*MUTANTS OF BACTERIOPHAGE λ UNABLE TO INTEGRATE INTO THE HOST CHROMOSOME**

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A temperate phage such as λ can provoke one of two responses upon infection of a host cell: the *lytic response*, in which the cell lyses and releases new virus particles, or the *lysogenic response*, in which the cell survives with the viral DNA associated with the bacterial DNA and replicating with it—the prophage state.[1]

The lysogeny process in the case of λ can be divided into two distinct events—*repression* of viral genes involved in lytic development[1] and *integration* of the viral DNA into the host DNA.[2-5]

The λ genes required for repression of lytic functions in the lysogenic response to infection are c_I and probably c_{II} and c_{III} (Fig. 1).[6] λ phage with mutations in these genes produce clear plaques indicative of failure of the self-repression required as a prelude to lysogeny; they undergo either no lysogeny (c_I) or drastically reduced lysogeny (c_{II} and c_{III}). The phenotype of a λ mutant defective in integration, but not repression, is exemplified by λb2, a deletion mutant characterized by Kellenberger, Zichichi, and Weigle (Fig. 1).[7] λb2 makes turbid plaques like wild-type λ because it is able to establish repression;[8, 9] however, the surviving cells are not stably lysogenic but continue to segregate sensitive cells as cell division proceeds. There are two possible explanations for the integration defect of λb2: λb2 might be missing either an enzyme required for specific integration or a region required for physical recognition of the proper site in the host DNA (or both); Campbell has shown that the latter (structural defect) explanation is sufficient to explain the inability of λb2 to integrate.[10]

We have sought mutants defective in an enzymatic step required for integration by looking for point mutants with the integration-defective phenotype of λb2. The following describes the isolation, genetic location, and some of the properties of seven mutants of λ defective in integration (*int*$^-$ mutants). Mutants with similar properties have been isolated independently by Zissler[11] and Gottesman and Yarmolinsky[12] in the case of λ and by Smith and Levine[13] in the case of phage P22.

Materials and Methods.—Strains and media: The *E. coli* strains used in this investigation and the relevant genetic characteristics were the following: C600 *su*$^+$, W3350 *su*$^-$ (permissive and nonpermissive hosts, respectively, for the Campbell λ *sus* nonsense mutants[14]); R19 *gal*$^+$ *rec*$^+$ and R20 *gal*$^+$ *rec*$^-_{13}$ (*gal*$^+$ derivatives of the Howard-Flanders strains AB1157 and AB2463[15]); AB2463 and R20 carry a mutation which reduces bacterial recombination to a very low level; MS5061 *gal*$^-$ (deletion *gal*$^-$ mutant[16]). The λ mutants used were the following: λ *sus* nonsense mutants[14] (from A. Campbell and C. Radding); λb2[7] (from W. Dove); the thermally inducible c_I mutant λc_{I857}[17] (from W. Dove); and the defective, transducing phage λ*dg*A–J, missing the A–J λ genes[18] (from J. Adler).

The liquid growth medium used in all experiments was T-broth (10 gm Difco tryptone, 5 gm NaCl per liter). Solid media used were T-broth agar for phage assays and EMBgluc agar (EMB plus 0.1% glucose) for scoring of the *int*$^-$ character.

Phage crosses: Phage crosses were carried out by lytic growth in C600 at 37°C after phage adsorption in 0.01 *M* $MgSO_4$. A multiplicity of infection of 10 for each parental phage was employed. A drop of $CHCl_3$ was added 80 min after dilution from the $MgSO_4$ adsorption medium

into T-broth, and the lysate was plated on W3350 su^- for sus^+ recombinants. The int^- character was determined by stabbing W3350 plaques onto EMBgluc (see below). Low revertant stocks (sus^+ revertant fraction $< 10^{-5}$) were used in the crosses, and self-crosses showed that reversion did not interfere with the mapping.

