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Superinfection in Bacteriophage S13 and Determination of the Number of Bacteriophage Particles Which Can Function in an Infected Cell

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Bacteriophage S13 shows exclusion of superinfecting homologous phage, but the exclusion is only partial. The superinfecting phage can form infectious replicative form deoxyribonucleic acid (RF), can direct protein synthesis, and can form progeny particles even at a superinfection time as late as 60 min after the first infection. Exclusion is also only partial for the closely related phage ϕX174. Seven min after the first infection, the exclusion mechanism begins to operate, requiring continuous phage-specified protein synthesis. The gene A protein (required for synthesis of progeny RF) appears to be involved in the exclusion mechanism. In superinfection experiments, it was found that at least 40 phage particles per cell can replicate and can carry out protein synthesis, though the number of sites for binding of RF to the membrane is only about 15 per cell. The results suggest that attachment of RF to a binding site is not required for protein synthesis. Evidence is presented that non-attached parental RF can serve as a template for single-stranded deoxyribonucleic acid synthesis.

Exclusion of superinfecting homologous phage has been found to occur for bacteriophage ϕX174 by Hutchison and Sinsheimer (2) and Knippers, Salivar, Newbold, and Sinsheimer (6). We have found that the exclusion mechanism requires phage-specified protein synthesis. Therefore, we wished to determine which of the eight known phage genes is responsible for the exclusion process. We used phage S13, very closely related to phage ϕX174 (3), because of the availability of lysis-defective derivatives of almost every known phage gene, though ϕX174 was also used in some of the experiments. (Genes are designated in this paper by the new S13-ϕX174 nomenclature. The correspondence between the old S13 nomenclature and the newly established S13-ϕX174 nomenclature is as follows, with the new designation being first in each pair: A, IV; B, II; C, VI; D, VII; E, V; F, I; G, IIIa; H, IIIb.)

While trying to identify the gene responsible for exclusion, we have found that though exclusion occurs, it is only partial. Even at a superinfection time of 1 hr after the first infection, the superinfecting phage can form an appreciable amount of infectious parental replicative form deoxyribonucleic acid (RF), can replicate, and can rescue mutant phage, i.e., it can direct protein synthesis. These results are contrary to the findings of Hutchison and Sinsheimer (2) and Knippers et al. (6) who found essentially no conversion of superinfecting deoxyribonucleic acid (DNA) to RF. Hutchison and Sinsheimer (2) found no yield of superinfecting phage in the progeny.

The finding that a superinfecting phage can replicate itself and can rescue preinfecting mutant phage has made it possible to determine the number of phage particles per cell that can replicate and the number that can participate in protein synthesis and to correlate these results with the number of RF-binding sites per cell.

[It has been shown by Knippers and Sinsheimer (5) that binding of parental RF to certain cellular sites that are probably on the cell membrane is necessary for synthesis of progeny RF.]

Previously, Yarus and Sinsheimer (14) found that in starved cells, only one or two phage particles could replicate, though in unstarved cells at least four could replicate. Stone (10) found that only one particle per cell could replicate in unstarved cells. It will be shown by us

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that in unstarved cells at least 40 phage particles can replicate together in the same cell and that at least 40 per cell can participate in protein synthesis. Since this number is considerably greater than the number of RF-binding sites, attachment of RF to a membrane replication site is apparently not essential for protein synthesis.

MATERIALS AND METHODS

Media. The media used included: broth and agar, *see* Tessman (12); modified M9 medium, *see* Shleser et al. (8); HFCC medium, *see* Gelfand and Hayashi (1); tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) is 0.05 M Tris-0.003 M EDTA (*p*H 8.0), and lysing medium is 100 μg of lysozyme per ml in 0.005 M EDTA-0.1 M Tris, *p*H 8.1.

Bacteriophage. Phages included S13 wild type; *su*N15, a lysis-defective mutant (gene E); *su*100, a gene A mutant (blocked in progeny RF synthesis).

The double mutants used are lysis-defective amber derivatives of the following mutants: *su*100 (A); *su*86 (B); *su*205 (C); *su*61 (F); *su*62 (G); *su*66 (H). All these are amber mutants except for *su*205, which is an ochre mutant. A mutant (H81) of gene D of ϕX174 was provided by M. Hayashi. A lysis mutant of ϕX174, *am*3, was provided by R. Sinsheimer. To prepare stable lysates of phage mutants, the method of Hutchison and Sinsheimer (2) was used, except that the lysates were always finally dialyzed against 0.05 M ammonium acetate, *p*H 7.8.

Bacteria. The nonpermissive host was *Escherichia coli* C. An HCR$^-$ derivative of *E. coli* C, strain AP1, was used after ultraviolet (UV) irradiation for measuring phage protein synthesis. A host range mutant of *E. coli* C, S26, was used in mixed indicators to distinguish mutant from wild-type plaques. The amber-suppressing host strains were *Shigella dysenteriae* Y6R, *E. coli* C600.1, and *E. coli* CR63.1. The ochre-suppressing strain was *E. coli* CA165.2.

