JOURNAL OF VIROLOGY, Oct. 1968, p. 992-998

Vol. 2, No. 10
Printed in U.S.A.

Second Locus of Bacteriophage P22 Necessary for the Maintenance of Lysogeny

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Received for publication 19 June 1968

A temperature-sensitive allele of a locus of phage P22, known to be involved in establishment of lysogeny, has been isolated. This mutant, P22 *ts mnt*, forms stable lysogens at 30 C which are induced by heating to 43 C. This shows that this locus is involved in the maintenance of lysogeny. The *ts mnt* locus is about 18 recombination units away from the *c* region. The wild allele, *mnt*$^+$, is dominant over *mnt* and is responsible for a cytoplasmically diffusible product.

The functioning of the c_2 locus of phage P22 is required for the maintenance of lysogeny (4, 5, 9). Zinder (12) described another mutant, P22 v_1, which is unable to form a stable lysogen when it infects *Salmonella typhimurium*. As one explanation for the behavior of P22 v_1, he suggested that it might be able to initiate lysogeny but lack the ability to maintain it.

A temperature-sensitive (*ts*) allele of the v_1 locus has been isolated. This mutant forms stable lysogens at temperatures below 30 C, but elevation of the temperature to 43 C causes prophage induction. Therefore, the function of the v_1 locus, as well as the c_2 locus, is required to maintain lysogeny. Because of its properties and the accepted use of "*v*" to designate virulent mutants, I have renamed the v_1 locus *mnt*. Experiments which show that *mnt*$^+$ is dominant over *mnt* and that *mnt*$^+$ is not repressed by an active c_2 locus in the prophage are described. The *ts mnt* locus has been mapped near the m_3 region of the P22 chromosome.

MATERIALS AND METHODS

Media. Liquid cultures were grown in M9-CAA medium (9) and platings were done on indicator agar (1, 4) and tryptone agar. All dilutions were made in buffered saline (9). Some experiments were scored on EMB, (Difco) galactose-agar by using a lawn of *S. typhimurium* LT 2 Gal$^-$ bacteria. These platings allow the determination of infective centers and lysogenic and sensitive colonies on the same plate (4). The number of mature phage was determined by diluting samples into buffered saline containing $CHCl_3$ before plating.

Phage strains. Wild-type P22, P22 c_2, P22 m_3, P22 $m_3c_1h_{21}$ (4), and P22 *int* [called "P22 L" (8, 10)] have been described. The P22 *mnt* used as a reference is the original P22 v_1 of Zinder (12). The mapping of the conditional-lethal loci *ts* 1.1, and *ts* 11.1 has been described (2).

Bacterial strains. The strain of *S. typhimurium* LT 2 used in these experiments is wild-type LT 2 cured of a defective prophage [strain SA1 (12)]. A Gal$^-$ strain of LT 2 (4) was used as plating bacteria. R. Nagaraja Rao prepared a P22 *ts mnt* lysogen of *S. typhimurium* F$^-$ *pur* E66 *pro* AB47 (6). The normal attachment site (att^{I}_{P22}) for P22 is deleted in this strain and the P22 *ts mnt* prophage is integrated at a secondary attachment site (att^{II}_{P22}) on the *S. typhimurium* chromosome. *S. typhimurium* att^{II}_{P22} (P22 *ts mnt*) was crossed with *S. typhimurium* Hfr Su576 *pur* C7 *str* A att^{I}_{P22} (P22 $m_3c_1h_{21}$) (7) to isolate double lysogens. Purine prototrophs were selected and streaked on tryptone-agar plates which were overlaid with soft agar containing sensitive bacteria. Recombinants which were *S. typhimurium* att^{I}_{P22} (P22 $m_3c_1h_{21}$) att^{II}_{P22} (P22 *ts mnt*) liberated phage which produced both C and Mnt type plaques.

Phage crosses. The method of Gough and Levine (2) was used for phage crosses.

RESULTS

Isolation of P22 ts mnt. H. O. Smith isolated 40 P22 *mnt* phages from a 5-bromodeoxyuridine mutagenized phage stock. All 40 displayed the "bullseye" plaque morphology typical of v_1-type mutants (12) at 37 C. One produced wild-type plaques at 25 C. This mutant was designated *ts mnt*.

