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Integration-negative Mutants of Bacteriophage Lambda

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Mutants of bacteriophage lambda unable to form stable lysogens have been obtained by a simple screening procedure. These mutants, designated *int*, form turbid plaques. The procedure used for their isolation also allows the titration and selection of rare lysogens in a predominantly non-lysogenic population. The recombinational behavior of *int* mutants in wild-type and recombination-deficient (*rec*) bacteria suggests that the *int*$^+$ gene determines a site-specific function involved in normal prophage integration and detachment. The Int function can be provided in *trans* by an *int*$^+$ phage. The expression of the *int*$^+$ gene is subject to repression by the phage immunity substance.

Single *int* lysogens give a low yield of active phage upon induction, whereas double lysogens, when tandem, give nearly normal phage yields. Both single and double *int* lysogens give large yields of transducing particles. Recombination between vegetative *int* phages and productive induction of *int* lysogens, both of which occur in recombination-deficient bacteria, may be attributed to a separate function (Red) operative in phage-infected or induced cells. The Red function is not available for use in integration of a superinfecting phage, a function which the product of the bacterial *rec*$^+$ gene can accomplish.

1. Introduction

Bacteriophage λ engages in two kinds of genetic recombination: (1) mating between λ genomes and (2) localized recombinations between λ and the bacterial chromosome. Recombinations of the first kind occur during vegetative growth of phage and involve the substitution of genetic regions of one phage genome by homologous regions of another (Meselson & Weigle, 1961; Kellenberger, Zichichi & Weigle, 1961*a*). Thus, if a sensitive bacterium is mixedly infected with genetically marked λ, the burst will contain recombinant particles in which the genotypes of the input phage have recombined.

Recombinations of the second kind occur in the formation of stable lysogens following infection and in the transition to the vegetative state following lysogenic induction. Lysogenization by λ entails the linear integration of λ DNA into the bacterial chromosome (Calef & Licciardello, 1960; Campbell, 1963; Franklin, Dove & Yanofsky, 1965; Rothman, 1965; Signer, 1966; Hoffman & Rubenstein, manuscript in preparation). The integration process is visualized as recombination between closed forms of the two genomes at the b2$^+$ region of λ and a specific, perhaps homo-

logous, region of the *Escherichia coli* chromosome (Campbell, 1962; Zichichi & Kellenberger, 1963). The reciprocal event, occurring early in the process of phage induction, can account for prophage detachment (Campbell, 1962; Weisberg & Gallant, 1967).

Bacteria deficient in chromosomal recombination have been described (Clark & Margulies, 1965). When these *rec* bacteria† are mixedly infected with genetically marked bacteriophage λ, the mating of phage genomes is unimpaired (Brooks & Clark, 1967; Zissler, 1967). Likewise, the defect in *rec* bacteria does not interfere with lysogenization and prophage detachment (Brooks & Clark, 1967; Hertman & Luria, 1967). Thus, λ can provide recombination-deficient bacteria with one or more phage-determined functions enabling its own genome to engage in recombination of both kinds.

The existence of a site-specific phage function involved in the process of integration has been adduced from the behavior of deletion mutants of φ80 unable to lysogenize normally unless "helped" by wild-type phage with the appropriate attachment specificity (Signer & Beckwith, 1966). Such mutants differ from λb2 deletion mutants, which have been reported not to form stable single lysogens in *E. coli* K12 under any conditions (Kellenberger, Zichichi & Weigle, 1961*b*; Zichichi & Kellenberger, 1963). Whereas the defect in λb2 appears to be structural (Campbell, 1965), resulting in an inability to pair appropriately for lysogenization (Fischer-Fantuzzi, 1967), the φ80 mutants of Signer & Beckwith (1966) appear deficient in a diffusible enzyme.

We describe here the isolation and properties of non-integrating mutants of λ the characteristics of which suggest that they are point mutants deficient in a site-specific integration function. These mutants also exhibit defects in prophage detachment upon induction and in superinfection curing. Thus, *int* mutants appear defective in performing recombinations of the second kind. However, they do recombine with one another in a recombination-deficient host, i.e. the *int* defect does not markedly impair recombinations of the first kind. The two kinds of genetic recombination, therefore, are resolved genetically. The existence of recombinations between *int* phages in a *rec* host requires that there be an additional recombination function. Mutants of λ defective in this function have been isolated recently (Signer; Echols, personal communications) and they are designated *red* for recombination-deficient. Deletion mutants defective in the same function have been isolated as well in φ80–λ hybrid phage by Franklin (1967). In what follows we distinguish the influence which each of the three recombination functions, Int, Rec and Red, exerts upon recombinational events in which λ participates.

The isolation of phage mutants with similar properties has been achieved independently in several laboratories (phage P22: Smith & Levine, 1967; phage λ: Zissler, 1967; Gingery & Echols, 1967). Deletion mutants of φ80 and φ80–λ hybrid phage, which appear similar in some respects, have been studied by Signer & Beckwith (1966), and Franklin (1967). In addition, deletion mutants of φ80–λ hybrid prophage, defective in prophage detachment, have been studied by Franklin *et al.* (1965), Gratia (1966), Dove (1967) and Franklin (1967).

† In conformity with the nomenclature proposed by Demerec, Adelberg, Clark & Hartman (1966), the following abbreviations are used: *rec*, *rec*$^+$ refer to mutant and wild-type alleles of a bacterial gene the Rec function of which is required for recombination. Similarly, *int*, *int*$^+$ and Int are used in reference to a phage-determined integration function and *red*, *red*$^+$ and Red in reference to a function involved in generalized recombination, which is found in phage-infected bacteria. The allele of supII conferring non-permissiveness towards amber mutants is designated supII (Signer, Beckwith & Brenner, 1965).

2. Materials and Methods

(a) *Media*

Tryptone broth (TB): 1% Tryptone, 0·5% NaCl, 10^{-3} M-$MgSO_4$ and 1·0 μg thiamine hydrochloride/ml. Tryptone agar: 1% Tryptone, 0·25% NaCl, 1·0 μg thiamine hydrochloride/ml. and 1·1% agar. Tryptone top agar: 1% Tryptone, 0·5% NaCl and 0·7% agar. Tryptone soft agar: 1% Tryptone, 0·5% NaCl and 0·35% agar. Luria Broth (LB) (Luria, Adams & Ting, 1960): 1% Tryptone, 0·5% NaCl and 0·5% yeast extract (Difco). MacConkey–galactose agar is the MacConkey agar of the *Difco Manual* (Difco Laboratory, Detroit, Mich.) except that galactose was substituted for lactose. All of the above media were adjusted to pH 7·0 with NaOH. EMBO agar is the EMB agar of Campbell (Campbell, 1957) with sugar omitted. The minimal medium, M56 (Monod, Cohen-Bazire & Cohn, 1951) was used diluted and appropriately supplemented with amino acids, thiamine and 0·2% glucose and solidified with 2% agar. Phosphate buffer: 0·7% Na_2HPO_4, $7H_2O$, 0·3% KH_2PO_4, 0·4% NaCl and 10^{-3} M-$MgSO_4$.