Measurement of infectious superinfecting RF. A 200-ml broth culture of *E. coli* C was grown with aeration at 37 C to 2×10^8 cells/ml. $CaCl_2$ was added to 10^{-2} M, which results in 98% attachment in 2 min. The culture was divided into a number of 20-ml samples which were prewarmed at 37 C for 3 min before infection. The preinfecting phage was added at an multiplicity of infection (MOI) of 7, and aeration at 37 C was continued. The superinfecting phage (the gene A mutant) was added at an MOI of 30 at the desired times. The cultures were chilled 5 min after superinfection, which is sufficient time for complete formation of parental RF by the gene A mutant. The DNA was extracted by sodium lauryl sulfate (SLS) lysis, and infectious RF was determined after hydroxylamine inactivation of the extracts, which under the conditions used eliminates infectious single-stranded DNA, leaving the RF unaffected. The procedures used for extraction, hydroxylamine inactivation, and assay were described by Tessman (13). Plaques of the superinfecting gene A mutant were distinguished from plaques of the preinfecting mutant phage by plating on a 1:1 mixture of C + C600.1, except in the case of preinfection with the A-E mutant, when plating was done on CA165.2.

The experiment involving preinfection with the gene D mutant of ϕX174 was done by using UV-irradiated cells of the HCR$^-$ strain *E. coli* C AP1, which fail to lyse after infection. The cells were grown in HFCC medium to 10^8 cells/ml, UV-irradiated at 3.6×10^5 ergs/cm^2 with a germicidal lamp, concentrated twice by centrifugation, and made 2×10^{-2} M in $MgSO_4$. The cells were infected with an MOI of 7.5 of the ϕX174 gene D mutant or the S13 lysis mutant and then at various times were superinfected with the S13 gene A mutant at an MOI of 30. Cultures were chilled at 10 min after superinfection.

Measurement of rescue and replication of the superinfecting phage. *E. coli* C was grown in broth to 2×10^8 cells/ml and made 2×10^{-2} M in $CaCl_2$. Samples of 2.5 ml were prewarmed for 3 min at 37 C with aeration and then infected with the desired double mutant, and aeration at 37 C was continued. The superinfecting phages used were either wild-type S13 or the gene A mutant. After superinfection (6 or 7 min) several cultures from each experiment were assayed for nonadsorbed phage by shaking with chloroform. The nonadsorbed phage was always found to be only a negligible fraction of the burst size of the rescued phage or of the superinfecting phage. Several cultures in each experiment were left without superinfection to measure the amount of leakiness of the mutant alone. This always turned out to be negligible even when high MOI levels were used. Eight minutes after superinfection the cells were diluted 10^4 times into broth at 37 C and allowed to grow for 90 min from the time of superinfection to reach the maximum yield. Since artificial lysis was necessary to determine the burst size of the preinfecting phage in unmixed infection, artificial lysis was also used for the cultures to which superinfecting phage had been added for uniformity. Plating was also done without artificial lysis in each experiment.

Test for inhibitory effects of high MOI. In experiments where the preinfecting mutant phage was used at a series of increasing MOI levels, the mutant lysate was tested for inhibitory properties by determining burst size in CR63.1 as a function of MOI, up to an MOI of 50. For some old lysates, burst size was found to decrease with increasing MOI and such lysates were not used.

Artificial lysis. Cultures were diluted 10 times into lysing medium and frozen-thawed three times.

Measurement of "attached RF." A 200-ml broth culture of *E. coli* C was grown to 2×10^8/ml and made 10^{-2} M in $CaCl_2$. Samples (20 ml) were infected at 37 C with a gene A mutant at MOI levels ranging from 5 to 40. The cultures were chilled for 5 min after infection, divided in half, centrifuged, and resuspended in Tris-EDTA. One-half culture of each pair was lysed with SLS at 60 C for 10 min as described by Tessman (13) to measure total RF. The other half-culture was gently lysed by the Brij lysis procedure of Knippers and Sinsheimer (5). The Brij lysate was centrifuged at 16,000 rev/min for 30 min in the no. 34 Sorvall rotor. The pellet was resuspended in Tris-

EDTA and then treated with SLS as the first half-culture. All extracts were hydroxylamine-inactivated and assayed for infectious RF by the procedures of Tessman (13).

RESULTS

Exclusion was measured in two different ways: by the ability of the first phage to block the formation of infectious parental RF by the superinfecting phage and by the ability of the first (mutant) phage to block rescue by the superinfecting phage.

Formation of infectious RF by the superinfecting phage. To measure the amount of parental infectious RF formed by the superinfecting phage, the host cells were preinfected with a lysis-defective mutant at an MOI of 7, then superinfected at various times with a gene A mutant at an MOI of 30, and chilled and extracted 5 min later. A gene A mutant was used as the superinfecting phage because mutants of this gene are blocked in formation of progeny RF (9, 13). Since this defect is at most only slightly rescuable, the RF formed by the superinfecting phage will be largely parental RF. The superinfecting phage was used at a high MOI because of the low efficiency of the spheroplast assay; the higher the MOI, the more parental RF formed (up to an MOI of 40 to 50) and therefore the greater the plaque count in the infectivity assay.