Different colony morphologies on indicator agar are characteristic of clones of bacteria which harbor a stably or unstably integrated prophage (10). Stable lysogens produce faster-growing white colonies which may or may not be tan-centered. Unstable lysogens produce slower-

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growing green colonies, or colonies with dark green areas. Bacteria infected with P22 *mnt* yielded green colonies when incubated at any temperature, whereas LT 2 infected with P22 *ts mnt* made green colonies at 42 C and white colonies at 25 C. This observation confirms that P22 *ts mnt* makes a stable lysogen only at low temperatures.

To confirm that *mnt* and *ts mnt* are in the same cistron, a series of complementation tests was run. Cells were infected with mixtures of phage plated on indicator agar at either 42 or 25 C, and green and white colonies were scored. Mixed infections with P22 *ts mnt* and P22 wild type, P22 c_2, or P22 *int* produced white or tan-centered colonies at both temperatures. The same results were obtained when P22 *mnt* was used in place of P22 *ts mnt*. This shows that the *mnt* function can be supplied by a mnt^+ phage in a mixed infection. An infection was made with a mixture of P22 *mnt* and P22 *ts mnt* and plated at 42 and 25 C. At 25 C, white colonies were obtained. At 25 C, P22 *ts mnt* supplied the function missing from P22 *mnt*. At 42 C, on the other hand, green colonies were observed. The inability of P22 *ts mnt* to complement P22 *mnt* at 42 C is consistent with the two mutations being in the same cistron.

Since the isolation of the P22 *ts mnt* used in this report, two more mutants have been found. They display the same induction kinetics reported for P22 *ts mnt* in this paper, but have not been further studied.

Mapping of the ts mnt locus. Zinder (12) reported that *mnt* mapped about 25 units away from the *c* region of P22. H. O. Smith (*personal communication*) located *mnt* near m_3.

Phages carrying *ts mnt* and m_3 mutations were crossed with phages carrying a conditional lethal *ts* marker. Lysates from crosses were plated at 37 C on tryptone agar to select for nontemperature-sensitive phage. Mnt and Mnt^+ plaques can be distinguished on these plates (12). The distance between *ts mnt* and the conditional lethal *ts* is measured as the fraction of Ts^+ Mnt^+ phage at 37 C on these plates. Ts^+ Mnt^+ plaques were picked with needles onto indicator agar plates (1, 4) seeded with LT 2 to score M and M^+ plaque morphology markers. These three factor crosses ordered the *mnt* locus with respect to three *ts* loci and m_3. The four genes *ts* 1.1—*ts* 11.1—m_3—*ts* 9.1 are linked in the order shown in Fig. 1 (2).

Crosses were carried out in duplicate (Table 1). Cross 1 established that *ts mnt* is to the right of *ts* 11.1. Since mnt^+ and m^+ showed more than 80% linkage, mnt^+ and m^+ must have remained in parental linkage after the crossover event between *ts* 11.1^+ and *ts* mnt^+. Two gene orders

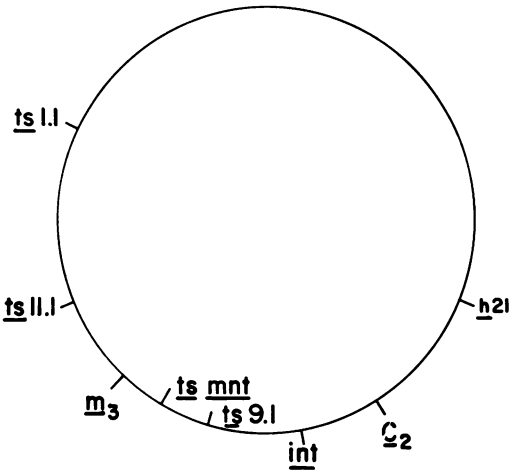

FIG. 1. *Linkage of some genetic markers of phage P22.*

are compatible with this finding: *ts* 11.1—m_3—*ts mnt* or *ts* 11.1—*ts mnt*—m_3. In either case, the conclusion is that the *mnt* locus is to the right of *ts* 11.1.

