identical replication regions. Overnight cultures of host cells were pelleted, suspended in TM buffer to 4 × 10^8 cells per ml for 1 h, mixed with λimm^{434} plus λimm^λ phages (each at an MOI of 3), and adsorbed for 15 min at 39°C. The mixture was transferred into 20 ml of prewarmed TB, shaken (39°C) for 80 min, chloroformed, diluted, and plated on host strains W3350, W3350(λ), and W3350(λimm^{434}T) at 30°C. These hosts score for total burst, λimm^{434}, and λimm^λ phages, respectively.

DNA sequencing. DNA was isolated from λ wt and the seven λse isolates (100a, 101b, 109b, 109e, 141e, 145c, 145d) and digested with *Bgl*II. The fragments were labeled in an end-fill reaction, with the Klenow fragment of DNA polymerase I, by addition of complementary [α-^{32}P]dGTP to 3′ OH ends of restriction fragments. The end-labeled fragments were digested with *Hae*III, and the 856-bp *Hae*III-*Bgl*II fragment (λ bases 37,247 through 38,103) was separated and subjected to Maxam and Gilbert (14) G, A (G+A), T (T+C), and C sequencing chemistries. The cleavage products were separated electrophoretically on 8% acrylamide gels.

RESULTS

Functional immunity analysis. Rare RK$^-$ survivor colonies arose at 42°C from each of the 6 RK$^+$ selector strains. The RK$^-$ survivors were subdivided into two broad classes by determining whether λvir could form plaques at 42°C on a cell lawn of each cultured clone. The clones that were incapable of supporting growth of λvir, termed Hd$^-$, were not further considered. They presumably had mutations in *E. coli* genes required for λ infectivity, replication, vegetative growth, or mutations within the defective λ fragment which caused them to negatively complement for an essential λ growth function.

The RK$^-$ survivor clones that permitted plaque formation by λvir at 42°C were screened for functional λ immunity, i.e., to determine whether the *imm*^{λ} region of the defective λ

fragment could be recombined into a heteroimmune phage and allow vegetative growth of the recombinant. This test was carried out by replicating the remaining RK$^-$ clones to agar overlay plates containing 6 × 10^8 W3350(λ*imm*^{434}T) cells plus 10^9 λ*imm*^{434}*cI*$^-$ phage and incubating overnight at 39°C. The appearance of a zone of lysis in the cell lawn surrounding a replicated clone indicated λ*imm*^{434}*cI*$^-$ could infect and exchange by a double recombination event the *imm*^{434} region on the infecting phage with the *imm*$^\lambda$ segment within the chromosome of the replicated clone. Only the resulting *imm*$^\lambda$ recombinants released from printed cells infected with λ*imm*^{434}*cI*$^-$ are capable of growing vegetatively and producing a lysis area on the W3350(λ*imm*^{434}T) cells in the overlay.

The RK$^-$ survivor clones not giving a lysis spot in the functional immunity test were considered either to have deletions into *imm*$^\lambda$, or to carry a mutation within an *imm*$^\lambda$ target site that was required for expression of the RK$^+$ phenotype, e.g., p$_R^-$. The replicated survivor clones with lysis spots in the functional immunity test were tentatively considered to carry spontaneous mutations outside of *imm*$^\lambda$ in either known or unknown genes or target sites involved in the initiation of lambda replication. These clones, which were defective in various functions required for the initiation of lambda replication, were collectively described as having an Ilr$^-$ phenotype.

Nie phenotype. A variety of phenotypic properties were observed among the Ilr$^-$ isolates. One phenotype exhibited by many isolates was nonimmune exclusion (Nie). An initial characterization of the ability of Ilr$^-$ clones to complement at 42°C for λ genes *N*, *O*, or *P*, i.e., by determining their ability to support the plaque formation of *imm*^{434}, *imm*$^\lambda$, and *imm*^{21} phage with amber mutations in these genes, revealed that many of the assayed Ilr$^-$ clones appeared to have a pleiotropic mutation that gave them an N$^-$O$^-$P$^-$ phenotype, suggesting the production of an inducible inhibitor of λ gene expression or activity. Accordingly, we examined the ability of λ wt and λ*imm*^{434} to plate at 42°C on lawns of randomly selected RK$^-$ Ilr$^-$ survivors from four RK$^+$ strains (Table 2). Some survivor clones were found to support the growth of both phage. Others permitted the plating of λ*imm*^{434}T but not λ wt and could represent clones where the mutation responsible for the RK$^-$ phenotype was either a reversion of the *cIts857* mutation, a second site mutation that restored repressor activity at 42°C, or one that produced the Hyp phenotype (6). A third type, having the Nie phenotype, prevented the plating of both the *imm*$^\lambda$ and *imm*^{434} phages at 42°C. In a detailed analysis of 261 RK$^-$ Ilr$^-$ survivor clones, the Nie phenotype was exhibited by 31% of 174 RK$^-$ clones derived from four N$^+$ *cro*$^+$ RK$^+$ or N$^+$ *cro*$^-$ RK$^+$ strains (Table 2) and by 65.5% of 87 RK$^-$ clones derived from N$^-$