Although high MOI levels were used in this experiment and in other experiments described in the present paper, no abnormal effects due to high MOI levels have been found with the lysates used here, in contrast to the results of Stone (11) who found that synthesis of total (cell plus phage) DNA, ribonucleic acid (RNA), and protein by infected cells was seriously impaired at high MOI levels. We have found that burst size and number of infective centers are independent of MOI up to a value of at least 50. We have also found that phage-specified protein synthesis (at least in UV-irradiated cells) is unaffected by MOI levels up to 50 (Fig. 1). Inhibitory effects on burst size and on amount of phage-specific protein synthesis were previously found by us when we used phage resuspended in borate buffer instead of ammonium acetate.

The amount of parental RF formed by the superinfecting phage remains constant until a superinfection time of at least 7 min after the primary infection and then decreases, and by a superinfection time of 15 min reaches a plateau (Fig. 2). The plateau value is about 15% of the RF formed by the same phage in simultaneous infection. The plateau remains constant for a

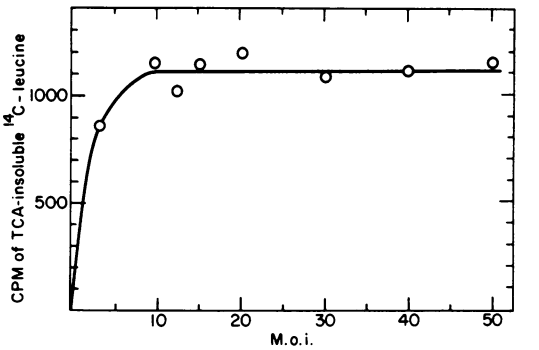

FIG. 1. *Amount of protein synthesis as a function of MOI. Strain AP1 (an HCR⁻ derivative of E. coli C) was grown to 10^8 cells/ml in HFCC medium, UV-irradiated, infected with different MOI levels of a lysis-defective mutant (suN15), and labeled with ^{14}C-leucine as described in the legend to Fig. 2 of Jeng et al. (3). At 60 min after infection, samples were removed from each culture, precipitated with cold 10% trichloroacetic acid, washed several times with cold trichloroacetic acid, collected on membrane filters, and counted in a gas flow counter.*

superinfection time of at least 60 min after the primary infection.

The observed exclusion requires phage-specified protein synthesis because if 30 μg of chloramphenicol (CM) per ml is added 3 min before infection, no exclusion takes place (Fig. 2). Continuous protein synthesis is required for the exclusion mechanism because if CM is added at late times, just before superinfection, partial reversal of the exclusion is observed. When CM was added at 17 min and the superinfecting phage was added at 20 min, a fourfold increase in infectious RF was observed.

To determine which phage gene is responsible for the observed exclusion, a mutant of each phage gene was used as the preinfecting phage, and then the cells were superinfected with the gene IV mutant at different times. Figure 2 shows the results for an A-E, B-E, C-E, F-E, G-E, H-E, and E mutant (E is the lysis gene). All the mutants gave approximately the same amount of exclusion, except for the case of preinfection with the A-E mutant, which gave less exclusion. In this case the plateau value was 35%, rather than 15%, of the value at time zero.

Since no mutant of S13 gene D had yet been isolated at the time of these experiments, a mutant of the homologous gene D of φX174 was used as the preinfecting phage in one experiment. Furthermore, since a lysis-defective derivative of the gene D mutant was not available, the experiment was carried out by using

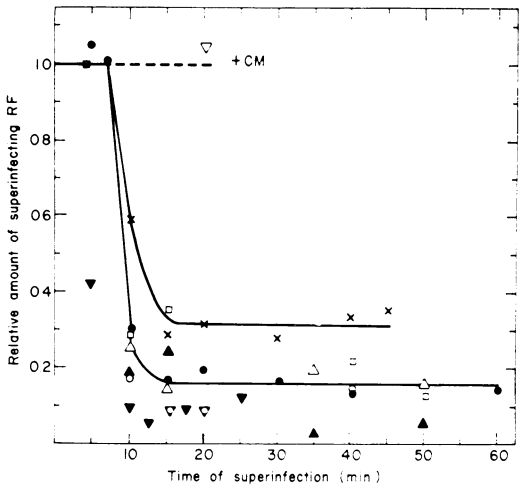

FIG. 2. *Relative amounts of infectious RF formed by superinfecting S13 at different times after preinfection with nonsense mutants of seven S13 genes. Samples of E. coli C in broth at 37 C were infected either with a lysis-defective mutant (gene E) or with lysis-defective derivatives of mutants of genes A, B, C, F, G, or H. The cultures were superinfected with a gene IV mutant at the indicated times. After superinfection (5 min) the cultures were chilled, and the DNA was extracted and assayed for infectious RF after hydroxylamine inactivation. The MOI of the preinfecting phage was 7.0 and of the superinfecting phage was 30. In one experiment chloramphenicol was added to 30 μg/ml 3 min before infection. At time zero, the cells were infected with the lysis mutant, at 20 min they were superinfected with the gene IV mutant, and at 25 min the cultures were chilled. This experiment was repeated twice with the same result. Symbols: ×, A-E; ○, B-E; ▼, C-E; ●, E; ▲, F-E; □, G-E; △, H-E; ▽, E plus chloramphenicol.*