Color test for integration defect and isolation of int^- mutants: A bacterial colony in which cell lysis is occurring gives a red color on EMBgluc.[19] λb2 produces repression, but bacterial growth will dilute out the repressed, nonreplicating phage genomes and eventually the supply of repressor, leaving a population of cells sensitive to λ infection.[8, 9] A bacterial colony arising from a λb2-infected cell will therefore give a red color on EMB gluc or a red and white mottled streak on EMBgluc.[10] Since an integration-defective mutant should produce this same response, we sought int^- mutants among turbid plaques produced by a stock of the standard strain of λ mutagenized by hydroxylamine;[20] cells from the center of plaques on strain W3350 were stabbed to EMBgluc and incubated at 37°C. Phage from resulting red colonies were spotted on W3350 lawns for incubation at 37 and 30°C. Cells from the center of the plaques were then streaked on EMBgluc plates and incubated at the same temperature as the plates from which the cells were taken; a mottled red and white streak was taken as an indication of an integration defect. One mutant (int_1) exhibited a mottled streak at 37°C and not at 30°C and therefore was presumably temperature-sensitive for the integration defect. In some cases the rec^- strain R20 was used instead of W3350.

Results.—Integration-defective (int^-) mutants: Seven mutants were isolated by the EMBgluc technique and their defectiveness in integration was verified. Because cell division dilutes out nonintegrated, repressed phage genomes, a colony arising from a bacterium infected with phage unable to undergo stable integration will contain many sensitive cells if steps are taken to prevent reinfection. High-frequency segregation of sensitive cells in a colony known to contain phage is, therefore, a test for failure of the phage genome to achieve stable integration into the genome of the host.[8]

This type of experiment was performed and is presented in Table 1. Anti-λ serum was present to minimize reinfection of sensitive cells. The behavior of int_1 in this experiment indicates that it has a temperature-sensitive integration system; this was suspected since no mottling of the EMBgluc streak was apparent at 30°C. The other mutants isolated exhibit the integration-defective phenotype at both temperatures. In order to demonstrate that the isolated mutants were not large deletion mutants similar to λb2, CsCle quilibrium density gradient centrif-

TABLE 1

DEFECTIVENESS OF *Int*$^-$ MUTANTS IN INTEGRATION

Mutant	Frequency of Phage-Containing Cells in a Colony Grown from an Infected Cell	
	30°C	37°C
+	1.0	1.0
b2	0.06	0.06
int_1	1.0	0.38
int_2	0.10	<0.05
int_3	<0.05	0.06
int_4	<0.05	<0.05
int_5	<0.05	<0.05
int_6	0.07	0.08
int_7	<0.05	<0.05

Phage were plated on host W3350 or R20 at 30 and 37°C and cells from the center of the plaques were streaked on T-broth agar containing anti-λ serum (0.1 ml of K = 147) and incubated at the same temperature as the plates from which the infected cells were taken. Approximately 20 colonies were first tested for the presence of phage by stabbing to a W3350 lawn; then a phage-containing colony was streaked on T-broth agar, and the frequency of phage-containing colonies among approximately 50 colonies in the second streak determined by stabbing to a W3350 lawn. The average of three such determinations is presented in the table.

ugations were performed on all the mutants. No detectable density difference from the parental λ could be observed.

Complementation between int⁻ mutants: We wanted to determine whether the *int*⁻ mutations affected a function distinct from the b2 deletion defect and whether more than one gene was involved. We therefore studied the ability of pairs of *int*⁻ mutants and *int*⁻ and b2 to complement for integration in mixed infection. Table 2 gives the results of these complementation experiments. Among int_1, int_2, int_4, int_5, int_6, and int_7, a positive complementation response was observed only when int_1 was one of the pair; this presumably reflects some residual function of the temperature-sensitive int_1 mutant at 37°, as indicated by the "self-complementation" result with int_1 and the data of Table 1. Int_3, however, gave a positive complementation response with *all* other *int*⁻ mutants and no "self-complementation." These data therefore suggest that int_1, int_2, int_4, int_5, int_6, and int_7 are defective in one integration function and int_3 in a second. Since all mutants except int_5 showed clear complementation with λb2, it would appear that the integration functions defined by these *int*⁻ mutations are distinct from the function or functions missing in the λb2 deletion. Poor complementation by int_5 with b2 indicates some peculiar properties of this mutation.