Cross 2 showed that *ts* 1.1 is farther from *ts mnt* than is *ts* 11.1. The *ts* 1.1 locus is known to be to the left of *ts* 11.1 (2) and the order *ts* 1.1—*ts* 11.1—*ts mnt* was established by these two crosses. Analysis of the frequency of recombination between *ts mnt* and m_3 in these two crosses lead to the conclusion that *ts mnt* is to the right of m_3.

If m_3 is to the right of *mnt*, then recombinants with ts^+ mnt^+ m_3 genotype would be the result of two crossover events: one between the wild allele of the conditional lethal *ts* gene and mnt^+, the other between mnt^+ and m_3. The frequency of crossovers in the first interval should have no effect on the frequency of crossovers between *ts mnt* and m_3. The frequency of crossovers between *ts mnt* and m_3 are different in crosses 1 and 2. These results were inconsistent with the *mnt* locus being located to the left of m_3.

On the other hand, if the linkage is conditional lethal *ts*—m_3—*mnt*, both ts^+ m_3mnt^+ and ts^+ m^+ mnt^+ recombinants are the products of single crossover events. As the distance between the conditional lethal *ts* mutation and *ts mnt* lengthens, the frequency of crossovers occurring in the m_3—*ts mnt* interval compared to the total between the conditional lethal *ts* mutation and *ts mnt* will decrease. The frequency of crossovers in the m_3—*ts mnt* interval in cross 1 was greater than that in 2. These data were consistent with the order *ts* 1.1—*ts* 11.1—m_3—*ts mnt*.

A third conditional lethal marker *ts* 9.1 has been mapped about 5 to 7 units to the right of

TABLE 1. *Mapping of ts mnt*

Cross no.	Phage genotypes	Ts^+ Mnt^+[a] recombinants/total plaques		M Mnt^+ recombinants/M Mnt^+ + M^+ Mnt^+[a]	
1	*ts* 11 × m_3 *ts mnt*	162/2,595	(6.2)[b]	18/104	(17)[b]
		77/965	(8.0)	11/71[c]	(16)
2	*ts* 1 × m_3 *ts mnt*	150/1,038	(14.5)	10/104	(10)
		277/2,058	(13.5)	14/156[c]	(9)
3	*ts* 9 × m_3 *ts mnt*	68/4,505	(1.5)	0/23	(<4)
		131/4,118	(3.1)	5/132	(4)

[a] Nontemperature-sensitive (Ts^+), wild-type plaques (Mnt^+) were picked from platings at 37 C on tryptone-agar. These were stabbed into indicator agar to score M and M^+ phenotypes.

[b] Numbers in parentheses indicate percentages.

[c] The probability that the difference between 29/175 and 24/260 is due to chance is <0.05.

m_3 (*unpublished results*). Cross 3 showed that *ts* 9.1 is to the right of *ts mnt*. The ts^+ mnt^+ recombinants are primarily m^+. Because m^+ and mnt^+ entered the cross in parental linkage and this linkage was preserved after the crossover between *ts* 9.1 and *ts mnt*, it was concluded that the m_3 and *ts mnt* linkage was not disrupted by the crossover between *ts* mnt^+ and *ts* 9.1^+. Since the frequency of crossovers between *ts mnt* and *ts* 9.1 is only about 2%, if m_3 were between those markers it should segregate as a middle marker and appear in recombination with *ts mnt* much more frequently than it does. This observation reinforces the conclusion that *ts mnt* is to the right of m_3.

Figure 1 shows the location of *ts mnt* with respect to some other markers of P22.

Temperature induction of LT 2 (P22 ts mnt). The kinetics of thermal induction of LT 2 (P22 *ts mnt*) are shown in Fig. 2. LT 2 (P22 *ts mnt*) was grown at 30 C to about 2×10^8/ml, treated with anti-P22 serum, and diluted into 43 C medium containing anti-P22 serum (K = 2). Samples were taken at the indicated times to determine colony-formers and infective centers. The number of mature phage particles was determined in samples from 43 C medium which contained no antiserum. For determination of cell number, 8 ml of the 30 C culture was diluted into 8 ml of 56 C M9-CAA and put at 43 C. At the indicated times, 0.5 ml of culture was removed, added to 1.0 ml of buffered saline containing 0.01 M NaCN, and subsequently counted with a phase microscope.