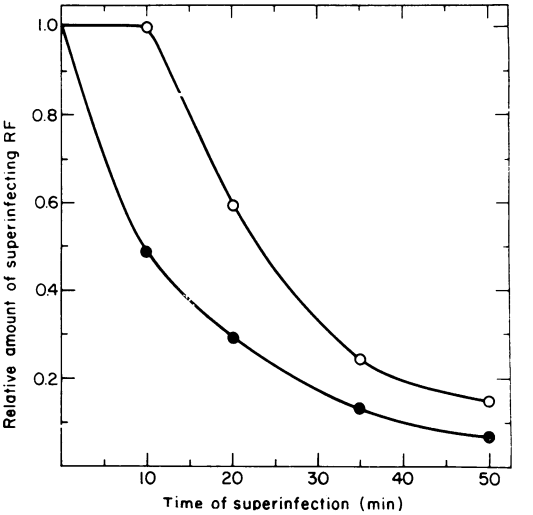

FIG. 3. *Relative amounts of infectious RF formed by superinfecting S13 as a function of time after preinfection with a mutant of φX174 gene D. Strain E. coli C AP1 was grown in modified M9 medium and then UV-irradiated at 3.6 × 10^5 ergs/cm^2 to prevent lysis after infection. The cells were infected with a mutant of gene D of φX174 (H81) or with the S13 lysis mutant. The cells were superinfected with an S13 gene A mutant at the indicated times and then chilled 10 min after superinfection. The MOI of the preinfecting phages was 7.0 and of the superinfecting phage was 30. Symbols: ●, preinfection with φX174 mutant; ○, preinfection with S13 lysis mutant.*

UV-irradiated HCR⁻ host cells to prevent lysis. Figure 3 shows that preinfection with a gene D mutant also results in partial exclusion of the superinfecting phage.

Rescue of mutants by the superinfecting phage. Mutants of seven phage genes were found to be rescued by superinfecting wild-type phage (Table 1). (The eighth gene, D, could not be studied for lack of a suitable lysis-mutant derivative.) The observed rescue indicates that the superinfecting phage can direct protein synthesis. The burst size of the rescued mutant varied from 5 to 30 particles per cell. Table 1 shows that rescue is not due to leakiness of the nonsense mutant.

The amount of rescue as a function of time of superinfection with wild type is shown in Fig. 4 and 5 for a B-E mutant and a G-E mutant, respectively. In some experiments the burst size of the rescued phage shows an initial rise, but this then decreases with increasing time of

TABLE 1. *Rescue of S13 nonsense mutants by superinfecting wild-type phage*[a]

Gene	Burst size of the mutants in unmixed infection	Burst size of the rescued mutant
A	0.5	6
B	0.07	15
C	2.0	23
E	1.8	30
F	0.01	4.8
G	0.03	8.8
H	0.01	16

[a] *E. coli* C grown in broth at 37 C to 2 × 10^8 cells per ml was infected with lysis-defective derivatives of mutants of the genes shown (MOI of 4) and then superinfected with wild-type at an MOI of 8. Superinfection was at 20 min, except in the case of preinfection with the C-E mutant, where, because of leakiness at later times, superinfection was done at 7 min. The burst of the mutant in unmixed infection was obtained after artificial lysis, except in the case of the gene E (lysis) mutant. The burst size of the rescued mutant was the same with and without artificial lysis, except for the case of the gene E mutant.

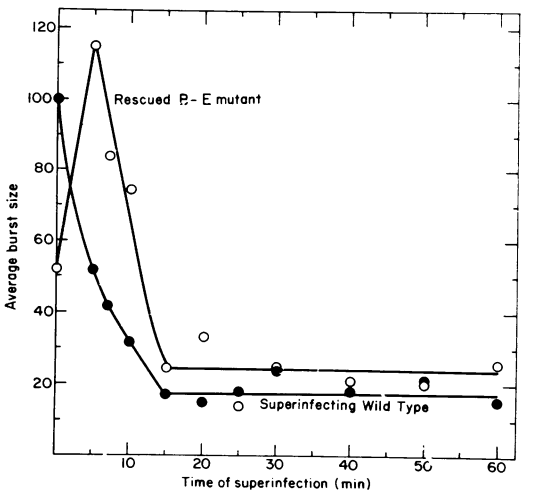

FIG. 4. *Rescue of a preinfecting mutant by superinfecting wild-type phage. Samples of a culture of E. coli C in broth at 37 C (2 × 10⁸ cells/ml) were preinfected with a B-E mutant at an MOI of 7.5. At the indicated times the cells were superinfected with wild-type S13 at an MOI of 7.5. The cultures were diluted 10⁴ times in broth at 8 min after superinfection and then allowed to grow until 90 min after superinfection.*

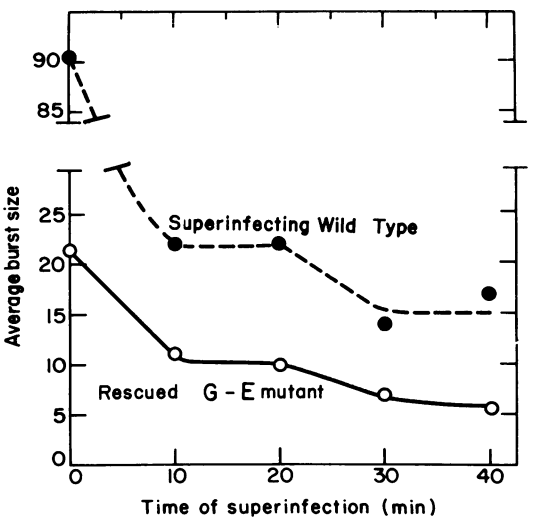

FIG. 5. *Rescue of a preinfecting G-E mutant by superinfecting wild-type phage. The procedure was the same as in Fig 4, except that the MOI of the preinfecting phage was 6.0 and of the superinfecting wild-type was 8.0.*

superinfection. By a superinfection time of 15 min, the burst size of the rescued mutant reaches a plateau which is constant until a superinfection time of at least 60 min. Controls for these experiments showed that the yield of the mutant alone in unmixed infection was only about 0.5% of the yield in mixed infection.