If an *int*⁺ phage provides a protein required for integration, then mixed infection by *int*⁻ and *int*⁺ phage should yield some cells lysogenic only for the *int*⁻ mutant. Such *trans* complementation would not be expected if the *int*⁻ mutation involves a site, as in the case of λb2. Examples of stable lysogens capable of yielding only integration-defective phage were looked for and found in the case of mixed infection by λ⁺ and λint_2. However, such lysogens were not found in the case of λ⁺and λint_3. Furthermore, mixed infection by λint_2 and λint_3 resulted in stable lysogens of λint_2 alone, or λint_2 and λint_3, but not λint_3 alone. Thus, λint_3 appears to be peculiar with respect to complementation for integration and if it can lysogenize at all by complementation, it does so with a markedly reduced frequency.

Mapping of int⁻ mutations: The general procedure in the mapping was to cross

TABLE 2

COMPLEMENTATION BY *Int*⁻ MUTANTS

Mutant	Frequency of Stable Lysogeny after Mixed Infection								
	+	b2	int_1	int_2	int_4	int_5	int_6	int_7	int_3
+	0.9	0.7	0.3	0.4	0.1	0.3	0.4	0.3	0.6
b2	0.7	0 (100)	0.1	0.1	0.1	0.02 (100)	0.2	0.1	0.4
int_1	0.3	0.1	0.1	0.1	0	0.1	0.1	0	0.2
int_2	0.4	0.1	0.1	0	0	0	0	0	0.2
int_4	0.1	0.1	0	0	0	0	0	0	0.1
int_5	0.3	0.02 (100)	0.1	0	0	0 (100)	0	0	0.1
int_6	0.4	0.2	0.1	0	0	0	0	0	0.2
int_7	0.3	0.1	0	0	0	0	0	0	0.4
int_3	0.6	0.4	0.2	0.2	0.1	0.1	0.2	0.4	0

Complementation to produce integration was determined by spotting approximately 2×10^5 phage of each mutant pair to form an overlapping plaque on a bacterial lawn of 10^8 C600. Bacteria were picked from the overlap region, grown for about seven generations in Tryptone broth at 37° and spread on EMBgluc plates. White colonies (either stable lysogens or nonphage-containing segregants—see *Methods*) were tested for immunity by stabbing to an EMBgluc plate seeded with approximately 10^9 λc_I. Immune colonies are white and nonimmune colonies are red after growth on the λc_I plate. The numbers in the table are the frequency of stably lysogenic (immune) white colonies among the population of white colonies on EMBgluc. Except where noted, approximately 25 colonies were scored in each case; therefore 0 in the table means <0.04. In every case of a positive complementation response between λ*int*⁻ pairs or λ*int*⁻ and λb2, the possibility of integration by a recombinant λ⁺ rather than true complementation was ruled out by the fact that stable lysogens produced by mixed infection yielded only integration-defective phage.

TABLE 3

MAP LOCATION OF *Int*⁻ MUTATIONS

Int mutation	Cross Result (% int^+/sus^+) Which Locates: Left or Right of N: $N_7 \times N_{53} int^-$	$N_{219} \times N_{53} int^-$	Right of J: A–J*del* $\times N_{53} int^-$	Between b2 and N: $J_6 b2 \times N_{53} int^-$
int_1	36 (25)*	70 (50)	74 (50)†	41 (50–200)‡
int_2	16	60	94	45
int_4	—	76	78	47
int_5	16	72	78	26
int_6	36	62	74	38
int_7	36	72	84	33
int_3	84	46	98	—
int^+	—	—	—	61
			Right of P: $P_3 \times P_{80} int^-$	Left of Q: $Q_{73} \times Q_{21} int^-$
int_3			56(100)	14(100)

Phage crosses were carried out by growth in strain C600 su^+ and plating on W3350su^- to select recombinant phage. Scoring for *int* was carried out by stabbing recombinant plaques to EMBgluc (see *Methods*). N_7, N_{53}, N_{219}, J_6, P_3, P_{80}, Q_{21}, and Q_{73} are *sus* mutants unable to grow on W3350; A–J*del* is a defective, transducing λ*dg* which has deleted the A–J genes.