The number of colony-forming units remained more or less constant for about 30 min, then fell precipitously, at which time the number of infective centers began to increase. The loss of colony-forming units and their conversion to infective centers showed that prophage P22 *ts mnt* is induced by the high temperature.

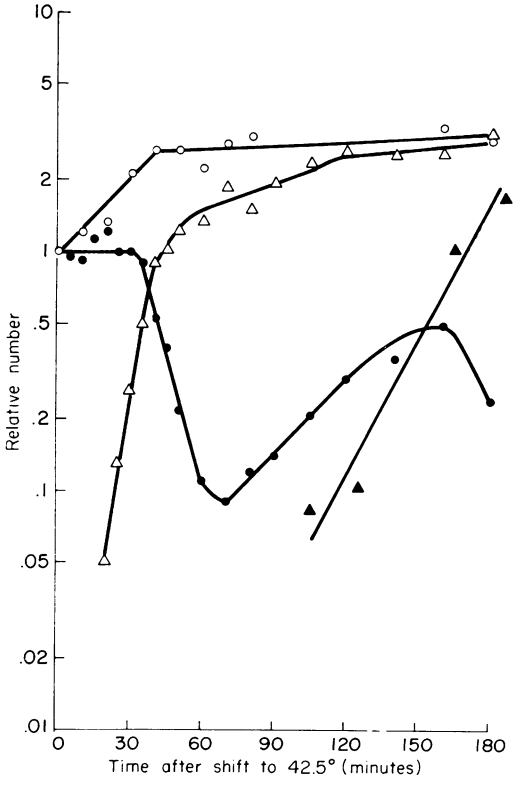

FIG. 2. *Thermal induction of prophage P22 ts mnt. LT 2 (P22 ts mnt) was grown in M9-CAA at 30 C, treated with P22-antiserum and diluted into 43 C medium. At the indicated times, samples were plated to determine infective centers (△) and colony-formers (●). Other samples were diluted into buffered saline containing CN^- and cells were counted with a phase microscope (○). Mature phage particles were counted by diluting samples from 43 C medium containing no antiserum into buffered saline containing $ChCl_3$, waiting 5 min, and plating on indicator bacteria (▲).*

By 120 min, the number of infective centers was three times the number of colony-formers at time-zero. Although there was no threefold increase in colony-formers, microscopic counts show that the number of cells in the culture did increase threefold. The increase in cell number was sufficient to account for the threefold excess of infective centers at 120 min, compared to colony-formers at time-zero.

The latent period for the induction of P22 *ts mnt*, estimated as the time between the appearance of infective centers and mature phage, was about 90 min. Although data are not shown in Fig. 2, the yield of infectious phage particles was about one per infective center. This small yield is consistent with the report of Israel (3) that the yield of infectious particles of P22 from induction of prophage at temperature higher than 40 C is less than 1 phage per infective center. Israel (3) found that the reduced yield of infective particles was due to the failure of P22 to produce a sufficient number of tails at high temperature.

The secondary increase in colony-formers, which begins at around 70 to 80 min and continues to about 120 to 150 min, has been observed in all thermal inductions of prophage P22 *ts mnt*. When LT 2 (P22 *ts mnt*) was held at 43 C for 360 min, no other "rounds" of increase of colony-formers was observed. However, the number of colony-formers remained constant at about 0.1 of that seen at time-zero. In another experiment, after 18 hr at 42 C, the number of colony-formers was still 0.14. A colony was picked from platings made at 80 min, 150 min, and 18 hr after a shift from 30 to 43 C. Each colony was grown at 30 C and induced by shifting to 43 C. All were induced with the same kinetics shown in Fig. 2.

Temperature induction of prophage P22 ts mnt when in the secondary P22 attachment site (att^{II}_{P22}). The normal attachment site for prophage P22 on the *S. typhimurium* chromosome is between *pro* A and *pro* C (8). At least one secondary attachment site exists on the LT 2 chromosome (R. N. Rao, *personal communication*).

