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Requirement for a Functional *int* Product in Temperature Inductions of Prophage P22 *ts mnt*

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Prophage P22 *ts mnt* was found to be inducible by high temperatures. This property was maintained when the *ts mnt* allele was recombined into a phage that cannot synthesize phage deoxyribonucleic acid at high temperature. Prophage P22 *int ts mnt*, on the other hand, was not inducible at high temperature. This result leads to the suggestion that the *int* enzyme, which is involved in excision, is regulated by the product of gene *mnt*.

Prophage P22 requires two repressors, *mnt* (5) and *c*2 (8), to maintain lysogeny (*mnt* is called *v*1 in reference 14). Phages with temperature-sensitive (ts) mutations in *mnt* or *c*2 can establish and maintain lysogeny at temperatures <30 C but are induced at >40 C. When a culture of *Salmonella typhimurium* LT2 (P22 *ts mnt*) is shifted from 30 C to >40 C, the number of microscopically observable cells increases for about 45 min. There is a smaller increase in the number of colony-forming units. Thus killing, defined as the loss of colony-forming ability, begins at time zero. At 30 to 45 min, the number of colony formers begins to decrease and reaches about 0.1 by 90 min. Infective centers appear at 30 to 45 min and increase in number until 120 min. The yield of infective centers is equal to the number of cells observed at 45 min (5).

The kinetics of temperature induction of LT2 (P22 *ts c*2) are quite different. The number of viable cells, as determined by ability to form colonies, decreases from time zero and reaches <0.01 after about 20 min; infective centers increase from time zero and reach a number essentially equal to the number of colony formers by 20 min (13; *unpublished observations*).

When LT2 (P22 *ts mnt*) was irradiated with ultraviolet light before being shifted from 30 to 43 C, there was no change in the kinetics of temperature induction (6). Since ultraviolet irradiation is thought to interfere with the functioning of *c*2 product and there was no cooperative effect of irradiation and heat, I concluded that the products of *c*2 and *mnt* do not interact and that they probably act separately to repress different phage functions (6).

Infections of LT2 leading to lysogenization require at least two phage-directed activities: (i) phage-induced deoxyribonucleic acid (DNA) synthesis is regulated (11) and (ii) the phage DNA is integrated into the host chromosome (10). The first of these two requirements is satisfied by the sequential action of products of genes *c*1 and *c*2 which repress DNA synthesis (11). Levine and Smith concluded that *c*2 continues to repress phage-directed DNA synthesis during lysogeny (9). The product of gene *int*, "integrase," catalyzes the required recombinational event which inserts the phage DNA into the bacterial DNA (10, 12). The gene specifying integrase must also be regulated during lysogeny, because it is involved in the reverse operation of excision as well as integration (10, 12). If *int* is not repressed, the prophage DNA can be cut from the chromosome, resulting in either curing or induction.

The results presented in this paper show that a functional *int* gene is required in the prophage in order for P22 *ts mnt* lysogens to be temperature inducible, as measured by host killing or infective center production. This is to be contrasted to induction of prophage P22 by ultraviolet irradiation, or prophage P22 *ts c*2 by heat (10, 12); these prophages do not require a functional *int* to kill their hosts. The essential requirement for *int* in *ts mnt*-mediated inductions suggests that the relationship of *mnt* repression and the *int* function is much more intimate than is *c*2 repression and *int*. This observation is

consistent with the hypothesis that *mnt* product directly or indirectly represses *int* in the prophage.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* LT2 #18 obtained from the collection of M. Levine was used for all experiments. This strain is a wild-type LT2 cured of a defective prophage by Zinder (14). A *gal*⁻ derivative of LT2 was used as indicator bacterium (8).

Phage. The isolation of P22 *ts mnt* (5) and temperature-sensitive conditional lethal mutants (2, 7) has been described. Mutations in four cistrons of P22, e.g., 6, 12, 18, and 21, are known to interfere with DNA synthesis (2; Levine and Botstein, *unpublished data*). Phages with temperature-sensitive alleles in these cistrons are designated P22 *ts* 6.1, P22 *ts* 12.1, etc. P22 *int*4, *int*18, and *int*32 were isolated by Smith and Levine (12). The plaque morphology markers *m*3 and *h*21 have been described (8). Figure 1 represents the order of genes mentioned in this paper.