(b) *Sensitive bacteria*

The following *E. coli* K12 derivatives were used: *rec* strain 152 (Meselson, unpublished work) and its *rec*$^+$ parent, strain 28, Sm^r derivatives of W3102 galK2 (Lederberg, 1960) non-permissive towards *sus* phage mutants, from Dr M. Meselson; *E. coli* C600 (Appleyard, 1954) used in titrating *sus* phage mutants, from Dr F. Jacob; M5073, a supII homomerozygote (non-permissive) used as donor of F′1*gal*$^+$ (an F′ isolated by E. L. Wollman and described in Liedke-Kulke & Kaiser, 1967), from Dr E. Signer; PL2, defective in uridine diphosphoglucose 4-epimerase (epimerase) from Dr G. Buttin (Buttin, 1963*b*) used as recipient in *gal* transduction; W602, defective in biotin synthesis (Wollman, 1963), from Dr J. Rothman, used as recipient in *bio* transduction.

Bacterial counts were done by plating in soft agar.

(c) *Phages*

	Phage	Derivation or reference	Source
(1)	λ*susA11*	a	F. Jacob
(2)	λ*susN7,53*	a	J. Weil
(3)	λ*susP3*	a	D. Korn
(4)	λcI*72*	b	V. Bode
(5)	λcI*857*	c	F. Jacob
(6)	λcI*857susA11*	(1) & (5)	This work
(7)	λ*int6c*	λcI*857int6* spontaneous mutant	This work
(8)	λb2	d	C. A. Thomas, Jr.
(9)	λb2*c*	d	M. Gellert
(10)	λb2cI*857susA32susJ27*	(5), (8) & (15)	M. Yarmolinsky
(11)	434	e	F. Jacob
(12)	434hy	f	F. Jacob
(13)	434hycI*1*	(12) by ultraviolet mutagenesis	M. Yarmolinsky
(14)	434hycI*2*	(12) by ultraviolet mutagenesis	M. Yarmolinsky
(15)	434hy*susA32susJ27*	g	D. Korn
(16)	434hy*int6*	(12) & λ*int6*	This work
(17)	434hy*int6c*	(16) spontaneous mutant	This work
(18)	434hycI*1susP3*	(3) & (13)	M. Yarmolinsky
(19)	434hycI*1susP3int41*	(18) & λ*int41*	This work
(20)	434hy*susN7,53*	a & f	L. Pereira da Silva
(21)	21	e	H. Wiesmeyer
(22)	21gp	h	A. D. Kaiser
(23)	21*c*	(21) by ultraviolet mutagenesis	M. Yarmolinsky
(24)	21hy5	h	M. Liedke-Kulke
(25)	φ80	i	A. Weissbach

References: (a) Campbell, 1961; (b) Kaiser, 1957; (c) Sussman & Jacob, 1962; (d) Kellenberger, Zichichi & Weigle, 1961*a*; (e) Jacob & Wollman, 1956,1961; (f) Kaiser & Jacob, 1957; (g) Radding & Kaiser, 1963; (h) Liedke-Kulke & Kaiser, 1967; (i) Matsushiro, 1963.

Phage (13) was selected for the capacity to make a turbid plaque at 30°C and a clear plaque at 40°C. Lysogens of (13) are induced in Tryptone broth at temperatures above 36°C. The phage is not inducible by ultraviolet irradiation or by mitomycin C. Its properties will be described elsewhere.

Phage (14) forms a clear plaque at all normal incubation temperatures. It is complemented by phage (13) at low temperatures.

Lambda *cI857* was derived from λInd and is presumed to have retained this character in the several derivatives made from it.

(d) *Phage assays and phage stocks*

Assays of free phage and infective centers: pour-plates of indicator bacteria and phage on Tryptone agar were incubated at 39 to 40°C to distinguish the plaques of phages thermally induced at this temperature from those which are not. Higher temperatures were avoided because certain *sus* mutants do not plate efficiently on C600 above 40°C. Mixtures of 434 and λ were resolved by plating on C600(λ) and C600(434). Stocks of phage were obtained by mitomycin C (2 μg/ml.) or heat induction of appropriate lysogens grown in TB, by infection of bacteria in TB with 0·01 M-$MgSO_4$ added, or by the confluent lysis method using Tryptone soft agar.

Infection with phage was performed as follows: saturated overnight bacterial cultures were harvested, washed with phosphate buffer, suspended in 0·5 vol. of 0·01 M-$MgSO_4$, and aerated for 1 hr at 33°C. Bacteria thus treated did not lose viability or show altered sensitivity to phage for at least 4 weeks when stored at 5°C. The starved cells were mixed with phage suspended in TB, and adsorption was allowed to proceed for 20 min at 33°C. In all experiments, adsorption, measured as loss of free phage, was adequate to ensure infection of at least 80% of the cells.

(e) *Mutagenesis and preparation of* λint *mutants*

Mutagenesis of λ was performed essentially according to the method of Adelberg, Mandel & Chen (1965). C600(λcI*857*) was grown in 5 ml. TB at 33°C to about 10^8 cells per ml., and then aerated at 39°C for 15 min. The culture was chilled, harvested by centrifugation, and resuspended in 25 ml. of 0·05 M-Tris maleate buffer (pH 6·1) containing 10^{-3} M-$MgSO_4$ and 16 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wisc.). After incubation for 30 min at room temperature, the suspension was diluted 100-fold into TB, distributed among separate tubes, and aerated for 120 min at 38°C. Chloroform was then added and the phage plated at 33°C on strain 152 as indicator. The centers of normal-appearing, turbid plaques were picked to Tryptone agar and to EMBO seeded with about 10^9 co-immune clear-plaque phage. After overnight growth at 33°C, *int* mutants were identified by the dark mottled appearance of the colony arising on EMBO. The corresponding colony on Tryptone agar was transferred to TB, aerated for 2 hr at 39°C, and chloroformed. The *int* phage in the lysate was purified by repeated single-plaque isolations. Phage stocks were obtained by the confluent lysis method using Tryptone soft agar, or by induction of a tandem homogenotic lysogen constructed with the aid of helper phage.

Lambda *cI857susA11int21* was prepared by a similar technique from C600(λcI*857susA11*) using C600 indicator bacteria.

(f) *Construction of* int$^+$ *lysogens*

N103: 152(λcI*857susA11*), N173: 28(λcI*857susA11*), and N192: M5073 bearing an episomal 434hy prophage, were prepared by infection of *E. coli* strains 152, 28 and M5073, respectively.