A strain of LT 2 with prophage P22 *ts mnt* present at the secondary site was grown at 30 C then shifted to 43 C. The induction of prophage P22 *ts mnt* at the secondary site (Fig. 3) was similar to that seen when P22 *ts mnt* was present at the primary site (Fig. 2). The number of colony-formers remained almost constant for 20 to 30 min, then fell rapidly. At the same time, the number of infective centers began to increase and reached a number equal to two or three times the colony-formers present at time-zero. In these respects, the induction was the same as that shown in Fig. 2.

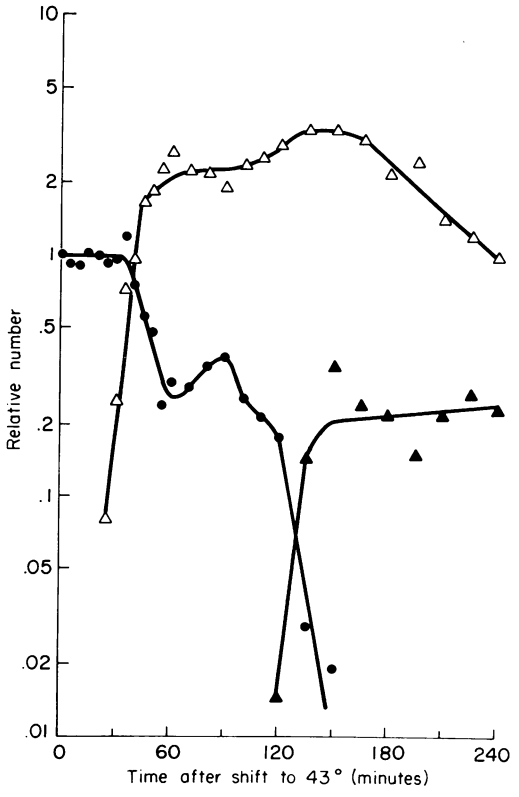

FIG. 3. *Thermal induction of prophage P22 ts mnt present at a secondary attachment site on the LT 2 chromosome. The experiment was run as described for Fig. 1. Symbols: infective centers, (△); colony-formers, (●); mature phage, (▲).*

There was a striking difference between inductions at the two sites, however. At 180 min, in the experiment shown in Fig. 3, there were fewer than 0.01 of the colony-formers present at time-zero. Such extensive loss of colony-formers has not been observed in any thermal induction of *S. typhimurium* att^{I}_{P22} (P22 *ts mnt*).

Although there are differences between induction of prophage P22 *ts mnt* at the primary and secondary attachment sites, a temperature shift causes induction at either site. Therefore, a double lysogen is suitable to test whether mnt^{+} or *ts mnt* is dominant at high temperature.

Dominance of mnt^{+} over ts mnt at high temperature. The doubly lysogenic strain LT 2 att^{I}_{P22} (P22 $m_3c_1h_{21}$) att^{II}_{P22} (P22 *ts mnt*) was prepared by crossing an Hfr att^{I}_{P22} (P22 $m_3c_1h_{21}$) with an F^{-} att^{II}_{P22} (P22 *ts mnt*).

The two parental strains and the double lysogen were grown at 30 C then shifted to 43 C.

Because the parental strains required purines for growth, the double lysogen was grown in medium with and without addition of adenosine. (Fig. 4).

As expected, LT 2 att^{II}_{P22} (P22 *ts mnt*) was induced by high temperature and the number of colony-formers decreased while infective centers were produced. The singly lysogenic strain LT 2 att^{I}_{P22} (P22 $m_3c_1h_{21}$) grows exponentially and no infective centers are produced. The doubly lysogenic strain containing a *ts mnt* and a mnt^+ prophage also grew exponentially in either the presence or absence of purines and produced no infective centers (<0.01 of time-zero colony formers). The presence of one mnt^+ allele in the double lysogen was sufficient to prevent thermal induction. Therefore, mnt^+ is dominant.