* The number of plaques stabbed to determine *int* for each cross is given by the number in parentheses at the head of the column (e.g., 70 (50) means 70% int^+ out of 50 plaques).

† Int_1 location to right of J was confirmed by int_1 $J_6 \times J_{27}$, which yielded 32% int^+/sus^+.

‡ Control $J_6 int_1 \times N_{53} b2$ gave <2% int^+.

two mutants unable to grow on W3350, one of which was also *int*⁻, select recombinants by plating on W3350, and score *int*⁻ by the EMBgluc technique (see *Methods*). The results of these crosses are given in Table 3. The mutations were first located to the left or right of the N gene by crosses of N_{53} *int*⁻ with N_7, which is to the left of N_{53}, and N_{219}, which is to the right (Fig. 1). These crosses placed six of the mutations to the left of N and int_3 to the right. The six mutations to the left of N were located to the right of J by crosses of N_{53} *int*⁻ with the λ*dg*A–J deletion phage (and in one case by J_6 $int_1 \times J_{27}$). Crosses of N_{53} *int*⁻ with J_6b2 then located the cluster of six mutations to the right of b2, distributed approximately 70 to 85 per cent of the way from J to N (Fig. 1). Since these mutations appear to define a single complementation group, it is likely that they all occur in a single gene, which we designate *int*A.

The int_3 site was shown to be to the right of P by a cross of P_{80} int_3 with P_3 and to the left of Q by a cross of Q_{21} int_3 with Q_{73} (Fig. 1). Since the int_3 mutation is not

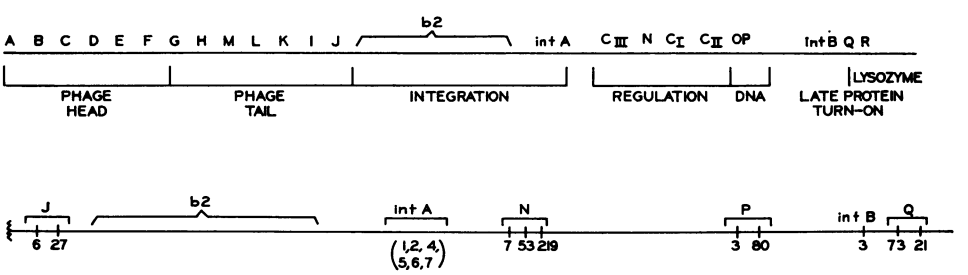

FIG. 1.—The vegetative genetic map of λ phage obtained from crosses during lytic growth is given above. Genes A–R (upper-case letters) are required for the production of λ phage in a lytic response to infection;[14] evidence for their role is summarized in Echols *et al.*[30] The repression genes c_I, c_{II}, and c_{III}; the integration genes *int*A and *int*B; and the b2 deletion are discussed in the text. The functional clustering of the λ genes is indicated below the vegetative map. The specific mutations used in this work and their genetic order are given on the "shortened" map below. The order of N_7 and N_{53}, P_3 and P_{80}, and Q_{73} and Q_{21} was determined by Campbell[14] and Amati and Meselson;[31] the order of J_6 and J_{27} and N_{53} and N_{219} was determined for this work.[32] The order of *int*A mutations has not been clearly established.

only genetically distinct from the *int* gene but is also blocked in a separate function, as judged by complementation analysis, we can tentatively conclude that another gene is necessary for effective integration; we designate this locus *int*B.