(g) *Construction of* int *lysogens*

Sensitive cells were infected with λ*int* and hetero-immune *int*$^+$ helper, each at a multiplicity of about 10, and the λ lysogens were recovered from an EMBO plate seeded with about 10^9 λ*intc*. Alternatively, rare *int* lysogens could be obtained from EMBO plates spread with *int*$^+$ clear-plaque phage when cells were infected with an *int* mutant alone. The high concentrations of infected bacteria which must be applied to the EMBO plates

when helper is omitted results in the appearance of λ-resistant clones on the plate. Such clones may be detected by their slimy character at 33°C and/or their ability to give a pink colony on EMBO seeded with a hetero-immune clear-plaque phage, e.g. 434hyc.

Lysogens prepared without added helper were: N111: 152(λcI*857int6*), a low yielder; and N123: 152(434hy*int6*).

N214: 28(λcI*857int6*), a low yielder, was prepared with helper 21hy5. N121: 152(λcI*857int6*), a normal yielder; N122: 152(λcI*857int6*, 434hy); N171: 152(λcI*857susA11int6*) and N172: 28(λcI*857susA11int6*) were constructed with helper 434hy.

N201: 152(434hy*int6*, λcI*857int6*) was constructed by superinfecting N123 with λcI*857int6*; N178: 152(λcI*857int6*, 434hy*int6*) was obtained by superinfection of N111 with 434hy*int6*. Both lysogens were recovered from EMBO plates seeded with *int*$^+$ clear-plaque phage.

N202: 152(λcI*857int6*)/F′ (434hy) was constructed as follows: N111 was infected with 434hy*int6* and then mated with N192 grown in LB to about 2×10^8 cells/ml. Double lysogens were detected by spreading the mating mixture on an EMBO plate seeded with 434hy*int6c* and λ*int6c*. The double lysogens were then streaked on MacConkey–galactose plates and galactose-positive clones which gave frequent galactose-negative segregants, owing to loss of the *gal*$^+$ episome, were selected. The galactose-negative segregants were found to have lost 434 but not λ immunity.

N229: M5073 bearing an episomal λcI*857int6* was prepared by infecting M5073 with λcI*857int6* and helper 434hy. Lambda lysogens in which the λ had integrated into the episome were selected by their behavior on MacConkey–galactose plates. At 33°C such clones gave galactose-positive colonies whereas at 39°C, only galactose-negative segregants, cured of the episome and its prophage, survived.

N237: 152(434hy*int6*)/F′(λcI*857int6*) was constructed by infecting N123 with λcI*857int6* and mating with N229. Double lysogens, obtained as described for N202, were transferred to MacConkey-galactose plates. Those which gave galactose–positive colonies at 33°C and galactose-negative, λ-sensitive segregants at 39°C, were selected.

(h) *Complementation between* int *mutants*

Cells infected with one or more *int* phages were transferred by means of a platinum wire to EMBO seeded with clear-plaque λ. The wire was first dipped into the cell suspension and then stabbed into the EMBO plate. The presence of 10 lysogens (N103) among about 10^4 non-lysogens transferred with each dip can be detected.

(i) *Measurement of lysogenization frequency*

Infected bacteria were spread on EMBO plates seeded with co-immune clear-plaque phage. Lysogens appeared as pink-staining colonies after about 20 hr of incubation at 33°C. To eliminate phage-resistant clones and to detect lysogens carying a second hetero-immune prophage, the putative lysogens were transferred to EMBO plates seeded with clear-plaque phage of the appropriate immunity and scored as before according to the appearance of the resulting colonies.

Lysogenization frequencies were also determined by plating infected bacteria on Tryptone agar, where no selection pressure favoring lysogens is exerted, and subsequently transferring the colonies to EMBO-indicator plates. Equivalent results were obtained with both methods.

(j) *Titration of* gal *and* bio *transducing particles*

The epimerase-negative strain PL2 or the biotin-negative strain W602 was grown to saturation at 34°C and starved in 0·01 M-$MgSO_4$ for 1 hr. Strain PL2 at 2×10^9 cells/ml. or W602 at 5×10^8 cells/ml. was mixed with helper phage at a multiplicity of exposure of 4. The helper phage used in these experiments was λcI*857* induced from the corresponding lysogen. The phage to be titrated was added, and the mixture incubated for 20 min at 34°C. For assays of *gal* transduction, the mixture was diluted at least fivefold, and 0·05 ml. spread on MacConkey–galactose agar plates. These were incubated 22 to 24 hr at 34°C, and the red colonies counted. For assays of *bio* transduction, the mixture was poured in minimal top agar onto supplemented minimal plates and the colonies counted after 40 hr at 32°C. Assays were shown to be linear with respect to quantity of tranducing lysate added.

(k) *Measurement of* gal *escape synthesis*

The activity of UDP glucose: α-D-galactose-1-phosphate uridylyl transferase was assayed in centrifuged samples of cell suspensions essentially by the method of Buttin (1963*a*).

(l) *Superinfection curing*

A thermo-inducible *rec* lysogen was superinfected with a hetero-immune phage, diluted at least 50-fold in TB and aerated for 2 hr at 33°C to allow segregation of non-lysogenic nuclei. Prophage bearing *sus* mutations were used to minimize the possibility of re-infection of cured cells. The culture was then incubated at 41°C for 15 min to induce and kill lysogens, and plated for survivors at 39°C. The frequency with which these survivors had acquired the immunity of the superinfecting phage was determined as described above.

3. Results

(a) *Isolation of non-integrating* (int) *mutants and the selection of rare lysogens*

Plaques of wild-type λ are turbid because lysogenic immune cells arise and grow during formation of the plaque. Phage mutants which are unable to establish true lysogeny may form turbid plaques, the turbid centers of which are comprised of abortive lysogens. These continually give rise to non-immune progeny, which may become re-infected. If such abortive lysogens are picked from the center of a mutant plaque to a plate previously seeded with about 10^9 clear-plaque type co-immune phage, infection of the non-immune progeny by the clear phage kills these bacteria as they are formed. If the indicator phage are seeded on EMBO agar, the resultant partially lysed colonies are easily scored by their small dark and irregular appearance (Lederberg & Lederberg, 1953). True lysogens, which transfer immunity to all their progeny, invariably form a large pink colony. Plaques of the deletion mutant λb2, which cannot lysogenize, may be distinguished readily from plaques of λb2$^+$ by this test (Plate I, upper two rows).

The test may also be used to screen for *int* mutants in stocks of heavily mutagenized phage λ. As many as 5% of the turbid plaques score as *int*. Fifteen independent mutants have been isolated by this test (e.g. Plate I, rows 3 and 4).

In the majority of experiments described below, EMBO plates which had been spread with clear-plaque *int* phage were used in selecting or titrating infrequent true lysogens in a predominantly non-lysogenic population. Lysogenization frequencies have been corrected for the contribution of λ-resistant bacteria which appear on the plates with a frequency of about 3×10^{-4}.

