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Control of the Replication Complex of Bacteriophage P22

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A replication complex for the vegetative synthesis of the deoxyribonucleic acid (DNA) of the temperate phage P22 previously has been described. This complex is an association of parental phage DNA, most of the newly synthesized phage DNA made during pulses with ^{3}H-thymidine, and other cell constituents, and has a sedimentation rate in neutral sucrose gradients of at least 1,000*S*. The complex is one of the intermediates, intermediate I, in the synthesis and maturation of phage P22 DNA after infection or induction. Evidence supporting the replicative nature of intermediate I is presented. Phage replication is repressed in lysogenic bacteria. On superinfection of P22 lysogens with nonvirulent phage, little association of the input phage DNA with a rapidly sedimenting fraction is demonstrable. However, after induction with ultraviolet light, the superinfecting parental phage DNA quickly acquires the rapid sedimentation rate characteristic of intermediate I; phage DNA synthesis follows; and progeny phages are produced. Infection with a virulent mutant of P22 produces progeny phages in lysogens. Its DNA associates with intermediate I. In mixed infection with the virulent phage, replication of nonvirulent phage P22 is still repressed, even though the virulent replicates normally. The nonvirulent input DNA does not associate with intermediate I. The repressor of the lysogenic cell prevents replication by interfering with the physical association of template material with intermediate I. A phage function is required for association of phage template with the replication machinery.

Phage P22 is a temperate phage of *Salmonella typhimurium*. On infection, some bacteria lyse, giving rise to a yield of progeny particles, but some survive and give rise to lysogenic cells. Lysogenic bacteria are immune to subsequent infection by the phage, but they possess the ability to produce the originally infecting phage on induction. Genetic control of the lysogenic condition lies in the prophage, which is the phage hereditary material integrated into the bacterial genome (12, 13). The immunity of the lysogenic cell to reinfection is due to the action of a specific repressor encoded in the prophage genome (9). Virulent mutants of temperate phage which plate on lysogenic bacteria have been isolated (M. J. Bronson and M. Levine, Bacteriol. Proc., p. 201, 1970). They are insensitive to repressor.

A number of intermediates in the synthesis and maturation of phage P22 deoxyribonucleic acid (DNA) have been described (1-3). When phage DNA is synthesized after infection or induction, most of the radioactivity incorporated into the viral DNA during pulses with ^{3}H-thymidine is recovered at the bottom of a sucrose gradient in a position indicating a sedimentation rate of at least 1,000*S*. This rapidly sedimenting fraction is called intermediate I. If an infection is initiated with ^{32}P-labeled phage, some parental phage DNA is always found associated with the pulse-labeled material in intermediate I. Both labels chase from intermediate I in parallel and are found in intermediate II, which is a collection of concatemers of multiple phage genomes, and finally in progeny phage particles. Intermediate I appears to be a complex of parental and replicating phage DNA with other cell components, possibly including the cell membrane.

This report examines whether repression in lysogenic cells is expressed after superinfecting phage DNA associates with intermediate I, or whether this association is prevented in immune cells. Evidence is also presented that a phage-specific function is required for association of phage DNA with intermediate I.

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MATERIALS AND METHODS

Wild-type *S. typhimurium* LT2, a uracil-requiring mutant (*pyr* B16, supplied by K. E. Sanderson) and a lysogen carrying a *sie* mutant prophage (10) were used as hosts in these experiments. Superinfecting phage genomes can rarely be recovered on induction of lysogens carrying wild-type P22 prophage (10, 16). The P22 mutant designated *sie* (*superinfection exclusion*), used in these experiments, was isolated by Rao (10). As prophage, such mutants do not exclude superinfecting phage, although they still immunize the lysogen.