Phage production after induction of an int⁻ mutant prophage: Campbell has proposed that the process of prophage induction involves a direct reversal of the integration event, leading to an excision of the prophage DNA from the host DNA.[2] If this proposal were correct, *int*⁻ mutants defective in integration might also be defective in excision and therefore produce few, if any, phage after repression is released by an inducing treatment.

The int_1 mutant with a temperature-sensitive integration defect can effectively establish stable lysogeny at 30°C; at 37°C integration is inefficient, although possible (Tables 1 and 2). We have asked whether a stable lysogen of int_1 produced at low temperature will yield λ phage after repression is released by inducing treatment at elevated temperatures. Release of repression was effected by temperature elevation, using int_1 and *int*⁺ prophages carrying the c_{I857} mutation in the c_I repressor gene, which renders the repression system senstive to high temperature.[17] Production of viable (plaque-forming) λ phage and defective, transducing λ*dg* at 37 and 43°C is given in Table 4 for two bacterial hosts—one normal and one defective (*rec*⁻) in bacterial genetic recombination.

TABLE 4

PHAGE PRODUCTION AFTER INDUCTION OF AN *Int*⁻ PROPHAGE

Lysogenic strain	Phage/Cell at 37°		Phage/Cell at 43°	
	λ	λ*dg*	λ	λ*dg*
Rec⁺ (*int*⁺)	47	2×10^{-6}	36	2×10^{-6}
Rec⁺ (int_1)	14	2×10^{-6}	2×10^{-3}	3×10^{-5}
Rec 13 (*int*⁺)	45	4×10^{-8}	12	6×10^{-7}
Rec 13 (int_1)	6	6×10^{-7}	3×10^{-4}	3×10^{-5}

Each phage contained the c_{I857} temperature-sensitive mutation in the c_I repressor gene; this mutation causes wild-type λ to undergo prophage induction by thermal treatment, presumably because the repressor is heat-labile. The bacteria were grown at 30° in T-broth to approximately 2×10^8/ml and repression released by heating at 43° for 10 min. The cultures were then incubated at either 37 or 43° until lysis of rec⁺ (*int*⁺) had occurred, as determined by optical density measurements. The cultures were then treated with chloroform and assayed for λ and λ*dg* phage. λ*dg* phage were assayed by transduction of the *gal*⁻ deletion strain MS5061 to *gal*⁺, using normal λ as "helper." This assay tends to underestimate the number of λ*dg* particles, since a lysogenic response producing integration of λ*dg* is normally required for a λ*dg* to yield a *gal*⁺ transductant. Data presented are the average of three separate experiments.

At 37°C a bacterial population lysogenic for int_1 is only slightly impaired in viable phage production compared to *int*⁺; however, at 43°C viable phage production is reduced to a very low level. In contrast to this result, the absolute number of λ*dg* particles from the cells lysogenic for int_1 increases at 43°C relative to either int_1 at 37°C or *int*⁺ at 43°C; in the case of the *rec*⁻ host the number of transducing particles actually becomes comparable to the number of plaque-forming particles, and it is possible that nearly all of the phage produced are defective in some way. Loss of cell viability after transfer to 43°C occurred at the same rate for *int*⁺ and int_1 lysogens.

Discussion.—Possible nature of the defect in int⁻mutants: Campbell has provided evidence that the λb2 deletion mutant is missing at least a region of DNA required structurally for integration, possibly a phage-host recognition region.[10] The *int*⁻ mutants appear to define at least one and perhaps two λ genes which provide contributions to the integration event distinct from the physical requirement deleted in λb2. Our evidence is suggestive, but not conclusive, for an enzymatic role for *int*A. In contrast to the case of λb2, *int*A mutants can be complemented by normal