DISCUSSION

Shifting a culture of *S. typhimurium* (P22 *ts mnt*) from 30 to 43 C results in induction of the prophage. This demonstrates that the *mnt* locus is involved in the maintenance of lysogeny.

There are two general models for the induction of prophage P22 *ts mnt* at high temperature. Either the higher temperature (i) causes the *ts mnt* to synthesize a "new" product which results in induction, or (ii) heat stops the synthesis or functioning of a product normally provided by the *mnt* locus. Model 2 fits the results found when a double lysogen is heated (Fig. 4). Heating P22 *ts mnt* normally results in induction, but the presence of a mnt^+ gene in another prophage in the same cell prevents such induction. The observation that the product from the mnt^+ locus prevents induction of either prophage shows that it is diffusible and works as a cytoplasmic agent. The conclusion that the mnt^+ cistron produces a cytoplasmic agent which is necessary for the maintenance of lysogeny is the basis for the following discussion of the thermal induction of prophage P22 *ts mnt*.

After shifting LT 2 (P22 *ts mnt*) from 30 to 43 C, the cell number increases threefold in about 40 min. Even though cell number increases, not all cells are competent to form colonies, as shown by an increase in colony-formers of <1.3 in the same time. I define the loss of ability to form a colony as a killing event.

Three assumptions can be used to construct a model for the first 60 min after the shift of LT 2 (P22 *ts mnt*) from 30 to 43 C. (i) There is an excess of *mnt* product in lysogenic cells at the time of the shift. (ii) Synthesis of *mnt* product stops immediately at 43 C. (iii) Reduction of the amount of *mnt* product in a cell below a critical level results in cell death.

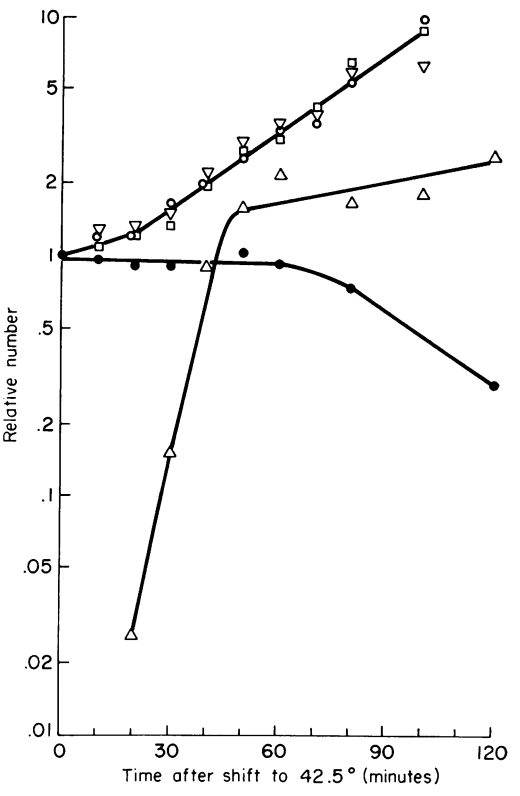

FIG. 4. *Failure of a temperature shift to induce a double lysogen LT 2* att^{I}_{P22} *(P22* $m_3c_1h_{21}$*)* att^{II}_{P22} *(P22 ts mnt). The experiment was run as described for Fig. 1. Symbols: colony formers, LT 2* att^{I}_{P22} *(P22* $m_3c_1h_{21}$*),* ○*, LT 2* att^{I}_{P22} *(P22* $m_3c_1h_{21}$*)* att^{II}_{P22} *(P22 ts mnt) grown in the presence,* ▽ *and in the absence* (□) *of purine; LT 2* att^{II}_{P22} *(P22 ts mnt),* ●*; infective centers from LT 2* att^{II}_{P22} *(P22 ts mnt),* △.

When the culture is shifted, synthesis of *mnt* product stops and the preexisting *mnt* product segregates between the daughter cells of each cell division. The distribution of *mnt* product is such that, on the average, one daughter of each pair is killed because of a lack of *mnt* product. During this time, the number of colony-formers will remain constant. The viable cells go on to divide again, but the amount of *mnt* product is now so reduced that neither daughter receives enough *mnt* product to prevent killing. These conjectures are consistent with the number of colony-formers remaining constant for 30 min, while cell number increases three times. By 30 min, the *mnt* product per cell is so reduced by the threefold dilution that most cells are killed.