Rescue of a gene A mutant by superinfecting wild type is distinctly different from rescue of mutants of other genes (Figure 6). The rescue of gene A mutants by wild-type phage in simultaneous infection is always poor (12), but it is seen in superinfection experiments that this amount of rescue remains the same even at late superinfection times, unlike the situation observed for mutants of other genes. It might be thought that rescue of gene A mutant particles occurs by wrapping parental mutant DNA in new coats, thus requiring no protein synthesis by the rescuing phage. However, it has been found by Alvaro Puga (*personal communication*) that the rescued gene A mutant particles contain newly synthesized DNA. Therefore the rescue requires protein synthesis by the superinfecting phage.

It is further seen (Fig. 6) that after preinfection with a gene A mutant the burst size of superinfecting wild type remains fairly constant with time, unlike the decrease seen after preinfection with mutants of genes B and G (Fig. 4 and 5) or with mutants of genes C, E, F, and H (*data not shown*).

Although the yield of superinfecting wild type does decrease sharply after preinfection with mutants other than gene A, the yield of wild type is two times greater than the input of wild type, and therefore the superinfecting phage is replicating even in these cases (Fig. 4 and 5).

The experiments presented so far give no information on whether exclusion is occurring in all the cells or in only a fraction of the cell population, and this was studied next.

Measurement of rescue of the lysis function

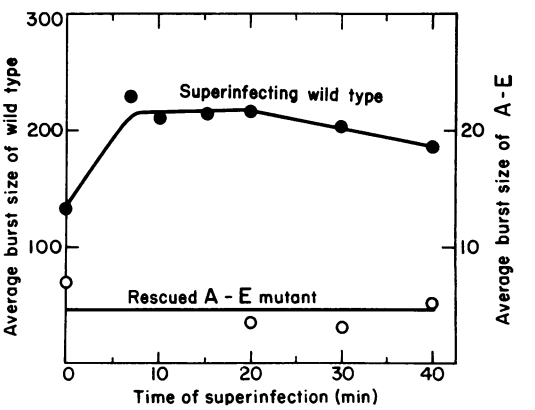

FIG. 6. *Rescue of a preinfecting A-E mutant by superinfecting wild-type phage. The procedures were the same as in Fig. 4.*

by the superinfecting phage provides a determination of the fraction of cells containing functional superinfecting RF at late superinfection times. Figure 7 shows the yield of lysis-defective phage in cells superinfected with wild type at different times, with and without artificial lysis. It is seen that the fraction of cells that can be lysed by the superinfecting phage decreases after a superinfection time of 7 min and by 15 min reaches a plateau at about 20%. It was found that the yield of superinfecting wild-type phage in the same experiment also decreased with time until a superinfection time of 15 min and was the same with and without artificial lysis, implying that only those cells that lysed spontaneously yielded wild-type phage. Therefore, it is concluded that the superinfecting RF is functional in only about 20% of the cell population at late times. This result suggests that the cell population is heterogeneous with respect to exclusion.

To test whether the heterogeneity is genetic, a single colony was isolated, and the experiment of Fig. 7 was repeated. The same results were obtained. Also the infectious DNA experiment of Fig. 2 was performed by using the culture derived from an isolated colony, and again a partial exclusion was observed. Therefore the cell population is not genetically heterogeneous with respect to exclusion, though it may be physiologically heterogeneous.

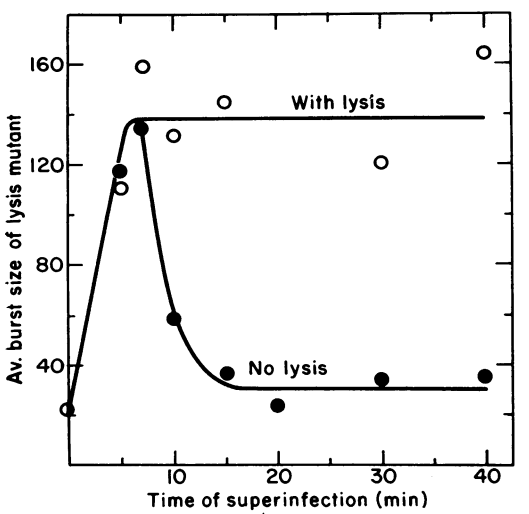

FIG. 7. *Ability of superinfecting wild-type phage to supply the lytic function. Samples of a culture of E. coli C at 2 × 10^8/ml at 37 C were infected with a lysis mutant at an MOI of 7, and then at the indicated times the cells were superinfected with wild type at an MOI of 25. After superinfection (8 min) the cells were diluted 10^4 times in broth and allowed to grow 90 min from the time of superinfection. Cultures were chilled and plated with and without artificial lysis to determine the yields of the mutant and the superinfecting wild type. The yield of wild-type phage is not shown in this figure. The yield of the lysis mutant in unmixed infection without artificial lysis was about five particles per cell.*

The burst size of the wild type at superinfection times of 15 min and later is about 10% of the total burst after artificial lysis. This value remains constant until late superinfection times.