TABLE 2. Exclusion characteristics of RK$^-$ Ilr$^-$ survivors[a]

Phage plating ability at 42°C[b]		Clones from selector strains:	
λ*imm*^{434}T	λ wt	N$^+$*cro*$^+$O$^+$P^{+c}	N$^+$*cro*$^-$O$^+$P^{+d}
+	+	69	22
+	−	11	18
−	−	10^e	44^e

[a] All RK$^-$ survivor isolates were spontaneously derived.

[b] Minus values are indicated where the EOP was <0.001. No clones were observed that enabled plating at 42°C of λ wt but excluded λ*imm*^{434}.

[c] 50 RK$^-$ isolates from RK$^+$ strain Y832 and 40 RK$^-$ isolates from Y836.

[d] 44 RK$^-$ isolates from Y834 and 40 RK$^-$ isolates from Y838.

[e] Clones with the Nie phenotype.

TABLE 3. Nie phenotypes

Plating phage	EOP on the following RK$^-$ survivor isolate class[a]:			
	A		B	
	30°C	42°C	30°C	42°C
λ wt	<2 × 10^{-9}	<2 × 10^{-9}	<2 × 10^{-9}	≤10^{-6b}
λ*imm*^{434}T	1.0	<3 × 10$^{-9c,d}$	1.0	<10$^{-3c,d}$
λ*vir*	1.0	1.0	1.0	1.0
λ*se*^{e}	<2 × 10^{-9}	1 × 10 to 5 × 10^{-7f}	<2 × 10^{-9}	0.4^g

[a] Class A RK$^-$ isolates included Ilr125d (parent strain, Y832), Ilr201a, Ilr208a (parent strain, Y836). Class B RK$^-$ isolates included Ilr100a, Ilr101b, Ilr109b, Ilr109e (parent strain, Y838), Ilr141e, Ilr145c, Ilr145d (parent strain, Y843).

[b] The precise EOPs vary because of difficulty counting minute plaques at low dilution; the average on seven class B Ilr$^-$ isolates was 8.5 × 10^{-7}.

[c] 10 of 12 screened *imm*^{434} phage plated with EOPs as low as or lower than that of λ*imm*^{434}T.

[d] EOPs, which varied for reasons described in footnote *b*, were <9 × 10^{-6} on isolates Ilr100a, Ilr109e, Ilr145c, and Ilr145d, 1 × 10^{-6} on Ilr100b, 9 × 10^{-4} on Ilr109b, and 5 × 10^{-5} on Ilr141e.

[e] Similar results were obtained with phage containing *se* mutations 100a, 101b, 109b, 109e, 145c, 145d, 145e.

[f] Range in EOPs of seven λ*se* phage on isolate Ilr125d.

[g] Average for 49 platings (seven λ*se* phage on seven class B isolates) compared with EOPs on hosts TC600 or W3350.

cro$^+$ RK$^+$ or N$^-$ *cro*$^-$ RK$^+$ strains (Hayes and Tuer, unpublished data).

Among the RK$^-$ Ilr$^-$ clones with a Nie phenotype, two classes were identified (Table 3). Each survivor class exhibited typical λ immunity at 30°C, preventing the plating of λ*imm*$^\lambda$ but not λ*imm*^{434} phage. Neither class A nor class B isolates supported plaque formation by these phage at 42°C. The class B survivors were distinguished because they permitted the formation of rare plaques at a somewhat higher frequency than did type A survivors (rare plaque formers were not observed on A isolates). We chose the class B RK$^-$ survivor clones to isolate λ mutations suppressing the Nie phenotype. These class B clones all arose from N$^+$ RK$^+$ selector strains carrying either the λ fragments *c*III *cI857cro27* P or N *cI857cro27* P. None formed an antiimmune state, indicating that they retained a Cro$^-$ phenotype (8). Because of the difficulty of plating on the class A isolates, it is unclear whether they remained *cro*$^+$. The ability of an RK$^-$ survivor to express the Nie phenotype appears not to be simply dependent upon its Cro activity, since survivor isolates exhibiting Nie activity were isolated from both *cro*$^+$ and *cro*$^-$ selector strains (Table 2) (see also Discussion).