The above model says nothing about induction, which is defined here as the production of an

infective center. It is clear that the killing event and induction are temporally separated. The removal of *mnt*$^+$ function results in immediate killing, but a train of events, set in motion by the removal of *mnt*$^+$ function, requires several minutes to cause induction. The killing event is irreversible by a shift to low temperature. Since many cells between 0 and 120 min produce neither colonies nor infective centers, some process necessary for induction must be inhibited by low temperature. If a killed cell is plated before the induction is complete at high temperature, the low temperature would stop the induction and no infective center would be formed. However, the cell could not form a colony, since killing is irreversible. This makes understandable the failure to recover all cells as colony-formers or infective centers before 120 min.

After 60 min, the continued survival of lysogenic colony-formers in a culture of LT 2 att^{I}_{P22} (P22 *ts mnt*) cannot be explained on the basis of any product with a discrete half-life. Because of the round of secondary growth between 70 and 120 min (Fig. 2), it seems likely that the lysogenic cells are dividing but not all the daughters are viable. The residual colony-formers could be explained if 0.1 of the population at time-zero had a completely stable *mnt*$^+$ product and this was not synthesized at the high temperature. However, a colony-former picked from a plating 18 hr after the shift to high temperature, grown at 30 C and shifted to 43 C, displayed the same induction kinetics as shown in Fig. 2. The same number of colony-formers (about 0.1) survived 360 min at 43 C. Therefore, if 0.1 of the population has a stable *mnt*$^+$ product, not all of the progeny of these cells produce such a product and the survivors are not genetically determined. Any discussion of residual colony-formers is complicated, because essentially no colony-formers in a culture of LT 2 att^{I}_{P22} (P22 *ts mnt*) survive after 180 min at 43 C.

The other gene of P22 known to be involved in maintenance of lysogeny, c_2, makes the P22 repressor (4, 5, 9). A thermal inducible allele of c_2, *ts* c_2, has been isolated (5). When LT 2 (P22 *ts* c_2) is shifted from 30 to 43 C, the prophage is induced. Colony-formers decrease and infective centers appear from time-zero. Induction is essentially complete in 20 min, when all colony-formers are lost and a number of infective centers equal to the number of colony-formers at time-zero is observed (Wing, Levine, and Smith, *in press* and *unpublished results*). These kinetics are quite different from those observed with P22 *ts mnt*. Removal of c_2 function or product may be a critical step in thermal induction of P22 *ts mnt*. Experiments are underway to investigate this possibility.

At least five genes are involved in establishment and maintenance of lysogeny by P22. The three clear genes, c_1, c_2, and c_3 (4) are required to establish lysogeny and c_2 is also necessary to maintain it. The *int* locus, called "L" (8, 10), is necessary for integration. I have shown a fifth gene *mnt*, known to be involved in establishment of lysogeny (12), which is also necessary to maintain lysogeny. The three *c* genes are clustered (4) and *int* is near them (10) on the P22 chromosome. However, *mnt* is some distance away from *int* and at least one "late" gene, *ts* 9.1, which is the structural gene for the P22 tail protein (Botstein et al., *unpublished results*), is between *int* and *mnt*.

It is now known that at least four genes of P22 are active in the prophage state: the c_2 repressor; the gene *a*1, which is responsible for an antigenic change in the host (11, 13); *sie*, which excludes superinfecting phages (R. N. Rao, *in press*); and *mnt*. It will be interesting to find out where *sie* maps, and a careful study may reveal that these three genes, *a*1, *sie*, and *mnt*, which are not under the control of c_2 repression, are clustered. An intriguing possibility is that these three genes are of host origin.

ACKNOWLEDGMENTS

I thank M. Levine, R. N. Rao, and H. O. Smith for gifts of strains, time, and advice.

This study was supported by Public Health Service grants GM 09252-05 and 5T01 GM 00071 from the National Institute of General Medical Sciences.

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