(b) *Lysogenization by* int *phages on single and mixed infection*

Of fifteen independent *int* mutants obtained by *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine mutagenesis, 12 have been examined for complementation with λ*cI857int6* and all 15 for complementation with λb2. Typical results are presented in Plate I. Whereas all *int* mutants were complemented by λb2, no demonstrable complementation occurred among the pairs of *int* mutants tested. We have also tested our *int* mutants (6, 21 and 41) for complementation with *int4* isolated by Zissler (1967). No complementation was observed. This complementation behavior is consistent with the interpretation that all the mutations tested belong to a single cistron lying outside the region deleted in λb2.

When lysogens prepared by complementation between λb2 and 434hy*int* are analyzed, it is found that the complementation is non-reciprocal (Table I). When only

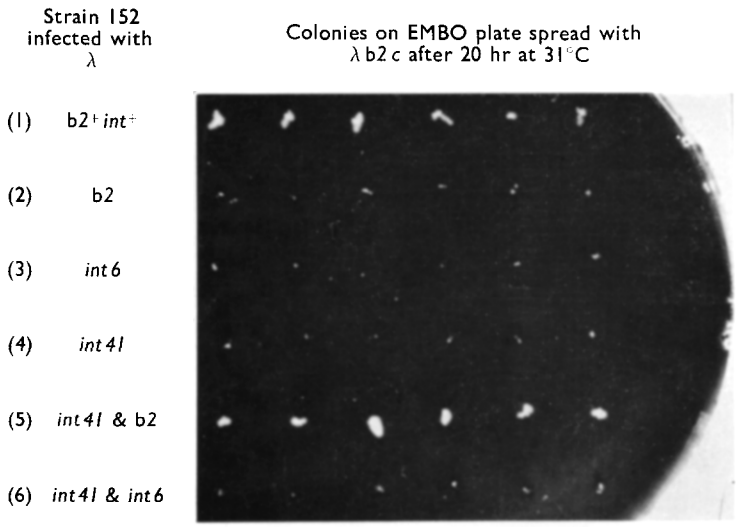

PLATE I. Spot test for the Int character.

Phages of each type were adsorbed to samples of strain 152 (10^8 viable cells/ml.) at a multiplicity of exposure of 10. After adsorption, a platinum wire was repeatedly dipped into the suspension of infected cells and stabbed into the EMBO plate, previously spread with 10^9 λb2*c*, to form a row. Incubation was as indicated. The relevant genotypes of the phages, which are all derivatives of λc*I857*, are also presented. Additional, and irrelevant, mutations present are *susA11* in λb2$^+$*int*$^+$, and *susA32 susJ27* in λb2.

The large spots are uniformly pink; the dots are surrounded by irregular darkened areas of cell lysis.

[*facing p. 492*

TABLE 1

*Non-reciprocal complementation between λb2 and 434hy*int41 *for lysogenization*

Infecting phage	Percentage of input cells having acquired immunity of		
	λ	434	λ and 434
λb2	0·01	—	—
434hy*int41*	—	<0·001	—
λb2 and 434hy*int41*	<0·02	5·4	3·0

Strain 152, at 5×10^8 cells/ml., was infected with the indicated phages at multiplicities of exposure of 4·4 for λ and 2·4 for 434hy. Dilutions of the infected cell suspensions were spread on EMBO plates previously seeded with 10^9 λb2*c* and EMBO plates seeded with 434hy*int6c*. After 24-hr incubation at 31°C, pink colonies were picked from these plates and tested for both immunities. Additional, and irrelevant, mutations present are *cI857susA32susJ27* in λb2 and *cI1susP3* in 434hy*int41*.

one phage has integrated (identified here by its immunity), invariably the phage is 434hy*int*. This non-reciprocality is interpreted to mean that λb2 lacks a structural element, acting *cis*, which is involved in integration (cf. Campbell, 1965), but that this mutant can provide in *trans* the function missing in an *int* mutant.

When either a *rec*$^+$ or *rec* host is used, rare colonies, stably lysogenic for λb2 alone, can be isolated. At even lower frequencies, *rec*$^+$ lysogens of λ*int*, obtained without exposure to *int*$^+$ phage, may be recovered. The location of the prophage is normal in the b2 lysogens, abnormal in the *int* lysogens. The properties of these λb2 and λ*int* lysogens will be the subject of separate communications.

Evidence concerning the nature of the Int function has been obtained from a comparison of the effect of various *int*$^+$ phages in helping to form stable lysogens of *int* phages (Table 2). The genomes of most temperate phages are attached to the bacterial chromosome at a specific site (Jacob & Wollman, 1961). It is possible that the prophages of λ, 434 and 434hy are inserted at identical sites (Liedke-Kulke & Kaiser, 1967), between bacterial markers *gal* and *bio* (Rothman, 1965). The hybrid between 21 and λ used here (21hy5) (Liedke-Kulke & Kaiser, 1967) is inserted at the λ site. Prophages 21 and φ80 are located in the vicinity of the *trp* region (Jacob & Wollman, 1958; Matsushiro, 1961; Signer, Beckwith & Brenner, 1965). Of these λ-related phages, only those which are located at (or adjacent to) the λ site serve as helpers for λ*int* integration. These results imply that the Int function is site-specific. We propose that the *int*$^+$ locus of λ supplies an enzyme or some other diffusible gene product, the existence of which was inferred by Signer & Beckwith (1966), which promotes recombination between the bacterial λ attachment site and a locus within or adjacent to the b2$^+$ region of λ.

The preceding experiments were performed with a *rec* strain of *E. coli* to avoid complications which the bacterial Rec function might introduce. However, the presence of a *rec*$^+$ gene in the host does not raise the lysogenization frequency of an *int* mutant to a value above 10^{-3} per infected cell (Table 3). The host Rec function does not appear capable of promoting recombination between the genetic elements involved in normal phage integration.

TABLE 2

Helping effect of int$^+$ *phages in lysogenization by* λint6

No.	Bacterial host	Helper phage	Lambda lysogens per 100 infected cells
1	*rec*	none	<0·05
2	*rec*	φ80	<0·05
3	*rec*	21gp	<0·05
4	*rec*	434	5
5	*rec*	434hy	23
6	*rec*	21hy5	21
7	*rec*(21gp)	none	<0·05
8	*rec*(21gp)	21hy5	<0·05
9	*rec*(21gp)	434hy	0·80†

Strain 152 was infected with *λint6* at a multiplicity of exposure of 15 and simultaneously with the indicated helper phage at the following multiplicities of exposure: φ80, 24; 21gp, 15; 434, 20; 434hy, 16; 21hy5, 15. Similarly, the lysogenic derivative of strain 152 was simultaneously infected with *λint6* at a multiplicity of exposure of 12, and helper phages 21hy5 and 434hy at multiplicities of 10 and 18, respectively. The starved cultures were infected at a final cell density of at least 2×10^8/ml. After adsorption, the infected cell suspensions were diluted appropriately and spread on plates of EMBO–*λint6c* for determination of λ lysogens. In the experiments of lines 4, 5 and 6, the λ lysogens obtained were tested for whether they were also lysogenic for the helper phage. The proportion of lysogens harboring λ alone was 0·83, 0·74 and 0·85, respectively.