In another experiment, a ϕX174 lysis mutant, *am3*, was used as the preinfecting phage instead of the S13 lysis mutant. The MOI of the preinfecting phage was 7.5 and of superinfecting wild-type S13 was 7.5. In this experiment a similar result was obtained; the burst size of the superinfecting phage was about 14% of the total burst at late superinfection times or about 30 particles per cell. Thus exclusion after infection with ϕX174 is only partial.

In experiments involving preinfection with lysis-defective mutants of genes A, B, C, F, G, and H, the burst sizes of the rescued mutant phage and of superinfecting wild-type phage were always the same with and without artificial lysis, except for the experiment shown in Fig. 9. In this experiment a B-E mutant is rescued by a superinfecting gene A mutant. In this case artificial lysis increased all burst sizes by a factor of 5.

Genetic recombination by the superinfecting phage. The superinfecting phage can recombine with the preinfecting phage. The amount of recombination has the same dependence on time of superinfection as does rescue. Figure 8 shows the amount of recombination between a B-E mutant and a superinfecting gene A mutant after superinfection at different times.

Number of phage participating in protein synthesis per cell. The finding that a superinfecting phage can rescue made it possible to study the questions of how many phage per cell take part in protein synthesis and in replication. Host cells were preinfected with increasing numbers of B-E mutant particles up to an MOI of 40 and then at 7 min were superinfected with a gene A mutant (Fig. 9) or with wild type (Fig. 10). [It is known that the maximum number of parental RF molecules which can be formed per cell is 40 to 50 (Tessman, *unpublished results*).] The rescue of the mutant B-E phage is not much affected by filling up the cell with increasing numbers of the mutant phage. Even at an MOI of 40 of the first phage, there is appreciable rescue by the superinfecting phage

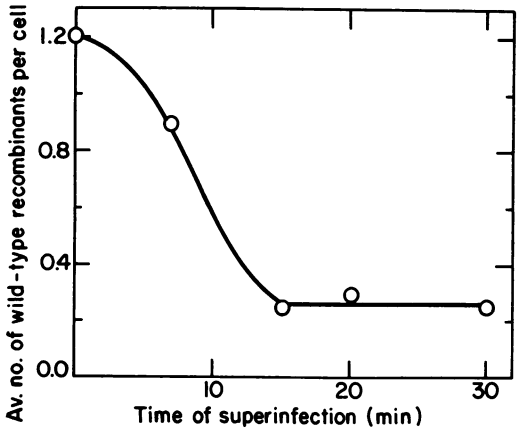

FIG. 8. *Formation of recombinants by the superinfecting phage. Samples of a culture of E. coli C in broth at 37 C (2 × 10⁸ cells/ml) were infected with a B-E mutant at an MOI of 7 and then at the indicated times each culture was superinfected with a gene A mutant at an MOI of 20. The cells were diluted 10³ times in broth 8 min after superinfection and allowed to grow for 90 min. Plating was done on E. coli C to assay for wild-type recombinants after artificial lysis.*

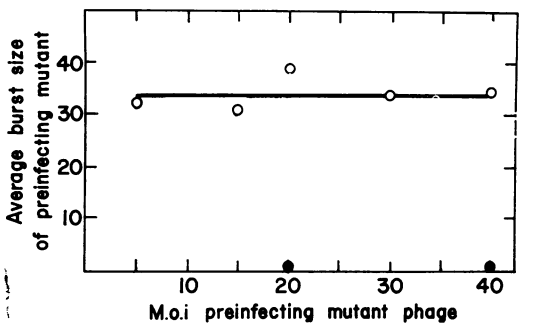

FIG. 9. *Rescue by the superinfecting phage as a function of MOI of the first phage. Samples of a broth culture of E. coli C (2 × 10⁸ cells/ml) at 37 C were infected with a B-E mutant at MOI values ranging from 5 to 40. At 7 min the cultures were superinfectioned with a gene A mutant at an MOI of 20. After superinfection (8 min) cultures were diluted 10 times in broth and allowed to grow until 90 min from the time of superinfection. Plating was done after artificial lysis. Symbols: ○, burst size of B-E mutant in mixed infection; ●, burst size of B-E mutant in unmixed infection.*

(Fig. 9 and 10). This rescue is not due to leakiness of the first phage, since controls showed that the burst sizes in unmixed infection (after artificial lysis) were negligible, even at high MOI