λ for integration in an event which produces a cell lysogenic *only* for the *int*A mutant; it is probable therefore that the *int*A function can act *trans* through the cytoplasm, consistent with an enzymatic role in integration. As discussed above, *int*B appears to act *cis*. Further experiments will be required to clarify the nature of the *int*B defect. The temperature-sensitive integration property of the int_1 mutant also supports the concept that the *int*A gene specifies a protein. A variety of other experiments have previously suggested an enzymatic requirement for integration.[21-23]

Campbell has proposed that integration involves a reciprocal recombination between paired phage-host recognition regions which provides for linear insertion of the prophage DNA into that of the host.[2] Within the framework of this model, an integration enzyme would probably be involved in this specific recombination. Efforts to identify the *int* genes with a specific localized recombination function are complicated by the existence of a separate λ-specified system for normal vegetative recombination between phage DNA molecules.[24-26] Recombination experiments with int^- mutants and mutants defective in vegetative recombination will be reported separately.[24]

Excision and defective phage formation: In his proposal of a recombinational mechanism for integration, Campbell suggested that prophage induction might involve a direct reversal of the integrative recombination, resulting in excision of the prophage DNA from the host DNA. He also noted that rare errors in the excision event would explain the production of defective, transducing λ*dg* phage.[2] The finding that an *int*A mutant defective in integration is also defective in phage production after an inducing treatment supports the concept that the integration process is normally reversed in prophage induction. This result suggests that the *int*A gene specifies a protein concerned with both integration and excision of viral DNA. Similar results have been obtained by others with integration-defective mutants.[12, 13, 27] Other evidence for prophage excision has been provided by Ptashne[28] and Eisen, Siminovitch, and Mohide.[29]

The increase in defective phage production found after inducing treatment of the int_1 lysogen is consistent with the error mechanism proposed by Campbell. Since the number of λ*dg* particles per cell is the same after an inducing treatment of both rec^+ and rec^- hosts lysogenic for int_1, the most likely source of the "erroneous excisions" in the case of int_1 would appear to be the λ enzyme for vegetative recombination; a defective integration enzyme is another possibility. However, "normal" λ*dg* production after int^+ induction drops to a very low level in a rec^- host, suggesting that the bacterial recombination system is more important than the phage system under these conditions. Further experiments with other int^- mutants and recombination-defective λ mutants will be required to clarify the situation.

Repression, integration, and excision: All of the available evidence is consistent with the point of view that the lysogenic response to infection is the culmination of two separate phage-directed events—repression of viral functions required for lytic development, and integration of the viral DNA into the host DNA. Similarly, the process of prophage induction to lytic growth appears to require two separate events—release of repression (accomplished by the inducing treatment) and phage-directed excision of the prophage DNA. Just as the establishment of repression

is necessary but not sufficient for stable prophage integration, the release of repression is necessary but not sufficient for excision. The requirements for integration appear to be a structural "recognition region"[10] and at least one phage-directed protein (specified by the *int*A gene). The requirements for normal excision appear to be a region at each end of the prophage[3] (a presumptive recombinant recognition region) and at least one phage-directed protein (the *int*A protein).

Summary.—Seven integration-defective (*int*$^-$) mutants of phage λ have been isolated and characterized genetically. Six of the mutations define a single complementation group and are located in the central region of the λ vegetative map; we have designated this cistron the *int*A gene. The seventh mutation complements all of the *int*A mutations and is located toward the right-hand terminus of the λ vegetative map, suggesting a second gene involved in integration; we have therefore tentatively defined an *int*B gene. None of the *int*$^-$ presumptive point mutations lie within the region deleted in the integration-defective λb2 deletion mutant, which has probably lost a region of DNA required structurally for phage-host recognition. One temperature-sensitive *int*A mutant which can integrate efficiently at low temperature was tested for phage production from the prophage state after release of repression at high temperature; viable phage production was very low and defective phage production was increased. The properties of the *int*A mutants and other aspects of λ integration are most easily explained if the *int*A gene specifies a protein which catalyzes both integration of λ DNA into the host and excision of λ DNA from the host under conditions of prophage induction.

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