† The helping effect of 434hy is considerably reduced by the presence of 21 prophage. However, the reduction in helping effect is not complete and may be attributed to some partial exclusion of 434hy by 21. A comparable reduction by 21 prophage in the efficiency with which 434hy alone lysogenizes (from 64 to 2%) was observed in this same experiment.

TABLE 3

Helping effect of bacterial Rec function in lysogenization by a superinfecting hetero-immune int *phage*

No.	Genotype		Lysogens with 434 immunity per 100 cells superinfected with	
	host	λ prophage	434hy*int6*	434hy
1	*rec*	none	<0·1	41
2	*rec*$^+$	none	<0·1	92
3	*rec*	*int6*	<0·1	32
4	*rec*	*int*$^+$	<0·1	55
5	*rec*$^+$	*int6*	1·4	81
6	*rec*$^+$	*int*$^+$	1·4	67

The *rec*$^+$ strain 28, the *rec* derivative (strain 152), and the indicated λcI*857* lysogens of these strains, at a cell density no less than $1{\cdot}2 \times 10^8$/ml., were infected with 434hy*int6* or with 434hy at the following respective multiplicities of exposure: (1) 4, 7; (2) 4, 6; (3) 3, 2; (4) 5, 9; (5) 8, 5; (6) 5, 8. After adsorption, appropriate dilutions of the infected cell suspensions were spread on EMBO–434hy*int6c* and incubated at 33°C. Cells were also plated at 39°C to determine if prophage substitution rather than double lysogenization had occurred. It was found that of *rec*$^+$ cells which acquire 434 immunity by superinfection with 434hy*int6*, less than 10% lost the immunity of the prophage.

(c) *Control of expression of the Int function*

Evidence is also presented in Table 2 that the Int function, like other phage functions, is under control of the immunity repressor. Whereas 21hy5 is an effective helper for integration of *λint* into a non-lysogen, the helping effect of 21hy5 is entirely eliminated when the host is lysogenic for 21.

A comparison of lines 4 and 5 of Plate I also demonstrates that the Int function is under immunity control. When cells are infected with *λint41* first, and immunity is established, the Int function of the superinfecting λb2c on the plate is not expressed, and λ lysogens are not produced (line 4). When infection is simultaneous (line 5), complementation occurs because the helping phage is not repressed initially.

Early defective mutants of lambda bearing amber (*sus*) mutations in the N cistron are deficient in a variety of lambda functions for which the N product may act as an inducer (Protass & Korn, 1966; Thomas, 1966; Dambly, Couturier & Thomas, 1968). These mutants are capable of efficient lysogenization at a high multiplicity of infection (Brooks, 1965). To test the possibility that this lysogenization might derive from "leakiness" of the N mutation, accentuated at high multiplicity, or might be due to the action of the bacterial recombination system, a phage bearing two mutations in the N cistron was assayed for lysogenization of a *rec* host. Twenty-five per cent of the infected cells of the *rec*, non-permissive strain 152 were lysogenized by 434hy*susN7,53* at a multiplicity of infection of five. Similar results were obtained with *λsusN7,53*. To eliminate possible phenotypic suppression by high concentrations of magnesium ions (Dahl, 1967), the standard lysogenization assay was altered. Cells were not starved in $MgSO_4$, and the concentration of Mg^{2+} throughout was reduced to $< 10^{-4}$ M. No significant difference in the lysogenization frequency of *λsusN7,53* was observed under these conditions. These results indicate that the *int*$^+$ gene is expressed under conditions where N cistron function is eliminated.

(d) *Lysogenization by superinfecting* int *phages*

Unlike the formation of *int* single lysogens, the formation of *int* polylysogens by superinfection is affected appreciably by the bacterial Rec function (Table 3). The lysogenization frequency of an *int* superinfecting phage is increased more than tenfold by the presence of hybrid prophage of differing immunity. This increase is entirely dependent upon the presence of a *rec*$^+$ gene in the host. It is independent of the prophage *int* allele. Thus, the bacterial Rec function, while not promoting single lysogenization, appears to permit recombination between a prophage and a superinfecting hetero-immune phage genome, as illustrated in Fig. 1.

(e) *Recombination among vegetative* int *phages*

Zissler (1967) has reported that *int* phages recombine in a *rec* host. The results of Table 4 confirm Zissler's findings. The extent of recombination between two vegetative phages in a *rec* host does not appear to be affected by the absence of the Int function (Table 4, line 4) when distant *sus* markers spanning the b2$^+$ region are used.

(f) *Induction of* int *lysogens*

Lysogens bearing only *int* prophages may be constructed by using *int*$^+$ helper phage (see Materials and Methods). Two distinct classes of lysogens are observed (Table 5). The most frequently obtained *int6* lysogen is one which, upon induction, yields

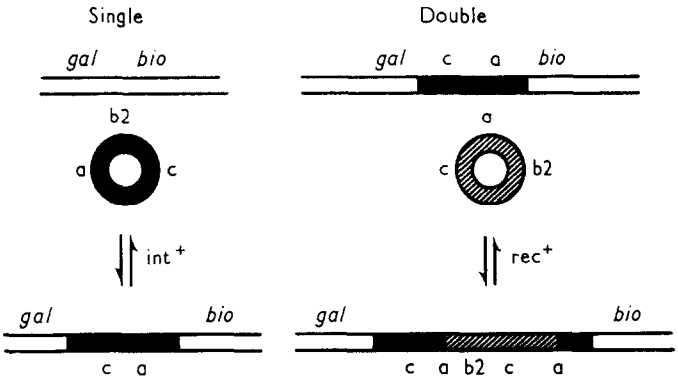

FIG. 1. Lysogenization.

TABLE 4

Recombination between int sus *mutants in a* rec *host*

Cross	*sus*+ recombinants per 100 progeny phage
λ*susA* × 434hy*susP*	14·0
λ*susAint21* × 434hy*susP*	14·6
λ*susA* × 434hy*susPint41*	12·6
λ*susAint21* × 434hy*susPint41*	16·5

The recombination-deficient non-permissive strain 152 was infected at 2×10^8 cells/ml. with phage at a multiplicity of exposure of 2·4 for each phage. After adsorption, the cultures were aerated at the inducing temperature of 39°C for 2 hr. The lysates were then chloroformed and the phage titrated on the non-permissive host, strain 28, and the permissive host, C600. The markers whose recombination is being assayed are *susA11* and *susP3*.

about 4×10^{-3} infective centers per bacterium and the same proportion of free phage, i.e. a burst size of about one. Although the phage yield is subnormal, heat-induction of these lysogens results in death and lysis of every cell.

The inability of certain *int* lysogens to produce normal phage yields is believed to be due to a defect in prophage excision. This interpretation is consistent with the finding that the lysates of these induced lysogens contain large numbers of transducing particles (Table 5). It is also supported by the observed inability of *int* mutants to promote superinfection curing (see below), which is known to involve prophage excision (Ptashne, 1965). It appears therefore that the same defect which impairs phage integration impairs prophage detachment; both processes rely at some stage on the same function.