Media. All liquid cultures were grown in M9-CAA (8), and M9 salt solutions without glucose or amino acids were used for dilutions. Platings were made on indicator agar (8) to determine phage plaque morphology. EMB-galactose-agar was used for plating of induction experiments. The use of EMB-galactose-agar permits the scoring of lysogenic colonies, sensitive colonies, and infective centers on the same plate (8). LT2 #18 is Gal⁺ and grows through Gal⁻ bacteria used as indicator on EMB-galactose plates. Dark Gal⁺ colonies that are surrounded by haloes of lysis in the Gal⁻ bacteria are scored as lysogenic bacteria.

Phage crosses. Phage crosses were carried out as described previously (7), with the exception that treatment of phage-bacteria mixtures with antiserum to P22 after phage adsorption was eliminated. This step is unnecessary, under the conditions used, because >95% of the input adsorb.

P22 *int ts mnt* was prepared by crossing P22 *ts mnt ts* 12.1 × P22 *int*4, P22 *int*18, and P22 *int*32. Lysates were plated out at 39 C on tryptone-agar to detect Mnt plaques (14); turbid centers of such plaques were picked with sterile toothpicks onto indicator agar and incubated at 25 C to determine which were Int (12). The genotype of P22 *int ts mnt* was verified by backcrosses.

Recombinants between *ts mnt* and *ts* conditional lethal genes were prepared as follows. The plaque morphology gene *m*3 (8) is closely linked to *ts mnt* (5), and *h*21 (8) is linked to *ts*12.1 (7; Fig. 1). To prepare P22 *ts mnt ts* 12.1, P22 m^+ *ts mnt* h^+ was crossed with P22 *m*3 *ts*12.1 *h*21, and M⁺H recombinants from plating at 25 C were picked onto plates that were incubated at 39 C. Phage that did not grow at 39 C were scored as recombinants that were m^+ *ts*12.1 *h*21. Lysates of P22 m^+ *ts*12.1 *h*21 were grown at 25 C and plated at 39 C on tryptone-agar. Phages that had reverted to *ts*12.1⁺ plated under these conditions and could be scored for the presence of *ts mnt* because of the distinctive plaque morphology of *ts mnt* a 39 C (5). Phages that had been scored as m^+ *ts*12.1 *h*21 whose revertants to *ts*12.1⁺ produced Mnt plaques at 39 C were identified as P22 m^+ *ts mnt ts*12.1, the desired result of this cross. Similar crosses were carried out to prepare P22 *ts mnt ts*6.1, P22 *ts mnt ts*18.1, and P22 *ts mnt ts*21.1.

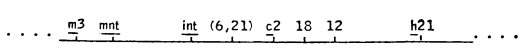

FIG. 1. *Partial genetic map of phage P22. (Data from references 5, 7, 8, 11, and unpublished experiments).*

Preparation of lysogens. Lysogens carrying P22 *int ts mnt* prophages were prepared by complementation. LT2 was mixedly infected with P22 *int ts mnt* and P22 *c*2 *mnt* (12) at 25 C in M9-CAA, and lysogenic bacteria carrying P22 *int ts mnt* were isolated and purified by repeated streakings. The genetic constitution of the prophage was verified by backcrosses. Other lysogens were prepared by picking turbid centers of plaques at 25 C.

Induction experiments. Cultures (10 ml) of lysogenic bacteria were grown in M9-CAA in 250-ml flasks shaken at 30 C. When a Klett-Summerson colorimeter reading corresponding to 10^8 bacteria per ml was reached, the bacteria were shifted to 43 C by dilution. Cultures were diluted to 10^3 to 2×10^3 bacteria per ml at the higher temperature in M9-CAA containing antiserum to P22 at $K = 2$. At intervals, 0.1-ml volumes were plated on EMB-galactose-agar. Colony-forming units and infective centers were scored after 2 days of incubation at 25 or 30 C.

Plating experiments. Lysogenic bacteria were grown to late log phase at 30 C, diluted, plated, and incubated at 25 and 43 C. Dilutions were made so that each plate at 25 C produced 75 to 125 colonies after 36 to 48 hr of incubation. Colonies on the plates incubated at 43 C were counted at the same time.

RESULTS

Experimental induction of prophage P22 *ts mnt* involves a period of time at 43 C which is followed by plating of the culture at 25 C on EMB-galactose plates (5). High temperature could (i) directly and completely eliminate the biological function of *mnt* product or (ii) reversibly inactivate it. If the former model is correct, all steps subsequent to heating, including killing of the host and production of an infective center, could occur on the EMB-galactose plate at 25 C. On the other hand, if inactivation is reversible, some other critical phage gene product would be required to act at high temperature to cause induction.