Lambda mutations suppressing nonimmune exclusion. Phage carrying spontaneous *se* mutations were obtained from λ wt as described in Methods. Each formed about 1.5-mm cocarde (clear center, turbid ring, clear periphery) plaques at 42°C on strains TC600 and W3350 and on λ*imm*^{434} lysogens of these hosts, but did not plate on TC600(λ). Their EOP at 42°C was about half that obtained when they were plated at 30°C. The results of coinfection of the RK$^-$ Ilr$^-$ survivor isolates with λ wt plus the corresponding λ*se* isolate (Table 4), suggested that each of the *se* mutations was recessive and appeared to be *cis* acting, since the λ*se* phage were unable to help λ wt give a burst at 42°C on Nie$^+$ Ilr$^-$ clones. Alternatively, examination of the progeny from these coinfections of Ilr$^-$ isolates with λ*se* and λ wt revealed that the bursts for the λ*se* phage were reduced about 10-fold (Table 4) when compared with single bursts of λ*se* phage on W3350 (see Table 7), suggesting that the coinfecting λ wt interfered with the process whereby λ*se* phage escape nonimmune exclusion. (The seven individual λ*se* phage

TABLE 4. *cis-trans* activity of *se* mutations[a]

Coinfected RK⁻ isolate	λ*se* phage	Ratio of turbid plaques to clear plaques[b]
Ilr145c	λ*se*145c	<0.0003
Ilr145c	λ*se*145d	<0.0002
Ilr145d	λ*se*145c	<0.0005
Ilr145d	λ*se*145d	<0.0001

[a] Test to determine whether λ*se* phage can help a coinfecting λ wt to grow vegetatively and escape nonimmune exclusion at 42°C in an induced RK⁻ Ilr⁻ Nie⁺ survivor isolate. Similar results were obtained for analogous coinfections of Ilr⁻ isolates 100a, 101b, 109b, 109e, and 141e with λ wt and each of the other λ*se* phages.

[b] The actual ratios of turbid plaques (λ wt) to clear plaques on TC600 were <1 × 10³/3 × 10⁶, <1 × 10³/5 × 10⁶, <1 × 10³/2 × 10⁶, and <1 × 10³/8 × 10⁶ for the coinfections shown. The bursts of 1 to 2 obtained on the host TC600 for λ*se* phage from the mixed infections (i.e., clear plaque formers) were reduced about 10-fold compared with those from single infections on Ilr⁺ hosts. Confirmation of λ*se* phage among progeny was determined by plating on Ilr⁻ host cells, i.e.; each coinfection was plated to TC600, Ilr145c, and Ilr145d. The observed PFU from coinfection were somewhat higher on TC600 than on Ilr⁻ host strains (see the text).

isolates plated at 42°C on class B Ilr⁻ isolates at about 0.1 or better [average, 0.4 times] their EOP on a permissive host (Table 3). A similar reduction in EOP was observed for λ*se* progeny from the coinfection with λ wt (Table 4) when plaque titers on Ilr⁻ and TC600 hosts were compared.)

The *se* mutations did not confer a *vir* phenotype since λ*se* phage were unable to form plaques on *imm*^λ wt or *c*Its857 lysogens. The formation of clear plaques by the λ*se* isolates on several suppressing and nonsuppressing hosts suggested that the *se* mutations negatively influenced lysogenization. The ability of these phage to lysogenize *E. coli* and to complement for λ genes *c*III, *c*I, and *c*II (Table 5) was examined. None of the λ*se* isolates was capable of lysogenization; whereas, the infecting λ wt phage from which they were derived lysogenized 60 to 70% of infected cells (data not shown). In the complementation assay measuring the ability of two coinfecting phage to establish lysogeny (measured by the appearance of turbid cell growth between two partially overlaying clear plaques), each λ*se* isolate complemented, as λ*c*I⁻ phage, for defects in genes *c*II and *c*III carried on *imm*^λ but not *imm*^434 phages. The lysogenization and complementation results revealed that λ*se* isolates were defective in either the λ repressor gene or