Induction of a λ lysogen, but not infection of a sensitive cell, leads to derepression of the adjacent *gal* operon. This phenomenon, discovered by Buttin and Yarmolinsky & Wiesmeyer (see Buttin, 1963*c*; Yarmolinsky, 1963), is referred to as "escape

synthesis". Since escape synthesis is found only on induction of a prophage, we expected it might be influenced by a defect in prophage excision. Accordingly *λint* lysogens were tested for their ability to exhibit escape synthesis on lysogenic induction. The experiments of Fig. 2, performed by Dr Elke Jordan, demonstrate that a *λint* single lysogen shows an even greater escape synthesis than the *λint*$^+$ control. On the other hand, cells doubly lysogenic for *λint* (described below), in which active phage yields are normal, show no more escape synthesis than the *int*$^+$ control (data not shown). Whatever the cause of escape synthesis, the Int function is clearly dispensable.

TABLE 5

Phage yields of λcI857 lysogens thermally induced

Genotype		Infectious centers per input cell	Active phage per infectious center	Yield per input cell ($\times 10^4$) of phage transducing	
λ prophage	Host			*gal*	*bio*
int$^+$	*rec*$^+$	1·0	130	0·22	—
int$^+$	*rec*	1·0	71	0·15	0·24
int6	*rec*$^+$ (3/3)	0·004	*ca.* 1	1·7	—
int6	*rec* (13/17)	0·004	*ca.* 1	9	0·68
	rec (4/17)	1·0	60	3·3	—

The indicated lysogens of strains 28 (*rec*$^+$) and 152 (*rec*) were grown at 33°C in TB to 10^8 cells/ml., washed by centrifugation, and portions were plated at 33°C for determination of viable counts. Infectious centers were determined by plating at 39°C with strain 28 as indicator bacteria. For determination of phage yield, lysogens were grown at the inducing temperature of 39°C for 2 hr, chloroformed and plated with strain 28 at 33°C. Transducing particle titration is described in Materials and Methods.

Int lysogens were prepared by mixed infection with *int*$^+$ hetero-immune helper. The lambda lysogens so obtained were selected for the absence of helper immunity and *int*$^+$ phage in the burst. Results typical of individual clones of each class are reported. The figures in parentheses represent clonal frequencies.

The presence or absence of the host Rec function does not affect the phage yield. This is consistent with the finding, reported above, that the bacterial Rec function does not permit, unless very rarely, single lysogenization by *int* mutants.

In addition to the low-yielding clones of *int* lysogens, clones are obtained in which every cell can yield an infective center and the average burst size is nearly normal. We propose that these normal yielders bypass the impaired Int function as a consequence of homogenotic polylysogeny. A tandem-double lysogen, in principle, can detach one complete phage genome by a recombinational event anywhere along the length of the two prophages (see Fig. 1). The presence of the prophage genome in duplicate provides large areas of homology. Crossing over in these areas, resulting in the excision of one prophage, could be promoted by any generalized recombination system. By this means, the absence of the site-specific Int function could be circumvented. In the case of *int* lysogens of a *rec* host which are normal yielders, the recombination system involved in prophage excision might be the same non-specific

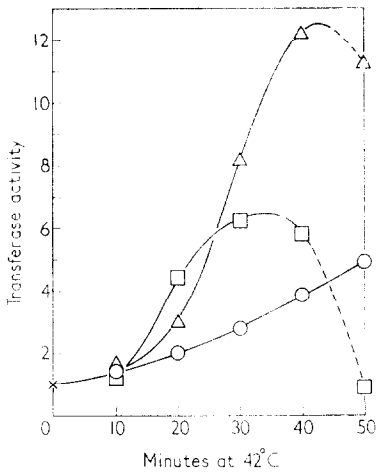

FIG. 2. Effect of *int* mutation on *gal* escape synthesis.

Derivatives of a *gal*$^+$ *rec* revertant of the *gal rec* strain 152 were grown at 34°C in TB and shifted to 43°C to induce the lysogens. The strains carry: no λ prophage (○); λc*I857int*$^+$ (□), λc*I857int6* low yielder (△). The activity of UDP glucose: α-D-galactose-1-phosphate uridylyl transferase per ml. of culture is normalized to unity at the time of the temperature change. Cell lysis is occurring where the lines are dashed. Results shown are the average values from two experiments performed by Dr Elke Jordan.

function (Red) which allows recombination between vegetative phage in a *rec* bacterium.

The hypothesis that homogenotic *int* polylysogens would give nearly normal phage yields upon induction was tested by constructing double lysogens, heterogenotic for immunity, in which the two prophages were located adjacent to, or separate from, each other. The results presented in Table 6 show that when the prophages are adjacent, every bacterium on thermal induction yields a burst of λ. When each prophage enjoys a separate location, e.g. one chromosomal, the other on an F′ episome, the yield of λ is low, generally no higher than from a single *int* lysogen.

The immunity marker of the second prophage is also recovered in the lysate (Table 6). The yield of 434hy*int6* is seen to be high in heat-induced lysates of N178 and low in lysates of the isogenic strain N201. This difference may reflect the sequence of the two prophages, and can be explained if we assume the order in N178 to be *gal*-λ-434hy-*bio* and in N201 to be *gal*-434hy-λ-*bio*. Random recombination between the two prophages would result in preferential excision of the centrally located immunity region of the prophage to the right.

The high yield of 434hy in the burst of N178 indicates that replication of this phage must occur. This result is unexpected, since an excised prophage should not replicate unless induced, despite multiplication of hetero-immune phage in the same cell (Thomas & Bertani, 1964). The mechanism of cross-induction of 434hy by induction of λ is under investigation.

(g) *Induction of superinfected* int *single lysogens*

We have seen earlier that an appropriate superinfecting *int*$^+$ phage allows integration of an *int* phage. Superinfecting phage can also aid in the excision of an induced *int* prophage. The data of Table 7 show that the yield of λ from an induced λ lysogen is reduced slightly by superinfection with the competing phage 434hy. However,

TABLE 6

Helping effect of adjacent prophage in thermal induction of λcI857int6 *heterogenotic* rec *polylysogens*

Strain	Second prophage		Total infectious centers per input cell	Lambda *cI857* per infectious center	Second phage per infectious center
	Name	Location			
N111	None	—	0·004	1·1	—
N158	21hy5	λ near *gal*	1	72	0·7
N159	21gp	21 near *trp*	0·004	1·1	<0·01
N157	434	434 near *gal*	1	25	2·5
N122	434hy	λ near *gal*	1	29	5
N202	434hy	on F'_1 *gal*$^+$	1	0·07	3
N178	434hy*int6*	λ near *gal*	1	7·7	86
N201	434hy*int6*	λ near *gal*	1	90	0·5
N237	434hy*int6*	on F'_1 *gal*$^+$	0·006	0·4†	0·02

Lysogens were treated as described in the legend of the previous Table. Phage immunities were distinguished by plaque type at 39°C, i.e. clear for lambda, turbid for all other phages used.