The double mutant phage c_1h_{21} (8) which is sensitive to repressor, and a virulent mutant, *vir-3* (M. J. Bronson and M. Levine, Bacteriol. Proc., p. 201, 1970) which is not, were used to infect sensitive bacteria and to superinfect the lysogens. Lysogens were induced by exposure to ultraviolet light (30-sec exposure to a 15-w germicidal lamp at a distance of 50 cm). This treatment produces a large number of particles which lack complete tails and are, therefore, inactive (6). These particles can be activated in vitro with purified tail parts (7). An excess of tail parts was added to each lysate to insure that accurate titers of progeny phage were determined.

The preparation and purification of ^{32}P-labeled phage, infection, pulse labeling of intracellular DNA with ^{3}H-thymidine, lysis of infected complexes, and zone sedimentation through sucrose density gradients have been described by Botstein (1). The only change was that the infections were carried out in supplemented M9 medium (8) instead of LCG20 (1).

Much of the data to be reported is given as per cent of parental DNA in intermediate I. In practice, this is the ^{32}P label recovered in the first six fractions of a sucrose gradient divided by the total amount of ^{32}P label recovered in the gradient times 100.

RESULTS

Association of parental DNA with intermediate I is under the control of repressor. Nonlysogenic strain 18 cells were infected with a multiplicity of 10 ^{32}P-labeled nonvirulent, c_1h_{21}, phage. At 10 min, a sample of the infected complexes was exposed to ^{3}H-thymidine. At 12 min, an excess of cold thymidine was added, and a sample was immediately treated with the lysis mixture. The results of sedimenting a sample of this lysate through a neutral sucrose gradient are shown in Fig. 1a. All of the newly synthesized DNA (labeled during the pulse with ^{3}H-thymidine) banded at the bottom of the gradient in a peak also containing a large share (36%) of the ^{32}P-labeled parental DNA. This is intermediate I as described by Botstein (1). The remainder of the ^{32}P-labeled DNA sedimented near the top of the gradient in the same position as DNA extracted from phage particles. This DNA served as a marker for the position of mature phage DNA in the sucrose gradients. As expected, this

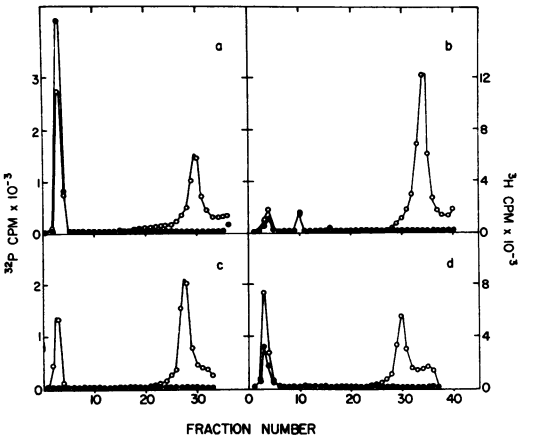

FIG. 1. *Association of DNA with intermediate I after infection of sensitive and lysogenic cells. Lysates were centrifuged through neutral 5 to 20% sucrose gradients with a cushion of high-density material at the bottom for 60 min at 35,000 rev/min at 21 C. (a) Infection of sensitive cells with ^{32}P-labeled nonvirulent phage. ^{3}H-thymidine was added at 10 min after infection, and lysate was made at 12 min. (b) Superinfection of P22-lysogen with ^{32}P-labeled nonvirulent phage. ^{3}H-thymidine was added at 10 min after infection, and lysate was made at 12 min. (c) Superinfection of P22-lysogen with ^{32}P-labeled nonvirulent phage followed by UV irradiation at 14 min after infection. ^{3}H-thymidine was added at 30 min and lysate was made at 32 min after infection. (d) Same as (c), except that ^{3}H-thymidine was added at 50 min, and the lysate was made at 52 min. Symbols: (○), ^{32}P radioactivity; (●), ^{3}H radioactivity.*

infection gave a large yield of progeny phage (Table 1, infection 1).

Strikingly different results were observed in a similar experiment in which a lysogen, strain 18 ($c^{+}h_{21}{}^{+}sie$), was superinfected with the same labeled phage, c_1h_{21}, at the same multiplicity (Fig. 1b). Very little ^{32}P-labeled DNA (8%) is found at the bottom of the gradient. Practically all of the parental material is found in the position for mature phage DNA. Very few progeny particles were produced in this infection (Table 1, infection 4).