TABLE 5. Complementation by λ*se* for λ markers

Phage marker	λ*se* isolates[a]
*c*I26, *c*I47, *c*I72	−
*c*II68	++++
*c*III67	+++
*c*y42	−
imm^434*c*Ids	−
imm^434*c*II68	−
imm^434*c*III67	−

[a] The phage were considered to complement for the designated marker (strong positive results indicated by +++ or ++++) if a turbid cell growth line was observed within the overlapping portion of the otherwise clear plaques. No complementation was indicated (−) by the absence of a turbid cell growth line between all overlapping plaques on plate. Negative complementation results were verified by repetitive assays. Identical results were obtained for λ*se* phage with mutations 100a, 101b, 109b, 109e, 141e, 145c, and 145d. In control experiments, λ*c*I⁻ gave identical results to those shown for λ*se* phage. λ*c*II68 complemented λ*c*I⁻, λ*c*III67, λ*imm*^434*c*I⁻, and λ*imm*^434-*c*III67. λ*c*III67 complemented λ*imm*^434*c*I⁻ and λ*imm*^434*c*II68 as well as λ*c*I⁻ and λ*c*II68.

in target sites (e.g., *p*RM, *o*R, *p*RE) required for the establishment or maintenance of repressor transcription.

Influence of *se* mutations on replicative inhibition. Upon coinfection of a λ lysogen with λ wt plus the hybrid phage λ*imm*^434 (sharing identical replication genes) the heteroimmune *imm*^434 phage was observed to predominate by 20-fold or more over the *imm*^λ phage in the burst (24). The impaired replication of the homoimmune *imm*^λ phage, described as replicative inhibition, was explained by the assumption that repressor molecules made by the prophage in the coinfected lysogenic cell prevented replication of the homoimmune phage, even when λ replication initiation proteins (gpO and gpP) were provided in *trans* by the heteroimmune phage. The explanation that the repressor blocked replication of the homoimmune phage by inhibiting its transcriptional activation was supported by observations showing that replication inhibition was suppressed (i) by mutation of the homoimmune phage in its rightward operator, *o*R, causing *p*R to become insensitive to repression, or (ii) by base changes creating new promoter sites downstream from *p*R, exempli-

TABLE 6. Phage sensitivity to replicative inhibition

imm^λ phage	Burst: *imm*^434 phage/*imm*^λ phage ratio
Test phages	
λ*se*100a	1.6
λ*se*101b	2.6
λ*se*109b	3.7
λ*se*109e	2.0
λ*se*141e	4.5
λ*se*145c	3.0
λ*se*145d	3.9
Control phages	
λ wt	29.7
λ*c*I72	26.2
λ*c*I90*y*c17	0.7
λ*c*y42	11.6[b]
λ*prm*116	4.8
λ*c*Its857*prm*116	10.0
λ*c*Its857(18,12)^P22	120.9[b]

[a] Results shown represent the average *imm*^434/*imm*^λ bursts for separate coinfections with (i) λ*imm*^434T plus λ*imm*^λ and (ii) λ*imm*^434*c*I⁻ (*c*Ids) plus λ*imm*^λ. The *imm*^434/*imm*^λ burst ratio from infection of W3350(λ) was divided by the *imm*^434/*imm*^λ burst ratio from infection of W3350 in order to take into account the relative efficiency for growth of each coinfecting phage in the equivalent nonlysogenic host. For a sample calculation, single bursts from infection of W3350 with λ*se*109b or λ*imm*^434*c*Ids were 13.09 and 20.16. Single bursts from infection of W3350(λ) with λ*se*109b and λ*imm*^434*c*Ids were 0.16 and 16.37. Upon coinfection of W3350 with each phage, the burst of *imm*^434 phage was 14.97 and that of *imm*^λ phage was 5.4. The *imm*^434/*imm*^λ burst ratio on W3350 was 2.77. Upon coinfection of W3350(λ) with λ*se*109b plus λ*imm*^434*c*Ids, the burst of *imm*^434 phage was 12.88 and that of *imm*^λ was 2.06. The *imm*^434/*imm*^λ burst ratio on W3350(λ) was 6.25. The *imm*^434/*imm*^λ ratio on W3350(λ) (i.e., 6.25) divided by the *imm*^434/*imm*^λ ratio on W3350 (i.e., 2.77) was 2.26. Similarly, the single bursts of λ*imm*^434T on W3350 and on W3350(λ) were 17.80 and 17.78. Upon coinfection of W3350 by λ*imm*^434T and λ*se*109b, the *imm*^434/*imm*^λ ratio of the progeny was 4.01/6.26 = 0.64, and in the analogous coinfection of W3350(λ), the ratio of bursts was 3.98/1.20 = 3.32. The *imm*^434/*imm*^λ ratio on W3350(λ) (i.e., 3.32) divided by the *imm*^434/*imm*^λ ratio on W3350 (i.e., 0.64) was 5.12. The average *imm*^434/*imm*^λ burst ratio was (2.26 + 5.12)/2 = 3.7. Single bursts for each λ*se* isolate on W3350 and W3350(λ) were about as noted for λ*se*109b. The single bursts on W3350 and W3350(λ) for representative *imm*^λ phage were, respectively: λ wt, 9 and <0.01; λ*c*I72, 40 and 0.08; λ*c*y42, 4.4 and 0.0004; λ*c*I90*y*c17, 13.1 and 8.6; λ*c*I857(18,12)^P22, 6.2 and 0.001.