The second prophage was introduced into the *rec* strain 152 lysogenized with λcI*857int6* previously (most strains), simultaneously (N122), or in the reverse order (N201). Preparation and identification of *int* double lysogens and of F′ heterogenotes are described under Materials and Methods.

† The observation that λ per infectious center is less than one is due to some replication of the lysogens prior to lysis in the infectious center assay. This leads to an overestimate of the number of infectious centers per input cell.

when the prophage is λ*int*, the same superinfecting phage increases the λ yield about four orders of magnitude. A comparable increase in λ yield is accomplished by superinfection with 21hy5. The yield of λ is also increased, although not to the same extent, when the superinfecting phage does not supply the appropriate *int*$^+$ function (e.g. 21gp, 434hy*int6*). This is probably a consequence of recombination between the two phages, providing an alternative mechanism by which superinfection can increase the yield of phage carrying prophage immunity. As seen in Table 7, this effect of marker rescue can account for only a small fraction of the helping by superinfecting *int*$^+$ phage.

(h) *Superinfection curing and prophage substitution*

Superinfection of a lysogen with a related hetero-immune phage can result in loss of the prophage immunity from the cells surviving superinfection. Some of these survivors have acquired the immunity of the superinfecting phage (prophage substitution), whereas others have not (superinfection curing) (Cohen, 1959; Six, 1960, 1961; Eisen, Siminovitch & Mohide, 1967; Liedke-Kulke & Kaiser, 1967). Homoimmune superinfection does not result in curing, suggesting that a repressible phage function is required for this process.

We have exploited the heat sensitivity of thermo-inducible lysogens to measure prophage loss after hetero-immune superinfection. Cells which have lost their thermo-inducible prophage will form colonies at 39°C. These colonies may then be tested for acquisition of the immunity of the superinfecting phage.

As seen in Table 8, prophage loss can be accomplished efficiently by superinfection with an *int*$^+$ phage having the same locus of attachment as λ. The curing function has the same site-specificity as the Int function. In these experiments, *rec* lysogens were used in order to minimize the frequency of recombinations between prophage and superinfecting phage resulting in the exchange of immunity regions.

TABLE 7

Yield of lambda from induced lambda single lysogens following hetero-immune superinfection

Induced lysogen	Superinfecting phage	Lambda per induced cell
rec(λ)	none	61
rec(λ)	434hy	17
rec(λ*int6*)	none	0·0002
rec(λ*int6*)	21gp	0·02
rec(λ*int6*)	434hy*int6*	0·1
rec(λ*int6*)	434	2·8
rec(λ*int6*)	434hy	5·7
rec(λ*int6*)	21hy5	21

Lysogens of strain 152, at no less than 3×10^8 cells/ml., were incubated with phage at the following multiplicities of exposure: 434hy, 5; 21gp, 2·4; 434hy*int6*, 2·7; 434, 9; 21hy5, 6. The infected cell suspensions were diluted in TB and shaken at 39°C for 2 hr. The lysates were chloroformed and the lambda titer determined with strain 152 as indicator at 39°C.

TABLE 8

Correlation of superinfection curing specificity with site of chromosomal attachment

Superinfecting phage		Prophage loss: survivors at 39°C per 100 survivors at 33°C
Name	Location	
none	—	<0·001
φ80	φ80 near *trp*	<0·001
21gp	21 near *trp*	<0·001
434	434 near *gal*	18
434hy	λ near *gal*	82
21hy5	λ near *gal*	70
434hy*susN7,53*	λ near *gal*	1·0

Strain 152 lysogenic for λ*cI857susA11int21* was infected at a density of 7×10^8 cells/ml. with phage at the following multiplicities of exposure: φ80, 8; 21gp, 2; 434, 30; 434hy, 7; 21hy5, 10; 434hy*susN7,53*, 2·5. The high multiplicity of exposure of 434 was used to obtain a multiplicity of infection of 3, as adsorption of this phage is poor. After adsorption, the infected cell suspensions were diluted at least 2×10^{-2} in TB, samples were plated for determination of the proportion of cells surviving infection and the remainder was grown 2 to 3 hr at 33°C to allow segregation of nuclei no longer lysogenic for λ. Samples were again plated for survivors at 33°C and the remainder incubated 15 min at 41°C to induce and kill λ lysogens, and then plated for survivors at 39°C. From 23 to 77% of the cells survived infection, except for φ80-infected cells, of which 15% survived.

TABLE 9

Roles of Int function in superinfection curing and of bacterial Rec function in prophage substitution

No.	Genotype: Host	Genotype: Prophage λcI857	Super-infecting phage†	Survivors at 39°C per 100 survivors at 33°C following superinfection with: none	434hy *int*	434hy *int*$^+$	Figures measure
1	*rec*	*susA11int21*	cI$^+$	<0·001	0·006	21	Prophage loss = curing and substitution
2	*rec*	*susA11int*$^+$	cI$^+$	0·009	0·041	24	
3	*rec*$^+$	*susA11int*$^+$	cI$^+$	0·042	0·38	28	
4	*rec*$^+$	*sus*$^+$*int6*	cI$^+$	<0·001	0·12	92	
5	*rec*$^+$	*sus*$^+$*int6*	cI*1*	<0·001	0·003	28	Curing alone

Lysogens of strain 28 or its *rec* derivative, strain 152, at cell densities of at least 3×10^7/ml., were superinfected with the indicated phages at multiplicities of exposure of 5 to 9. Superinfecting 434hy*int* phages were *int6* in experiments 1 through 4, and *int41susP3* in experiment 5. Following adsorption, the cell suspensions were diluted in TB and segregation of non-lysogenic nuclei was allowed to take place during incubation for 120 min (experiments 1 through 3), or on pour-plates incubated for 140 min (experiments 4 and 5). Survivors at 33°C were determined by sampling the broth cultures and plating at 33°C or, in experiments 4 and 5, by continuing incubation at 33°C of certain of the previously poured plates. Lysogens carrying thermo-inducible prophages were then killed by plating at 39°C directly (experiment 1), or after a preliminary incubation at 41°C for 15 min (experiments 2 and 3), or by transferring the remaining pre-incubated plates to a 39°C incubator (experiments 4 and 5). Surviving colony formers were scored after overnight incubation at 39°C.

† cI$^+$ phage are not thermo-inducible; cI*1* phage are induced at temperatures above 36°C.

The early defective 434hy mutant *susN7,53* unable to synthesize phage DNA (Brooks, 1965) was assayed for ability to cause prophage loss. The defect in N reduces the extent of prophage loss about 40-fold. Similar results have been obtained by Dr Naomi Franklin (personal communication).