To show that the failure to demonstrate intermediate I is due to immunity, a sample of the superinfected lysogen was exposed to an inducing dose of ultraviolet light at 14 min after superinfection. At 16 and 36 min after induction, samples were pulse labeled with ^{3}H-thymidine for 2 min and then lysed. The lysates were examined in sucrose gradients. At 16 min after the ultraviolet treatment (Fig. 1c), some 21% of the parental DNA appeared in intermediate I. There was, however, no incorporation of ^{3}H-thymidine. The inducing dose of ultraviolet light

TABLE 1. *Replication inhibition of nonvirulent phage*[a]

Infection	Phage	Host	Yield of infecting or superinfecting phage per cell		Per cent nonvirulent
			Virulent	Nonvirulent	
1	Nonvirulent	Sensitive		252	
2	Virulent	Sensitive	170		
3	Virulent + nonvirulent	Sensitive	170	140	45
4	Nonvirulent	Lysogen		0.5	
5	Virulent	Lysogen	110		
6	Virulent + nonvirulent	Lysogen	90	1.4	1.5
7	Nonvirulent	Induced lysogen		190	
8	Virulent	Induced lysogen	230		
9	Virulent + nonvirulent	Induced lysogen	199	61	23

[a] All infections were carried out in supplemented M9 medium at 37 C. The total multiplicity in each infection was 10. In the mixed infection, the MOI was 5 each for both the virulent mutant, *vir-3* and the nonvirulent, c_1h_{21}. Lysates were treated with chloroform 90 min after infection, and tails were added to detect incomplete phage particles. Lysates were plated on tryptone agar and indicator agar for progeny phage.

caused a 30-min inhibition in overall DNA synthesis. At 36 min after induction (52 min after superinfection), DNA synthesis had recovered, and, as expected, all of the pulse-labeled DNA was in intermediate I (Fig. 1d). Some 30% of the parental DNA was now found in intermediate I. The induction caused more than 90% of the exposed cells to yield progeny phages among which superinfecting (Table 1, infection 7), prophage, and recombinant types were represented. A preliminary report of this experiment was included in another report (3).

Similar experiments with phage λ have given rise to conflicting results. Hallick, Boyce, and Echols (5) reported that superinfecting λ DNA did not associate with a rapidly sedimenting "membrane" fraction until repression was lifted. In contrast, Salivar and Gardinier (11) reported that superinfecting λ DNA associated with the cell membrane of immune lysogens. Replication did not begin until induction.

A series of similar infections were undertaken with a virulent phage. *Vir-3* gave yields of virulent progeny in both sensitive and lysogenic hosts (Table 1, infections 2 and 5). The prophage was not induced in virulent infections of the lysogen. After mixed infection of the sensitive host with the nonvirulent phage (multiplicities of 5 each), nearly equal yields of virulent and nonvirulent progeny were obtained (Table 1, infection 3). This demonstrated that the nonvirulent is not at a competitive disadvantage with the virulent. In the lysogenic host, however, the nonvirulent progeny was less than 2% of the total yield and gave a burst size of about 1 (Table 1, infection 6). Thus, nonvirulent phage P22 exhibits the phenomenon of replication inhibition (15). It fails to replicate in a lysogen even though a virulent mutant replicates autonomously. When immunity was lifted by an inducing dose of ultraviolet light immediately before the infection, both superinfecting phages appeared in the yields (Table 1, infection 9).