[b] Results only for coinfections of W3350(λ) and W3350 with λ*imm*^434*c*I⁻ plus λ*imm*^λ.

TABLE 7. Deletion mapping of λ*se* mutations

λse mutation	Frequency of turbid recombinant λ wt plaques (10⁻⁵) with the following phage[a]									
	bio3h-1	bio30-7	biot124	cIΔKH54	spi173	spi275	spi113	spi274	imm434cI	spi156
100a	926	114	42	13	25	17	5	<1	<1	<1
101a	433	159	56	23				7	<1	<1
109b	500	126	67	22				31	<1	<1
109e	667	120	76	33				24	<1	<1
141e	397	105	55	46				27	<1	<1
145c	556	172	123	36				14	<1	<1
145d	667	166	47	46				39	<1	<1

[a] Ratio of turbid plaques (from λ wt recombinant) to clear plaques (formed by both the input λ*se* and λΔ phages). The order of markers is as follows: N *bio*3h-1 cI [*bio*30-7, *biot*124, *cI*ΔKH54, *spi*173, *spi*275] sM o_R3 *spi*113 *prm*116 *spi*274 o_R2 o_R1 cro *imm*434cIy cII *spi*156.

fied by *c*17 and by four *ri*ᶜ (replication inhibition constitutive) mutations (4, 5).

We examined whether the *se* mutations influenced o_R activity by determining if they could suppress replicative inhibition (Table 6). The *se* point mutations were found to reduce λ sensitivity to replicative inhibition by about 10-fold. Interestingly, the *prm*116 mutation, which falls between o_R3 and o_R2 (21) also reduced phage sensitivity to replicative inhibition to about the same extent as did the *se* mutations. As expected, both λ wt and a cI⁻ derivative were sensitive to replicative inhibition. The *c*17 mutation enabled the phage to completely overcome it. The results also revealed that when a coinfecting *imm*^λ phage depended upon the expression, in *cis*, of its own downstream replication genes (i.e., for synthesis of required replication functions, where they are not provided by the heteroimmune helper; for example, the coinfection of λcIts857(*18,12*)^P22 and λ*imm*434), the *imm*^λ hybrid phage was even more sensitive than λ wt to replicative inhibition.

Characterization of *se* mutations. The nature and map position of the *se* mutations were examined by analysis for phage deletion and by deletion mapping. Both λ wt and the λ*se* isolates were similarly inactivated by exposure to EDTA (number of phage [treated and untreated, respectively]: λ, 9 × 10⁵ and 9 × 10⁹; λ *se*100a, 1.5 × 10⁶ and 9.6 × 10⁹; similar results were obtained with *se* mutations 101b, 109b, 109e, 141e, 145c, and 145d). This treatment selects for phage with deletions representing (at least) about 3% less DNA than λ wt (16). A control phage λ*bio*30-7Δ*nin*5 tested in parallel was not inactivated by the same treatment (number of treated and untreated phage, respectively: 1.7 × 10¹⁰ and 1.5 × 10¹⁰). The EDTA inactivation of λ*se* phage isolates suggested that the *se* mutations comprised either point mutations or small deletions (i.e., less than 3% λ).

The map position of each *se* mutation was determined genetically by scoring for turbid λ wt recombinants in crosses between λ*se* and λ*bio* or λ*spi* substitution phages (Table 7). All seven *se* mutations were mapped within *imm*^λ, to the right of gene cI. The leftmost mutation, *se*100a, mapped between markers *spi*113 and *spi*274. Mutation *se*101b mapped just to the right of *spi*274, and the remaining mutations mapped even further to the right. The approximate rightward endpoint of *spi* deletions 113 and 274, assigned as including through bp 37,969 and 37,973, respectively, were determined by mRNA protection experiments (22) and considered accurate to within a few base pairs (20).