Table 9 shows the different roles of the Int and the Rec functions in prophage loss. When superinfection is by 434hy*int* in place of 434hy*int*$^+$, the number of cells which lose their thermo-inducible prophage is reduced almost two orders of magnitude (line 3). The residual prophage loss seen after superinfection with *int* mutants can virtually be eliminated in two ways: (1) by the use of a *rec* lysogen (lines 1 and 2), or (2) by making the superinfecting phage thermo-inducible as well (lines 4 and 5). When the superinfecting phage is also thermo-inducible, exchange of immunity markers with a thermo-inducible prophage will not result in the formation of a heat-stable lysogen. Lysogens in which an exchange of immunity markers has occurred, as well as those in which it has not, will both be induced at 39°C; only cured cells, bearing neither phage, will survive plating at this temperature. The results of Table 9 indicate that, of the two modes of prophage loss, only prophage substitution, utilizing the bacterial Rec function, occurs in the absence of the phage Int function.

4. Discussion

Three recombinational systems participate in genetic exchanges involving λ bacteriophage. The Rec function is bacterial; the other two functions, Int and Red, are phage-determined. The Int function (defective in *int* mutants) appears specialized for normal integration and detachment of the prophage genome.

We conclude that the *rec*$^+$ gene product participates in recombination between an *int* superinfecting phage and hetero-immune prophage, since stable lysogenization by the superinfecting phage requires a *rec*$^+$ allele. Presumably the recombination found to occur between prophages (Calef & Licciardello, 1960) is also catalyzed by enzymes of the bacterial host.

The existence of a second non-specific recombination system (Red), operative in phage-infected cells, is inferred from the finding that *int* mutants recombine efficiently during vegetative growth in a *rec* host. This same system functions also in recombination between vegetative phage and prophage (marker rescue) and in recombination between tandem prophages (induction from a double lysogen); both processes occur in the absence of either *int*$^+$ or *rec*$^+$ alleles. Cell survival is not required in any of these situations. Processes such as lysogenization of a hetero-immune lysogen or prophage substitution, involving similar recombinational events but requiring also that the infected cell survive, are not catalyzed by the Red function. Thus, the Red function is not efficiently expressed in the lysogenic pathway.

The erratic prophage excision which occurs following induction of an *int* single lysogen is probably due to expression of the Red function. The appearance of large numbers of transducing particles in the lysates of these induced lysogens and the low yield of viable phage can be accounted for by generalized recombination in the region of the induced prophage. Large areas of homology would not be involved and excision of a complete phage genome would be a rare event.

The phage-determined site-specific Int function participates in integration, prophage detachment upon induction, and superinfection curing. All of these functions are impaired by *int* mutations. In superinfection curing, the *int*$^+$ product of the incoming phage detaches the prophage in lieu of integrating the infecting phage. In subsequent cell divisions, both phages, each repressed, are diluted out. This model is supported by our observations and those of Signer & Beckwith (1966), and of Signer (cited in Signer & Beckwith, 1966), which indicate that the curing specificity of the superinfecting phage is identical to its attachment specificity. Further support is presented in a note added in proof to the article by Liedke-Kulke & Kaiser (1967).

The chromosomal location of phage 434 may be identical with the location of λ (Liedke-Kulke & Kaiser, 1967). The complementation which occurs between 434*int*$^+$ and λ*int*, and the ability of superinfecting 434*int*$^+$ to cure λ lysogens, is consistent with identity of the chromosomal attachment sites of 434 and λ.

The Int function is repressible. This is in agreement with the observation that integration of an *int*$^+$ superinfecting phage is known to be blocked by co-immune prophage (Dahl & Calef, 1966), even at an unoccupied site (Campbell & Zissler, 1966; Taylor & Yanofsky, 1966) or simply by repressor remaining in a non-lysogenic segregant of an abortive lysogen (Ogawa & Tomizawa, 1967). For normal integration to take place, therefore, the *int*$^+$ gene must be expressed prior to the expression of immunity, although integration itself may be delayed for as long as several generations (Smith & Levine, 1967).

There is no clear indication that Int function is under control of the N cistron. N-defective mutants efficiently lysogenize a *rec* host. The low efficiency of curing in the absence of N function, either upon transient induction as described by Eisen *et al.* (1966), or upon hetero-immune superinfection as described here, need not imply that Int is under N control. Instead it may reflect an additional N-dependent function in curing, e.g. host chromosome repair. Various functions affected by the N defect are expressed following hetero-immune superinfection with an N^+ phage. Thomas (1966) and Dambly *et al.* (1968) have proposed that these functions are under the positive control, *in trans*, of the N cistron product. Since integration of an *int* superinfecting phage is not complemented by an *int*$^+$ prophage, *trans* induction of the Int function does not appear to occur.

The function of the *int*$^+$ gene product is to promote, with high efficiency, integration of phage DNA within a specific region of bacterial DNA. It is generally assumed that this integration requires pairing of homologous regions. The fraction of the λ genome involved in such pairing should correspond to the frequency of detachment of λ prophage from a single lysogen relative to the frequency of detachment of a prophage from a tandem double lysogen, provided the recombination systems involved in each case do not exhibit site-specificity. This requirement is perhaps met in the case of induction of *int* phage from *rec* lysogens, where prophage excision involves only the Red function. Under inducing conditions, the ratio obtained is 4×10^{-3}/l. This ratio represents an upper limit for the relative size of the pairing region, first, because the denominator cannot exceed unity (no more than all induced lysogens can yield phage); second, because the numerator includes excisions which may lie outside the normal pairing regions yet result in the detachment of a viable phage. Calef, Marchelli & Guerrini (1965) have calculated a ratio of 10^{-2} using *int*$^+$ prophage. This value is probably overestimated, due to the bias introduced by the occasional derepression of the site-specific Int function. The Int product, appearing as a result of temporary derepression, may be largely responsible for the spontaneous curing of single lysogens; single *int* lysogens are very stable. Our calculations, as well as those of Calef *et al.* (1965), are based on the assumption that phage and bacterial copies of the attachment site are homologous.

Integration of λ by means of generalized recombination would require broad regions of homology and would result in unstable integration. The Int function, which allows specific recombination, obviates this requirement. Thus, the Int function, when expressed, permits efficient integration and excision. When repressed (in the lysogenic state), the absence of Int function permits prophage to be stable.

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Note added in proof: The inference that Red function, in the absence of Int or Rec functions, permits occasional prophage excision from single lysogens and relatively efficient prophage excision from tandem double lysogens, has been tested. Strain 152 (*rec*) was lysogenized with λcI*857int6red3*, using 21hy5 helper. The *int6* and *red3* mutations eliminate Int and Red functions, respectively. The *red3* derivative of λcI*857int6* was kindly supplied by Drs J. Weil and E. Signer who isolated and characterized this mutation (manuscript in preparation). Low yielders and normal yielders of λ*int red* were obtained; the phage yields did not differ by more than 50% from *int red*$^+$ controls. Accordingly, barring complementation between *red3* and the *rec* mutation in strain 152, excision may require very little expression of the non-specific recombination systems or alternative mechanisms of phage release may be responsible for the excision observed.