To decide between these two alternatives, LT2 (P22 *ts mnt*) was incubated at 43 C in M9 salts. Samples were removed at intervals and plated on EMB-galactose at 25 C. As is shown in Fig. 2, there was no decrease in colony-forming units nor any induction of infective centers for 150 min. All colony formers remained lysogenic,

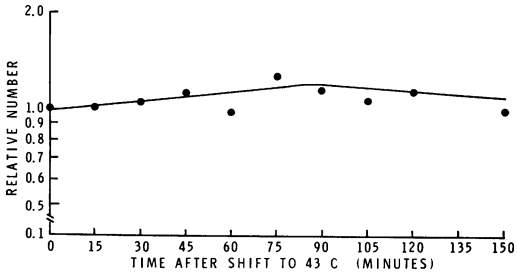

FIG. 2. *LT2 (P22 ts mnt) was grown at 30 C and shifted to non-nutritive medium at 43 C. At the indicated times platings were made at 25 C.* (●) *Colony-forming units.*

as determined by their appearance on EMB-galactose plates. This absence of effect of high temperature can be compared to Fig. 3, which shows results obtained when LT2 (P22 *ts mnt*) was heated in M9-CAA at 43 C. Heating LT2 (P22 *ts mnt*) in the absence of nutrients did not cause induction; this suggests that some metabolic activity and the functioning of some phage gene are necessary during the heat treatment in order for loss of *mnt* activity to result in host killing and prophage induction.

At least four genes of P22 are involved in DNA synthesis (2; Levine and Botstein, *unpublished data*). To see whether any of these are necessary for induction of prophage P22 *ts mnt*, temperature-sensitive alleles of each of these four genes were recombined into P22 *ts mnt*, and lysogens were constructed.

The results in Fig. 3 were obtained when LT2 (P22 *ts mnt ts* 12.1) and LT 2 (P22 *ts mnt*) were incubated under inducing conditions. After being shifted to 43 C, the number of colony formers in the culture of LT2 (P22 *ts mnt*) increased for about 40 min. At about 40 min, the number of colony formers began to decrease, and infective centers appeared. The results of heating the lysogen containing a prophage defective in DNA synthesis at high temperature were not markedly different from that of the control. The only observed difference was a slight delay in the beginning of the decrease in colony-forming units and the appearance of infective centers. Clearly, the inability of the *ts* 12.1 prophage to make DNA at high temperature does not prevent induction after removal of *mnt* product. Other experiments involving prophages P22 *ts mnt ts*6.1, P22 *ts mnt ts*18.1, and P22 *ts mnt ts*21.1 produced results comparable to those shown in Fig. 3. I concluded that none of these TS alleles of genes involved in DNA synthesis prevented or greatly retarded the induction of prophage P22 *ts mnt*. It is conceivable, of course, that the synthesis of the products of genes 6, 12, 18, and 21 is inhibited by high temperature or that the products are reversibly inactivated. If this is so, synthesis or functioning of these products could take place after the culture is plated at 25 C.

It is not known whether the synthesis of the products of these genes or the products themselves are temperature-sensitive, but the results of the plating experiments in Table 1 show that doubly mutant prophages carrying *ts mnt* and a TS allele of a gene involved in DNA synthesis kill their hosts at 43 C with essentially the same efficiency as does prophage P22 *ts mnt*.

Also shown in Table 1 is the much less efficient killing by prophages P22 *int*4 *ts mnt*, P22 *int*18 *ts mnt*, and P22 *int*32 *ts mnt* when their hosts were plated at 43 C. This result shows that a functioning *int* gene is of much more importance to induction after inactivation of *mnt* product than are the "DNA genes."

In contrast to the experiments shown in Fig. 3, and as expected from the results in Table 1,