DNA from λ wt and the λ*se* phages was extracted, digested with restriction endonucleases, and end-labeled, and the separated fragments carrying *se* mutations were sequenced (Fig. 1). All seven *se* mutations were base substitutions (Fig. 2). The *se* mutations 100a and 101b, (C:G →

A:T and G:C → T:A transversions, respectively) fell within o_R2 in bp 37,979 and 37,985 to the right of the polymerase recognition (−35 sequence) region for the repressor maintenance promoter p_RM. The five independent *se* mutations 109b, 109e, 141e, 145c, and 145d each were the same G:C → T:A transversion at bp 38,009 within o_R1 and the overlapping p_R promoter for rightward transcription. The *se* mutation 100a and the group typified by 109b, respectively, fell at the positions altered by operator mutation *v*1 within o_R2, and mutations *v*C1 and *v*s387 within o_R1, although they were not identical base substitutions (Fig. 2).

Each *se* mutation had the potential for destabilizing palindromes formed by o_R2 and o_R1 by being able to eliminate the formation of the topmost base pair between each stem and loop (Fig. 3). The p_RM mutation *prm*E93 within o_R2 (21), at the same site as *se*100a and operator mutation *v*1, also has this property.

DISCUSSION

Among the RK⁻ clonal isolates that support plaque formation by λ*vir* and have a functional immunity region, many
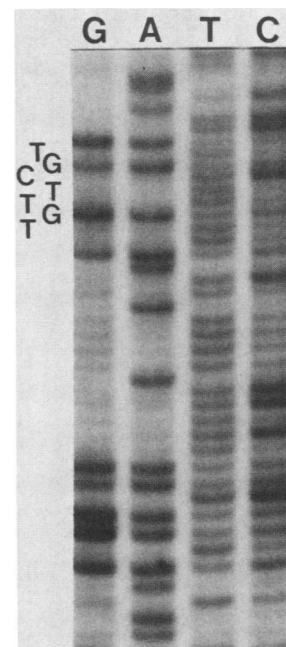

FIG. 1. DNA sequence change in λ *se* mutation 101b (G:C → T:A) within o_R2. Sequenced positions for the remaining *se* mutations are indicated in Fig. 2.

```
            oR3                      oR2                      oR1              sR
        _____              _____              _____        |--->
       /          \    /          \    /          \    /          \          |
                                              _____    _____      |
A TGCAATTTAGATAGTGGCGTTCCCTATTTATAGATTGTGGCACGCACAACTGATAAAATGGAGACCGCCACTATTACCAACG T 5'
  !      !      !      !      !      !      !      !      !
 -80    -70    -60    -50    -40    -30    -20    -10

            -10    -20    -30    -40    -50    -60    -70    -80
             !      !      !      !      !      !      !      !
5' T ACGTTAAATCTATCACCGCAAGGGATAAATATCTAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGC A
     !    _____  !      _____ ! !    !      !        !      ! ! !
 <---!              A            !  C!          !    C        !    T  ! ! !
                    T            !  G!          !    G      vs326___A T! !
  sM               E37           T  E104        !    x3      v3,UV8___A! !
                                 116 !          !                     T!
                                 U31 !          !           M36,U93___A!
                                 M104 37979   37985                  38009
                                      T         A                     A
                                      A         T                     T
                                      se100a  se101b       se109b, se109e, se141e
                                      v1                   se145c, se145d, vC1
                                      !                           !
                                      A                      G       T
                                      T                      vs387,    UV11
                                      E93                    C         A
```

FIG. 2. DNA sequence of the upper and lower strands of the p_{RM} o_R p_R region and changes caused by *se* mutations. The p_{RM} and p_R promoter regions are numbered (between strands) from their RNA start sites s_R and s_M, respectively. Base positions are as described by Daniels et al. (3). Mutations E37, 116, U31, M104, E104, UV8, U93, M36, and E93 were isolated as defects in p_{RM} (20, 21). The sequenced locations for o_R mutations *v*1, *v*C1, *vs*326, *vs*387, and *UV*11 and p_R mutation *x*3 are described by Daniels et al. (3). The Pribnow box and consensus sequence for each promoter are shown by dashed lines (8). The bottom and top (*l*- and *r*-strand) sequences are oriented with terminal 5' phosphoryls to the left and right, respectively.

are phenotypically N⁻ O⁻ P⁻ and express the Nie phenotype. The preponderance of these isolates led us to explore the mutational defects responsible for nonimmune exclusion.

