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Immediate-Early Expression of the Gene Causing Superinfection Breakdown in Bacteriophage T4B

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Superinfection breakdown appears to belong to the "immediate-early" functions induced by T4 phage.

It was reported by Lesley et al. (11) that cells infected with T-even phage break down about half of the superinfecting phage DNA into acid-soluble fragments. This acid solubilization, called superinfection breakdown, was recently shown (1, 7) to occur by the action of host endonuclease I. Results were also presented (1) indicating that superinfecting phage fail to inject their DNA successfully into the cell and, consequently, the DNA stays around the cell surface (probably in the periplasm). It is conceivable that such DNA near the surface has more chances for being exposed to the attack of periplasmic endonuclease.

Although a direct cause of superinfection breakdown may thus be the nuclease, a change in the cell envelope, due to which the secondarily infected phage DNA has difficulty pentrating into the cytoplasm, would be a more fundamental cause for this phenomenon. The following observations indicate that such a change in the envelope is brought about through phage-directed protein(s). (i) The extent of superinfection breakdown becomes greater with time during the initial stages of primary phage infection (1, 8). (ii) Antibiotics such as rifampin or chloramphenicol inhibit the development of this ability (1). (iii) Primary infection with phage ghosts does not cause the breakdown of the secondary phage DNA (1).

In this study, we show results which suggest that the synthesis of this protein, causing superinfection breakdown, is directed by an "immediate-early" mRNA, an RNA specified as being produced after phage infection even in the absence of protein synthesis.

To measure the extent of superinfection breakdown, we developed a "filtration method" for experimental convenience. The cells superinfected with thymidine-labeled phage were diluted with chilled nutrient broth and filtered

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TABLE 1. *Superinfection breakdown as measured by "filtration method"*[a]

Labeled phage[b]	Radioactivity on filter (counts/min)	
	^{3}H-leucine (coat protein)	^{14}C-thymidine (DNA)
Primary	973	700
Secondary	936	417

[a] *E. coli* B (0.5 ml; 5 × 10^{8} cells/ml), grown in medium E (15), was primarily infected with phage T4B (MOI = 5.5) in a shaking-bath at 37 C. The second phage (MOI = 5.5) were added 7 min after primary infection, and the cells were further incubated for 7 min. To this were added about 3 ml of chilled nutrient broth, containing 10 g of meat extracts, 10 g of polypeptone, and 2.5 g of NaCl per liter, and the mixture was immediately filtered through a glass fiber filter (Reeve Angel Inc. 984H or Whatman GF/F) under reduced pressure (obtained by connecting to a vacuum line). The tube was rinsed with nutrient broth and the filter washed two more times with about 2 ml of the same solution each time. The filter was dried and counted for radioactivity. This is the "filtration method."

[b] ^{14}C-thymidine- and ^{3}H-leucine-labeled phage were prepared in the following way. *E. coli* B *thy*$^{-}$ (5 × 10^{8} cells/ml), grown in medium E containing glucose (0.2%), Casamino Acids (0.1 %), and thymine (25 μg/ml), were washed and resuspended in fresh medium lacking thymine. The cells were infected with T4Bos (MOI = 5) in the presence of tryptophan (50 μg/ml). ^{14}C-labeled thymidine was added (final concn 2 μCi/26 μg/ml) at 10 min after infection, and at 3.5 h the cells were lysed by addition of chloroform. When ^{3}H-leucine-labeled phage were prepared, *E. coli* B, grown to 5 × 10^{8} cells/ml in medium E containing glucose (0.2%) and tryptophan (50 μg/ml), were infected with T4Bos (MOI = 5) and ^{3}H-leucine was added (final concn 2.5 μCi/0.067 μg/ml) at 15 min after phage addition. In both cases, the chloroform lysates were subjected to low- (2,000 × *g*, 15 min) and high- (25,000 × *g*, 2 h) speed centrifugation. The resulting phage were further purified by sucrose density gradient centrifugation (5 to 20% sucrose, Beckman SW25 rotor, 12,000 rpm, 1 h). Peak fractions

TABLE 1—*Continued*

were pooled and, after addition of gelatin (100 μg/ml), dialyzed against medium E. ^{3}H-leucine- and ^{14}C-thymidine-labeled phage thus prepared were mixed in an appropriate proportion for use in this experiment.

TABLE 2. *Superinfection breakdown in T4imm$_2$-infected cells at 30 C*

Procedures	Radioactivity on filter	
	counts/min	%
1[a] CM[b] · · T4imm$_2^+$, ^{3}H-T4 · · · · Filt.	11,365	100
2[c] T4imm$_2^+$ · · · CM, ^{3}H-T4 · · · · Filt.	6,914	60.8
3[c] T4imm$_2^d$ · · · CM, ^{3}H-T4 · · · · Filt.	11,091	97.5

[a] A 0.3-ml amount of *E. coli* B (3×10^8 cells/ml), containing chloramphenicol, was simultaneously infected with wild-type T4 (MOI = 7) and ^{3}H-thymidine-labeled T4 (MOI = 1) and 10 min later subjected to "filtration method."

[b] Chloramphenicol (125 μg/ml).

[c] Primary infection (imm$^+$ or imm$^-$), MOI = 7. Secondary infection (^{3}H-T4), MOI = 1. Interval between primary and secondary infection—13 min; interval between secondary infection and "filtration"—10 min.

[d] T4imm$_2$ (5) was prepared in this laboratory by crossing T4amE142 [=gene(39)–imm$_2$ (14), kindly supplied by W. B. Wood] with wild-type T4.

through a glass fiber filter. A typical result obtained by this method is presented in Table 1. The primary infection with phage mutant T4imm$_2$, which lacks most of the ability to make the cells resistant to ghost infection and to exclude the superinfecting phage (14), did not cause as much breakdown as wild-type T4B infection, as measured by the filtration method (Table 2). The addition of chloramphenicol or rifampin before phage infection inhibits the establishment of the condition of breakdown (Table 3). From these results, the filtration method is considered to be valid for measuring the degree of superinfection breakdown.