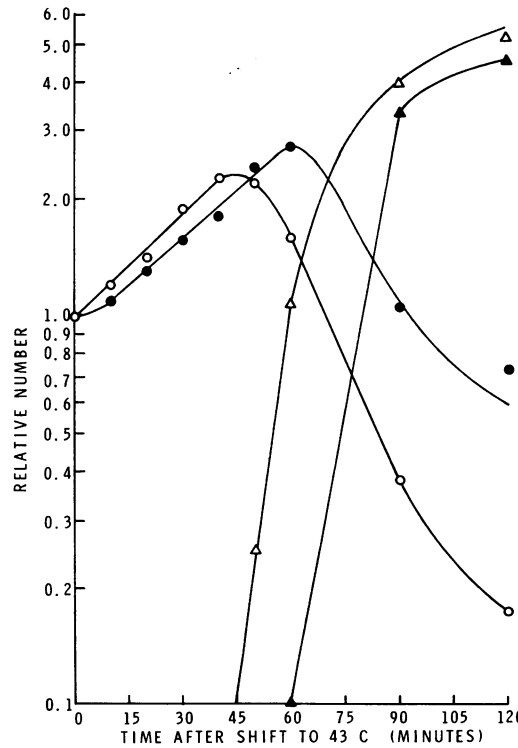

FIG. 3. *Lysogenic LT2 strains were grown at 30 C and shifted to 43 C in M9-CAA. At the indicated times, platings were made at 25 C. Strain LT2 (P22 ts mnt):* ○, *colony-forming units;* △, *infective centers. Strain LT2 (P22 ts mnt ts 12.1):* ●, *colony-forming units;* ▲, *infective centers.*

TABLE 1. *Number of colony formers found when equal volumes of lysogenic S. typhimurium cultures were plated and incubated at 25 and 43 C*

Genotype of prophage P22	No. of colony formers		Colony formers at 43 C/colony formers at 25 C
	At 25 C	At 43 C	
Wild type	351	371	1.06
ts mnt	921	28	0.03
ts mnt ts 6.1	267	22	0.08
ts mnt ts 12.1	243	0	<0.01
ts mnt ts 18.1	183	0	<0.01
ts mnt ts 21.1	1,608	13	<0.01
int 4 *ts mnt*	487	460	0.94
int 18 *ts mnt*	1,040	999	0.96
int 32 *ts mnt*	1,400	1,266	0.91

high temperature does not induce prophage P22 *int*4 *ts mnt*, as measured by decrease in colony-forming units or increase in infective centers. LT2 (P22 *int*4 *ts mnt*) was grown at 30 C to 10^8 bacteria per ml and diluted in quadruplicate to 10^3 bacteria per ml at 43 C. Samples (0.1 ml) were plated at intervals. The mean and standard error of the number of colonies at each time point is shown in Fig. 4. There was no decrease in the number of colony formers, and all remained lysogenic, as determined by plating on EMB-galactose plates at 25 C after exposure to 43 C. The number of infective centers produced was less than 0.01 times the number of colony formers. Very similar results were obtained when LT2 (P22 *int*32 *ts mnt*) was treated in the same way.

The absence of induction when prophage P22 *int ts mnt* (Table 1 and Fig. 4) is heated is in marked contrast to the induction of prophages P22 *ts mnt*, which cannot make phage DNA at high temperature (Table 1 and Fig. 3). The inability of thermal inactivation of the *ts mnt* product to cause induction in the absence of a functional *int* product suggests that "turning on" the *int* function is a necessary step in inductions following removal of *mnt* product.

The temporary lag in cell division observed in the growth curve of LT2 (P22 *int ts mnt*) between 30 and 60 min at 43 C has also been observed when nonlysogenic LT2 was subjected to the same temperature shift. Since the presence of a prophage is not necessary to cause this lag, it seems reasonable that the cell has to establish new equilibria of pool size or enzyme concentrations after the shift. The extent of the lag in division rate is variable in both lysogenic and nonlysogenic cells.

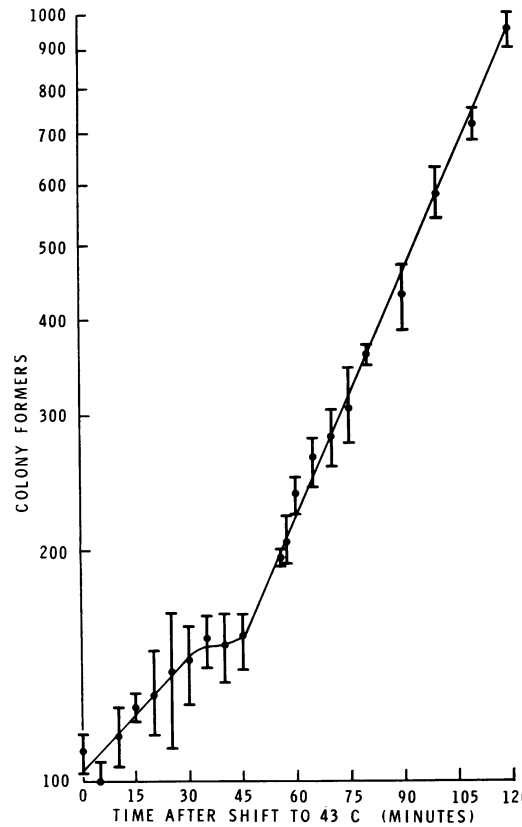

FIG. 4. *LT2 (P22 int ts mnt) was grown at 30 C and shifted to 43 C in M9-CAA. At the indicated times, platings were made in quadruplicate at 25 C. The average number of colony-forming units (●) and the standard error are shown.*