The Nie phenotype is only demonstrable when the Ilr⁻ λ prophage fragment is thermally induced, suggesting the synthesis of an inhibitor of λ gene expression or activity. The Nie inhibition would appear to be directed toward the initiation of transcription from p_R since both λ*vir* and λ*se* escape it, were it not for the exclusion of *imm*[434] phage (10 of 12 different *imm*[434] phage were excluded [unpublished data]). Blotting experiments with an *N* through *P* λ probe of *Bam*HI and *Bam*HI-*Eco*RV digests of DNA extracted from the seven class B RK⁻ Ilr⁻ isolates revealed that five strains have insertions. One insertion is near the rightward *imm*[21] marker; the remainder are within the fragment formed by

*Eco*RV cuts within *O* and the contiguous stretch of *E. coli* DNA downstream from *ren* (unpublished data). We conclude that mutational inactivation of the replicative killing capacity of an induced λ fragment (and by inference, its ability to initiate replication) can confer the Nie phenotype. The ilr⁻ mutations responsible for the Nie phenotype can occur at more than one site within the λ replicator region.

We examined the variability of the Nie phenotype by determining the plating ability of 61 λ and λ hybrid phage on culture overlays of each of the seven isolates (unpublished data). These studies and potential explanations for nonimmune exclusion are being reported elsewhere. However, the relevant observation with respect to the present report is that among 26 phages with missense mutations in gene cI (e.g., point, amber, conditional lethal, and *trans* dominant repressor mutations), 9 plated with about the same efficiency

```
      A               A               A             C             C
   C     C         C     C         C     C       C     G       C     G
   G     G         G     G         G     G       A     C       A     C
    G-C             T     C         G     A        G-C           G     A
    T-A             T-A             T-A            A     A        A     A
    G-C             G-C             G-C             G-C           G-C
    T-A             T-A             T-A            G  T          G  T
    T-A             T-A             T-A            T-A           T-A
   A     C         A     C         A     C        A-T           A-T

    oR2            se100a          se101b          oR1        se109b, 109e,
                                                              141e, 145c, 145d
```

FIG. 3. Influence of *se* mutations on palindromic structures that can be drawn for o_R2 and o_R1. Only the *r* strand for each operator region is drawn, with an N to P (left to right) orientation of the λ map (Fig. 2).

as did λ*se*, although most formed pinpoint plaques. Clearly, it is not essential that the plating phage be defective in o_R for it to escape nonimmune exclusion. Also, as a rationale for the plating results of λ*se* phage on the class A and B RK⁻ ilr⁻ isolates (each derived from *cro*⁺ and *cro*⁻ parental RK⁺ strains, respectively [Table 3] it was suggested to us that only a double mutant o_R^- *cro*⁻ might form plaques on a *cro*⁻ host (the *cro*⁺ host would complement for Cro). We do not view this hypothesis as tenable since, as mentioned, some *cro*⁺ λcI⁻ phage efficiently plate on the class B isolates, and, additionally, λcIts*cro27* was found to be totally unable to plate on the class B isolates at 30, 39, or 42°C (only Ilr145d permits reduced plating at 39°C [unpublished data]).

The *cis* dominant λ *se* point mutations confer several properties upon otherwise wild-type λ phage, making them defective for lysogenization of *E. coli*, insensitive to replicative inhibition, and able to escape nonimmune exclusion. The *se* mutations all map within o_R2 and o_R1 at −39, −45, and −69 bp from s_M, outside of the p_{RM} sequence regions necessary for RNA polymerase recognition and binding. Phage that acquire one operator mutation in either o_R2 or o_R1 cannot lysogenize because repressor occupancy of o_R1 and o_R2 (but not o_R3) is required for lysogeny (12). o_R1 mutants are not virulent because the interaction between repressors at o_R2 and o_R3 ensures that p_R expression is turned off by concentrations of repressor found in a lysogen (12). o_R2 mutants are not virulent because the binding of repressor to o_R1 is affected only slightly by mutation of o_R2 (18). By these criteria, each *se* mutation appears to inactivate o_R, since it prevents lysogeny yet does not confer virulence.