A lysate of the lysogen mixedly superinfected with ^{32}P-labeled *vir*-3 and unlabeled nonvirulent phage [multiplicity of infection (MOI) of 5 each] gave a banding pattern in a sucrose gradient shown in Fig. 2a. Thirty per cent of the parental virulent DNA banded in intermediate I, correlating well with the replication of the virulent in this host. Lysates of the reciprocal superinfection, ^{32}P-labeled nonvirulent phage and unlabeled *vir-3*, showed no more than 4% of the labeled nonvirulent parental DNA in intermediate I (Fig. 2b). The remaining fraction banded in the position for mature phage DNA near the top of the gradient. If, however, the lysogen was exposed to an inducing dose of ultraviolet light before the mixed infection, some 32% of the labeled nonvirulent parental DNA associated with intermediate I (Fig. 2c) 30 min after the ultraviolet treatment and the superinfection. These results show that replication-inhibited DNA does not associate with intermediate I in the immune cells.

Phage-specified function is required for association of parental DNA with intermediate I. A study of the kinetics of association of input phage DNA with intermediate I was undertaken. Nonlysogenic strain 18 cells were infected with a multi-

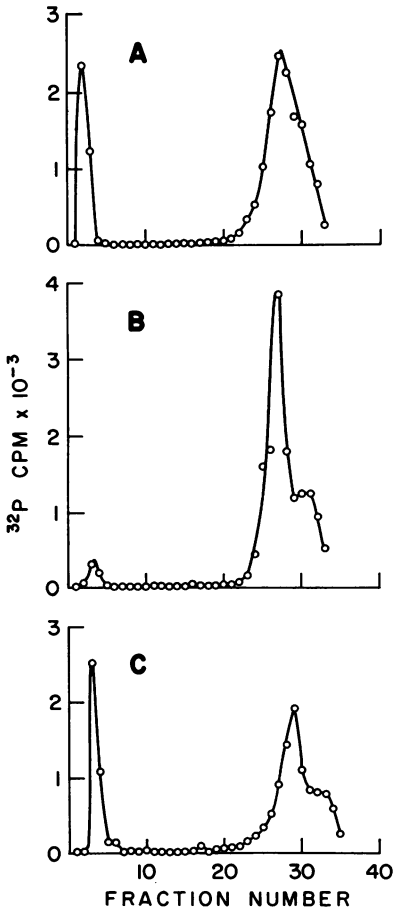

FIG. 2. *Association of DNA with intermediate I after infection of a lysogen. Lysates were centrifuged through neutral 5 to 20% sucrose gradients with a cushion of high-density material at the bottom for 60 min at 35,000 rev/min at 21 C. (a) Superinfection of lysogenic cells with ^{32}P-labeled virulent phage and unlabeled nonvirulent phage. The lysate was made at 15 min. (b) Superinfection of lysogenic cells with ^{32}P-labeled nonvirulent with unlabeled virulent phage. The lysate was made at 15 min. (c) Superinfection of ultraviolet-induced lysogenic cells with ^{32}P-labeled nonvirulent and unlabeled virulent phage. The lysate was made 30 min after the ultraviolet treatment and the superinfection. Symbols: (○), ^{32}P radioactivity.*

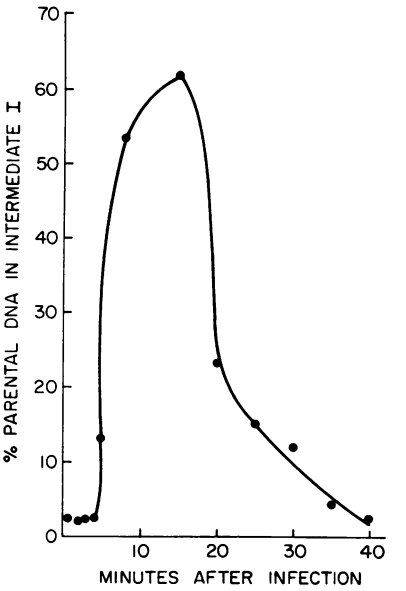

FIG. 3. *Kinetics of association of parental phage DNA with intermediate I. Nonlysogenic cells were infected with a multiplicity of 10 ^{32}P-labeled nonvirulent phage. At the indicated times, samples were lysed and run on sucrose gradients as described in the legend to Fig. 1. Each point represents the per cent of label recovered in the first six fractions of the gradient.*

plicity of 10 ^{32}P-labeled nonvirulent phage. At various times, samples were lysed and run in sucrose gradients, and the amount of label in the first six fractions was determined. Very little infecting DNA appeared in intermediate I during the first 4 min of the infection (Fig. 3). After this initial lag, labeled material rapidly accumulated in intermediate I, reached a peak at about 15 min, and then decreased. Lysis of the infected cells started at about 35 min. The observed lag suggests the need for a phage function for association of input DNA with intermediate I. The decrease is due to the release of DNA as intermediate II. Botstein and Levine (2) have demonstrated that a phage function is required for the release of labeled material from intermediate I.