DISCUSSION

The involvement of *int* product has been studied in three different avenues of induction of prophage P22. Prophage P22 *int* exposed to ultraviolet irradiation kills the host bacterium with the same kinetics as does P22 *int*$^+$ (10, 12). The yield of infectious phage following ultraviolet induction is, however, very much reduced by an *int*$^-$ allele (10, 12). Prophage P22 *int ts c*2 induced by a shift to high temperature also kills its host with the same kinetics as P22 *int*$^+$ *ts c*2, but produces a small number of infectious phage and a large number of defective particles which contain both phage and bacterial DNA (10, 12). The absence of *int* product in these two systems has little effect on host killing, but does affect the production of infective centers and phage.

The absence of *int*$^+$ function when prophage P22 *int ts mnt* is heated prevents both host killing and infective center production. This

suggests that *int* is directly involved in inductions following removal of *mnt*, and, in turn, suggests that *mnt* regulates *int*. The following discussion will be based on this assumption. If the primary function of *mnt* is to repress *int*, its inactivation would allow expression of the *int* gene present on each prophage-bearing chromosome. In the case of prophage P22 int^+ *ts mnt*, the expression of the int^+ is sufficient to cause killing of the host and results in formation of an infective center. When prophage P22 *int ts mnt* is induced, the expression of int^- is not sufficient to kill the host or cause induction. In the two other methods of induction, phage DNA replication may occur as a primary result of induction, and the increased gene dosage of the int^- genes may be sufficient for some *int* function. Such in situ replication of prophage DNA has been postulated to occur after induction of λ *sus* N prophages in *Escherichia coli* (4).

The two genes required for maintenance of lysogeny in prophage P22 appear to produce products affecting different operators (M. Bronson, and C. Swanson and D. Botstein, *personal communications*; M. Gough, *unpublished observations*). Two operators have been found which flank *c*2 and are most likely involved in control of the "DNA genes." Phages bearing an operator-type mutation which maps near *mnt* (C. Swanson and D. Botstein, *personal communication*; M. Gough, *unpublished observations*), can be used to superinfect LT2 (P22) lysogens. One result of such superinfections is to induce the prophage. My interpretation of this observation is consistent with the idea that *mnt* regulates the function of *int*. The conjecture is that the mutant operator on the superinfecting phage cannot bind the *mnt* product; therefore, the *int* function of the superinfecting phage could be expressed constitutively and excise the prophage DNA.

Induction after heating prophage P22 *ts mnt* proceeds through at least two stages, a killing event which begins at time zero and the appearance of infective centers which is delayed at least 15 min and lags behind the killing event until 90 to 120 min (5). The killing event could result from *int*-mediated excision which fails to restore the integrity of the host chromosome after the prophage DNA is cut out. The appearance of infective centers must involve irreversible changes leading toward the synthesis of mature phage. Why *c*2 product, which should exist after excision, fails to suppress DNA synthesis by the excised phage DNA is not clear. Perhaps in a cell killed by *int*-mediated excision some general effect of death results in removal of *c*2 repression.

In addition to a model such as this in which disruption of *mnt* function does not directly interfere with *c*2 function, models which involve interactions between *c*2 and *mnt* products are possible. Experiments are being designed to investigate such models.

The requirement of two repressors, *mnt* and *c*2, to maintain lysogeny in P22 is different from the one repressor needed in *E. coli* phage λ. In λ, the *c*1 product represses synthesis of DNA synthetic enzymes directly and *int* product synthesis indirectly (3). Whether one or neither of these systems for the maintenance of lysogeny is "typical" for bacteriophages is, of course, unknown.

Separation of the two repressor activities is probably responsible for the two immunity regulator systems found in P22 (1) as opposed to the one found in λ(3). Given that immunity is a desirable attribute, the two-repressor system of P22 may be an advantage, since evolution can take place in both. From the standpoint of maintaining lysogeny, however, the two-repressor system seems to confer no advantage, because removal of either repressor results in induction.

During the preparation of this manuscript, H. O. Smith (*personal communication*) isolated a mutation in P22 called *xis*. The product of gene *xis* is necessary but not sufficient for excision of prophage P22. Its possible relationship to the proposed *mnt*-mediated repression of *int* is now being investigated.

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