Rosen and Gussin (20) obtained P_{RM}^- mutants mapping within o_R2 (*prm*E104 and *prm*E93, −38 and −39 bp, respectively, from s_M) and o_R1 (*prm*UV8 and *prm*U93, −67 and −68 bp from s_M). The mutations E104 and E93 were circumstantially considered to be p_{RM} mutations, although E104 exerted a much stronger negative effect on *cI rex* expression than did E93 (21) and influenced the interaction of RNA polymerase with p_{RM} (8). The p_{RM}^- phenotype of the mutations within o_R2, and especially those within o_R1, was considered to be due entirely to an inability to bind repressor and, thus, to an effect on repressor activation of p_{RM} (21).

The *se* mutations within o_R2 and o_R1 each confer a p_{RM}^- (as well as p_{RE}^- ?) phenotype, since they eliminate *cI* expression, as measured by the inability of λ*se* phage to complement *imm*^λ phage with *cI*⁻ mutations or a *cis*-acting *cy42* mutation in p_{RE}. The inability of the *se* mutants to complement λ*cy42* distinguishes them from the p_{RM}^- mutants isolated by Rosen and Gussin (20). The latter selection was based upon the ability of λ*cI857* mutants to form clear (or partially clear) plaques, to complement λ*cy42* but not *cI*⁻ mutants for lysogenization, and to map to the right of *spi275*. The presence of the 857 marker in parental phage increases the selection sensitivity for weak p_{RM} mutations (20). The inability of λ*se* phage to complement λ*cy42*, λ*imm*⁴³⁴*cIII*⁻, or λ*imm*⁴³⁴*cII*⁻ suggests that the *se* mutations produce significant operator defects that prevent the repressor from activating p_{RM} and confer partial virulence, both for potential λ*cy* and λ*imm*⁴³⁴ lysogens.

Mutation of both o_R2 and o_R1 is required to eliminate repression of p_R (18). Nevertheless, we find that phage with *se* mutations escape repressor-dependent replicative inhibition and that the phage with *se* mutations in o_R2 are as insensitive to replicative inhibition as those with *se* mutations in o_R1. This latter result seems contrary to what would be expected (8) from studies of repressor action, where o_R1

is the preferred site of repressor binding. In addition, our finding that point mutation *prm116* suppresses replicative inhibition for an otherwise o_R2^+ o_R1^+ phage to about the same extent as do *se* mutations is not adequately explained by the model in which the repressor can prevent replication initiation by inhibiting transcriptional activation through the direct inhibition of p_R transcription. We question whether the ability of λ*se* phage to escape replicative inhibition is dependent upon their p_{RM}^- or o_R^- phenotype.

The ability of λ*se* phage to escape replicative inhibition suggests either that repressor inhibition of p_R transcription is significantly reduced by the *se* mutations in o_R2 or o_R1 or that the *se* mutations enhance p_R activity and in turn reduce p_{RM} activity. It is conceivable that o_R1 and o_R2 mutations confer a p_{RM}^- phenotype because they strengthen p_R by facilitating RNA polymerase binding. Accordingly, RNA polymerase bound to p_R (where either o_R1 or o_R2 was defective) would exclude binding of polymerase to the weaker promoter, p_{RM}. In the absence of o_R mutations, polymerase binding at p_R was found to have little inhibitory effect on binding of polymerase to p_{RM} (15).

There is no evidence that the *se* mutations negatively influence the initiation of transcription from p_R, even though the five independent *se* mutations represented by *se109e* also mutate p_R at a site 14 bp upstream from s_R. For example, each λ*se* phage gave good bursts on *E. coli* hosts W3350 and TC600. p_R can apparently function with any base at position −14, since each possible sequence variation is available among sequences for λ wt and mutations *se109e*, *vs387* and *UV11*. Rosen and Gussin (20) identified a p_{RM} mutation (*prm*E37) within o_R3 that fell 14 bp upstream from s_M. This mutation was reported to reduce p_{RM}-directed gene expression 20-fold in vivo and to be defective in transcription initiation from p_{RM} (8, 21). The powerful p_R promoter may be less sensitive to a single base change at position −14 than is the weaker p_{RM} promoter. Alternatively, the negative influence of a *se* mutation on the initiation of transcription from p_R might go unnoticed because the effect is counterbalanced by the mutation's inactivation of o_R1, which in turn reduces repressor expression and the consequent repression of p_R transcription. However, this interpretation is not in agreement with the observation that λ*se* phage escape *imm*^λ prophage-dependent replicative inhibition.

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