If a specific phage function is necessary for binding of input DNA to intermediate I, the conditions which inhibit messenger synthesis should interfere with the binding. In an attempt to show this, RNA synthesis was inhibited by starvation of a uracil-requiring auxotrophic strain for uracil.

Uracil-requiring cells were grown in M9-CAA medium without uracil. After 40 min of starvation, the culture was infected with ^{32}P-labeled nonvirulent phage (MOI 10), and split into two parts; uracil was added back to one. At various times, samples of the two cultures were lysed and analyzed on sucrose gradients. A significant but low level of input phage DNA associated with intermediate I in the starved cells (Fig. 4). Addition of uracil resulted in much more association with normal kinetics. It would appear that there is at least a partial requirement for newly synthesized messenger for binding of DNA to

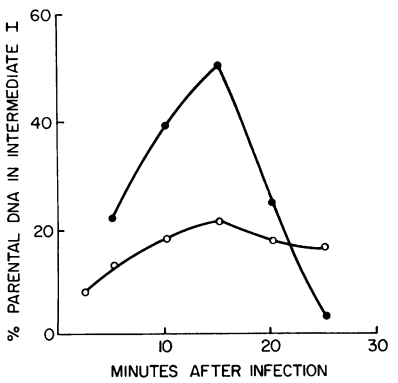

FIG. 4. *Effect of uracil starvation on association of parental phage DNA with intermediate I. ^{32}P-labeled nonvirulent phage (MOI of 10) was used to infect a starved uracil-requiring sensitive culture. Uracil was added to a sample of the culture at 5 min after infection. Samples were lysed at the indicated times and run on sucrose gradients as described in the legend to Fig. 1. Each point represents the per cent of radioactivity recovered in the first six fractions of the gradient. Symbols:* (○), *minus uracil;* (●), *plus uracil.*

intermediate I, supporting the idea of the need for a phage function.

Finally, a temperature-sensitive mutant phage has been isolated which does not associate its DNA with intermediate I at high temperature. Infection of nonlysogenic strain 18 cells at 39 C with ^{32}P-labeled *ts 25.1c*$_1$ phages gives sucrose gradient patterns similar to that described in Fig. 1b. Virtually none of the parental label sediments rapidly (less than 3%). A more complete characterization of this mutant will be presented later. Bezdek and Soska have isolated a similar P22 mutant (Mol. Gen. Genet., *in press*). Hallick et al. (5) have reported that the product of the N gene of λ is required for association of λ DNA with the membrane fraction.

DISCUSSION

Intermediate I undoubtedly represents a complex of phage DNA with other cell components, since its sedimentation characteristics cannot be accounted for by its DNA content alone. The complex has several properties expected of the replication machinery of phage P22. It contains parental DNA, it is preferentially labeled in pulses with ^{3}H-thymidine, and the DNA associated with it is precursor to the DNA in progeny particles. The data presented here extend the evidence for the replicative nature of intermediate I. There is a direct correlation between replication of a nonvirulent phage in a lysogen and association of its DNA with intermediate I. Under conditions of repression, DNA from a nonvirulent phage does not associate. Only after inactivation of repressor does the superinfecting DNA sediment rapidly and replication occur. Association of template DNA with the replication machinery is under control of repressor.