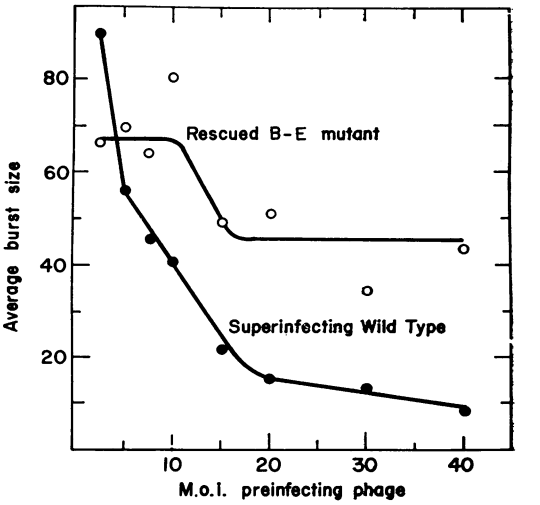

FIG. 10. *Rescue and replication by the superinfecting phage as a function of MOI of the first phage. Samples of a culture of E. coli C at 2 × 10⁸ cells per ml at 37 C were infected with a B-E mutant at MOI values from 2.5 to 40 and then at 7 min were superinfected with wild-type phage at an MOI of 20. After superinfection (8 min) the cultures were diluted 10⁴ times in broth and allowed to grow until 90 min from the time of superinfection.*

values. Therefore, at least 40 particles per cell can participate in protein synthesis.

In partial contrast to the results for rescue, the yield of the superinfecting phage does decrease sharply with an increasing MOI of the first phage, though it levels off at an MOI of 15 to 20 (Fig. 10). This same pattern of initial dependence on MOI is seen in Fig. 11, which gives the results of an experiment in which an A-E mutant was the preinfecting phage. In this experiment the burst size of the superinfecting phage was 30, even when the cell had been infected with 40 particles of the first phage. This burst size represents a true replication of the superinfecting wild type because the MOI of the wild type was only 7.5.

The decrease of the burst size of the superinfecting phage with increasing MOI of the first phage (Fig. 10 and 11) may reflect a decrease in available replication sites. Knippers and Sinsheimer (5) have shown that replication of progeny RF requires attachment of parental RF to a specific site, probably on the cell membrane. These authors determined that there were 9 to 15 RF-binding sites per cell. In agreement with this determination, we have found, by using infectivity measurements, that there are 15 RF-binding sites per cell (Fig. 12).

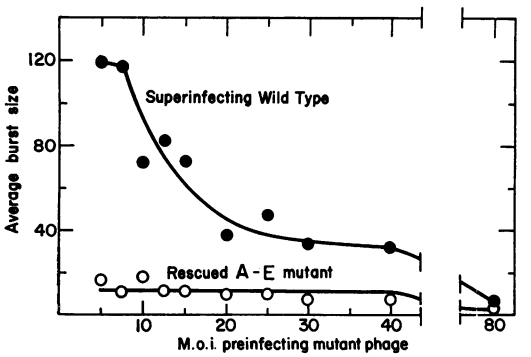

FIG. 11. *Rescue and replication by the superinfecting phage as a function of MOI of the first phage. Samples of a broth culture of E. coli C at 2 × 10^8 cells per ml at 37 C were infected with an A-E mutant at MOI values from 5 to 80 and then at 15 min were superinfected with wild-type phage at an MOI of 7.5. After superinfection (8 min) the cultures were diluted 10^4 times in broth and allowed to grow until 90 min from the time of superinfection.*

DISCUSSION

Infection with phage S13 results in exclusion of superinfecting phage, but the exclusion is only partial. Even at a superinfection time of 1 hr after the first infection, a superinfecting phage can form 15% of the infectious parental RF formed in simultaneous infection. This RF can replicate, recombine, and direct protein synthesis.

The exclusion mechanism begins to operate between 7 and 10 min after infection at 37 C. Experiments with CM show that exclusion requires phage-specified protein synthesis and that this synthesis must be continuous, at least for a time, to bring about maximum exclusion.

Attempts to identify the phage gene responsible for the exclusion mechanism have led to results that depend on which aspect of exclusion is studied. On the one hand, experiments measuring the ability of the first phage to block formation of infectious parental RF by the superinfecting phage show that mutants of all eight phage genes studied, including gene A, produce partial exclusion. On the other hand, experiments measuring the ability of the first phage to block rescue and replication by the superinfecting phage show that a gene A mutant fails to produce exclusion of these functions.

The experiments measuring exclusion of formation of infectious RF by the superinfecting phage lead to two alternative conclusions. Either more than one phage gene is required for exclusion, or a still undiscovered phage gene may be involved. [A ninth phage gene has recently been discovered for ϕX174 by M. Hayashi (*personal communication*) and has not yet been tested by us.]

The experiments measuring exclusion of the rescuing and replicating ability of the superinfecting phage by mutants of seven phage genes show that only the gene A mutant fails to produce any exclusion. (It has not yet been possible to test an eighth phage gene, D, because of technical difficulties.) Therefore, it is concluded from this type of experiment that the gene A protein is implicated in exclusion.