It has been observed that the establishment of superinfection breakdown occurs at an early stage of phage infection (1, 8), and we also confirmed this by the filtration method (Fig. 1). This fact can be interpreted to mean that the gene for causing superinfection breakdown may belong to an immediate-early class. To test this, the following experiments were undertaken. Phage infection of *Escherichia coli* was first carried out in the presence of chloramphenicol (125 μg/ml). After accumulation of immediate-early mRNAs of phage T4, rifampin (50 μg/ml)

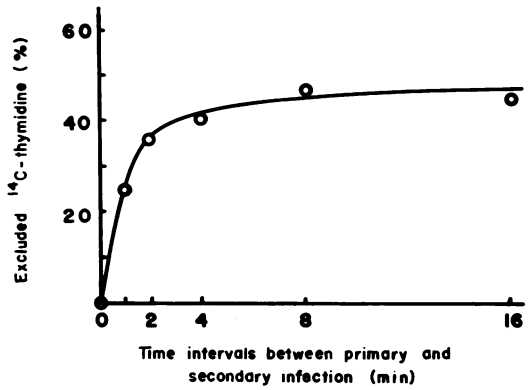

FIG. 1. *Development of "superinfection breakdown" at 30 C. At the times indicated, after primary infection of E. coli B grown in medium E (15) (5×10^8 cells/ml) with T4B (MOI = 5), the second phage, ^{14}C-thymidine-labeled T4B (MOI = 10), were added. Samples were incubated for 1 min and subjected to filtration method.*

was added to block further RNA synthesis. Chloramphenicol was then removed to allow protein synthesis from the accumulated mRNA, and we examined whether or not the establishment of superinfection breakdown could take place after these treatments. The data in Table 3 show that the breakdown does occur under this condition and thus lead us to conclude that an immediate-early mRNA is responsible for causing the majority, if not all, of superinfection breakdown.

A phenomenon, known as superinfection exclusion (6), that T-even phage-infected cells genetically exclude the superinfecting phage, has been considered (8) to be closely related to superinfection breakdown. In fact, if cells of endonuclease I$^+$ strains are used, acid solubilization of the secondary phage DNA (superinfection breakdown) parallels superinfection exclusion in a time-course experiment (8). From this and other observations, it has been postulated (1) that a change in the cell envelope, which is induced by the primary infecting phage, would be the basis for these two phenomena. This hypothesis is strongly supported by our present data (Table 2) that a mutant of T4imm, which lacks most of the ability of superinfection exclusion (4, 14), exhibits only a partial extent of superinfection breakdown. Thus, it is now likely that the expression of the imm gene takes place at an immediate-early stage.

Using a method similar to the one described here, Lembach and Buchanan (10) have already demonstrated that deoxycytidylate hydroxymethylase and dihydrofolate reductase are the products of immediate-early genes. In addition,

TABLE 3. *Immediate-early expression of "superinfection breakdown"*[a]

Treatments	^{3}H-T4 DNA (counts/min)	^{3}H-T4 DNA excluded (%)
1 · 2[b] · | · · · · · 10 · · · [F][c] · · 10 · · · [2°][d] 5 · · · [d][e]	2,706	10.5
2 · · [1°][f] · · · · · · · · · [F] · · · · · [2°] · · · [d]	1,406	53.5
3 CM[g] · | · · · 7 Rif · 3 · · [F] · Rif[h] -CM · · · · [2°] · · · [d]	3,022	= 0
4 CM [1°] · · · Rif · · · [F] · Rif -CM · · · · [2°] · · · [d]	1,942	35.7
5 CM [1°] · · · · · · · · · [F] · -CM · · · · [2°] · · · [d]	1,626	46.2
6 CM [1°] · · · · · · · · · [F] · CM · · · · [2°] · · · [d]	2,674	11.5
7 Rif CM [1°] · · · · · · · · · [F] · Rif -CM · · · · [2°] · · · [d]	2,863	5.3
8 Rif [1°] · · · · · · · · · [F] · Rif · · · · [2°] · · · [d]	2,861	5.3

[a] *E. coli* B was grown in medium E (15) to a concentration of 5×10^8 per ml. A 1-ml amount of the culture was taken into a tube containing chloramphenicol (125 μg/ml) and/or rifampin (50 μg/ml), where indicated, and incubated at 37 C. The primary phage T4B (MOI = 4), at 2 min, and rifampin, at 9 min, were added into the tube as indicated. The cells were, at 12 min, collected on nitrocellulose membrane filter (Sartorius, MF 50), and resuspended in 1 ml of chilled medium E containing antibiotics (as indicated), and, at 14 min, 0.5 ml of this solution was transferred to 37 C bath. After 10 min incubation, ^{3}H-thymidine-labeled T4B phage (MOI = 6) were added, and after an additional 5 min, the samples were subjected to "filtration method."

[b] Time interval in minutes.

[c] Filtration and suspension.

[d] Secondary infection with ^{3}H-thymidine T4B.

[e] Dilution with nutrient broth.

[f] Primary infection with T4B.

[g] Chloramphenicol (125 μg/ml).

[h] Rifampin (50 μg/ml).

until now messengers for a phage internal protein (3), DNA ligase (9), D2 protein (13), and the protein that makes cells resistant to "lysis from without" (12) have been shown to be immediate-early transcripts, by the use of in vitro assays of specific mRNA or by some other methods.

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