The kinetic data show very little parental DNA in the machinery before the fifth minute of the infection. This correlates very well with the time of onset of phage DNA synthesis (14). The product of gene 25 seems to be necessary for appearance of parental DNA in the replication machinery. Preliminary evidence shows that gene 25 product must act early in the infection for phage DNA synthesis to occur. Whether it acts directly to induce part of the phage replication machinery, by binding parental DNA to the machinery, or has a regulatory function as does gene N product in λ infections is not clear.

Binding of parental phage DNA to the machinery does not appear to be a consequence of replication. Temperature-sensitive mutants in two P22 loci, genes 12 (2) and 18 (Levine, *unpublished data*), do not synthesize measurable amounts of phage DNA at restrictive temperature, yet the mutant DNA molecules associate with intermediate I. In line with its replicative function, these data suggest that gene 12 and gene 18 products are usually expressed in intermediate I.

The virulent mutant induces all functions necessary for an active replication machinery and for binding of template DNA to it. Still, a nonvirulent phage does not replicate in the mixed superinfection, and its DNA does not associate. The virulent cannot complement nonvirulent DNA into the active machinery in the presence of repressor. Only after inactivation of repressor do both types of DNA bind to intermediate I and are both replicated. Repressor appears to play a direct role in replication inhibition by preventing repressed DNA from associating with the replication machinery. This suggests that the action of repressor may be a physical one. Perhaps the site for binding to the replication machinery is blocked by bound repressor. Alternatively, repressor may block transcription of a critical region of each DNA molecule which is a prerequisite for binding to the machinery. Dove et al. (4) have suggested a role for transcription of a particular region of the λ genome in release from replication inhibition.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Botstein, D. 1968. Synthesis and maturation of phage P22 DNA. I. Identification of intermediates. J. Mol. Biol. 34:621–641.
2. Botstein, D., and M. Levine. 1968. Synthesis and maturation of phage P22 DNA. II. Properties of temperature-sensitive phage mutants defective in DNA metabolism. J. Mol. Biol. 34:643–654.
3. Botstein, D., and M. Levine. 1968. Intermediates in the synthesis of phage P22 DNA. Cold Spring Harbor Symp. Quant. Biol. 33:659–667.
4. Dove, W. F., E. Hargrove, M. Ohashi, F. Haugli, and A. Guha. 1969. Replicator activation in lambda. Jap. J. Genet. 44:11–22.
5. Hallick, L., R. P. Boyce, and H. Echols. 1969. Membrane association by bacteriophage λ DNA: possible direct role of regulatory gene N. Nature (London) 223:1239–1242.
6. Israel, V. 1967. The production of inactive phage P22 particles following induction. Virology 33:317–322.
7. Israel, J. V., T. F. Anderson, and M. Levine. 1967. *In vitro* morphogenesis of phage P22 from heads and base-plate parts. Proc. Nat. Acad. Sci. U.S.A. 57:284–291.
8. Levine, M. 1957. Mutations in the temperate phage P22 and lysogeny in Salmonella. Virology 3:22–41.
9. Levine, M., and H. O. Smith. 1964. Sequential gene action in the establishment of lysogeny. Science 146:1581–1582.
10. Rao, R. N. 1968. Bacteriophage P22 controlled exclusion in *Salmonella typhimurium*. J. Mol. Biol. 35:607–622.
11. Salivar, W. O., and J. Gardinier. 1970. Replication of bacteriophage lambda DNA associated with the host cell membrane. Virology 41:38–51.
12. Smith, H. O. 1968. Defective phage formation in lysogens of integration deficient phage P22 mutants. Virology 34:203–223.
13. Smith, H. O., and M. Levine. 1965. Gene order in prophage P22. Virology 27:229–231.
14. Smith, H. O., and M. Levine. 1965. The synthesis of phage and host DNA in the establishment of lysogeny. Virology 25:585–590.
15. Thomas, R., and L. E. Bertani. 1964. On the control of the replication of temperate bacteriophages superinfecting immune hosts. Virology 24:241–253.
16. Walsh, J., and G. G. Meynel. 1967. The isolation of non-excluding mutants of phage P22. J. Gen. Virol. 1:581–582.