We have shown that exclusion is blocked by the inhibition of over-all protein synthesis with CM (30 μg/ml) as well as by a mutation in gene A. These two findings appear contradictory when previous experiments on the effect of CM on gene A function and gene A protein synthesis are considered. It was shown by Tessman (13) that 30 μg of CM/ml permits appreciable gene A function, i.e., synthesis of progeny RF. It was

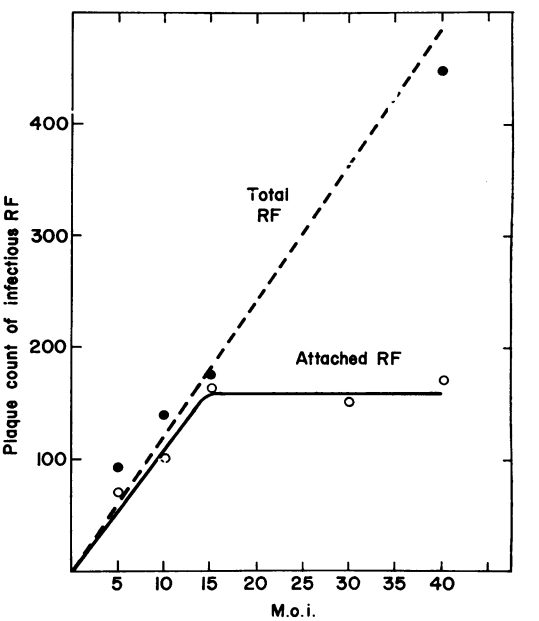

FIG. 12. *"Attached RF" and total RF formed by a gene A-E mutant as a function of MOI. Samples of a broth culture of E. coli C at 2 × 10^8 cells/ml at 37 C were infected with an A-E mutant at MOI from 5 to 40. Five minutes after infection (which is sufficient time for formation of parental RF), each culture was divided in half, centrifuged, and resuspended in Tris-EDTA. One half-culture of each pair was lysed by the Brij lysis procedure of Knippers and Sinsheimer (5). The other half-culture of each pair was lysed with SLS for total RF. RF in both fractions was determined after hydroxylamine-inactivation and assay on protoplasts. Symbols: ○, RF in pellet of Brij lysate; ●, total RF.*

reported by Levine and Sinsheimer (7) that the synthesis of the gene A protein is CM-resistant. Thus, it is difficult to understand why CM should block exclusion, if the gene A protein were indeed being formed in nearly normal amounts in CM.

We resolve this apparent contradiction by noting that the experiments of Levine and Sinsheimer (7) do not show that the amount of gene A protein made in the presence of 30 μg of CM/ml is comparable to the amount made normally. In fact, unpublished experiments of Shleser and Tessman on gel electrophoresis of phage-specified proteins indicate that the synthesis of the gene A protein is actually CM-sensitive. Therefore, it appears that the progeny RF synthesis observed in CM requires only a very small amount of gene A protein, whereas exclusion may require a normal amount of gene A protein synthesis and therefore could not occur in the presence of CM.

Experiments on rescue of the lysis mutant show that 20% of the cells contain functional superinfecting RF at superinfection times of 15 min and later. Thus, exclusion appears to be operating in only about 80% of the cell population rather than in the total population. This heterogeneity is not genetic.

Superinfection experiments have made it possible to determine the number of particles per cell that can participate in protein synthesis. This was done by measuring the amount of rescue of a preinfecting mutant as a function of MOI of the mutant, since rescue requires protein synthesis by the superinfecting phage. Rescue by the superinfecting phage was found to be quite independent of the MOI of the first phage, up to MOI levels of at least 40. Thus, a superinfecting phage particle can carry out protein synthesis when there are already 40 phage particles present in the cell.

The same experiments (Fig. 10 and 11) have also provided information on the number of particles which can replicate together in the same cell and they furthermore indicate that there is more than one route leading to synthesis of single-stranded (SS) DNA. Figures 10 and 11 show that as the MOI of the first phage increases, up to an MOI of 15 to 20, the burst size of the superinfecting wild-type phage decreases sharply. However at higher MOI levels of the first phage, there is very little further decrease in wild-type burst size. Figure 11 shows that even when there are 40 phage particles already infecting the cell, the superinfecting phage particles are still able to form about 30 progeny particles per cell. Since the MOI of the superinfecting phage was only 7.5 in this experiment, it is clear that the superinfecting phage is replicating several times.

The following model can account for the results of Fig. 10 and 11: Knippers and Sinsheimer (5) have shown that attachment of parental RF to a fast-sedimenting cell component, probably the membrane, is necessary for progeny RF synthesis. They found a value of 9 to 15 for the number of RF-binding sites. We have found a value of 15 (Fig. 12). We assume that at low MOI levels of the first phage the superinfecting RF molecules attach to vacant RF-binding sites and form progeny RF normally, which then serve as templates for SS synthesis (4). When the RF binding sites are all filled up, we assume that the unattached superinfecting parental RF molecules serve as templates for SS formation without the intermedate step of progeny RF synthesis.

Thus, it appears that parental and progeny nonattached RF are equivalent in acting as precursors for SS synthesis. This is the conclusion reached previously by Knippers et al. (6) when they found upon infection at high MOI values that DNA from parental nonattached RF appeared in the SS DNA of phage progeny particles. However, it is not yet known whether the mechanism of this SS synthesis is identical with that of the extensive SS synthesis observed for superinfecting RF in our experiments.

It is of interest that the superinfecting phage can direct protein synthesis when there are already 40 phage particles in the cell, since at this MOI the RF-binding sites should be all filled. Therefore, it is likely that messenger RNA synthesis does not require attachment of the RF to a replication site.

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