Ribonucleic Acid Bacteriophage Release: Requirement for Host-Controlled Protein Synthesis

HANNA ENGELBERG AND ESTHER SOUDRY

Department of Molecular Biology, Institute of Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Received for publication 18 May 1971

The release of the ribonucleic acid (RNA)-containing phage MS2 from *Escherichia coli* is accompanied by cellular lysis at 37 C, whereas at 30 C phage are released from intact cells. Chloramphenicol or rifampin prevents the release of progeny phage particles at both temperatures. Neither drug causes an immediate cessation of phage release and after inhibition of protein synthesis by chloramphenicol phage release proceeds for about 17 min at 37 C and about 35 min at 30 C. Rifampin does not inhibit phage release from mutant cells possessing a rifampin-resistant deoxyribonucleic acid-dependent RNA polymerase. The results indicate that a short-lived host-controlled protein(s) is essential for the release of RNA phage particles at both temperatures.

Phage development terminates with the release of newly formed particles from the bacterial cell. Despite intensive investigation of ribonucleic acid (RNA) phages, this last stage in the infection cycle has been little studied, and the mechanism of phage release is not well understood. The liberation of RNA phage at 37 C is accompanied by cellular lysis (15), and it was speculated that this may involve the phage coat protein (21). On the other hand, phage particles are released without lysing the host when the temperature is reduced to 31 C (11). The ability of RNA phage to pass out of the intact bacterial cells may represent an interesting model to elucidate the mechanism by which macromolecules escape from a cell into the surrounding medium.

Rifampin inhibits RNA synthesis and subsequently protein synthesis in *Escherichia coli* (8, 9, 14, 17) by acting directly on the core of the enzyme deoxyribonucleic acid (DNA)-dependent RNA polymerase (6, 18, 20). Recently, we demonstrated that this antibiotic prevents the release of MS2 phage from the host bacteria (7). This effect could be separated from the inhibitory effect of the drug on RNA phage synthesis (8, 16). Now we have studied further the effect of rifampin and chloramphenicol on MS2 phage release and have found that host-controlled protein synthesis is required to permit phage liberation both at 37 and 30 C.

MATERIALS AND METHODS

Bacterial strains and phage. Phage MS2 and its host bacterium *E. coli* AB301 (λ) met⁻ ribonuclease

I-less, rifampin-sensitive (kindly supplied by R. Gesteland) were used. For experiments with rifampin-resistant cells, a partial diploid was prepared by crossing an F'gal episome into a rifampin-resistant derivative of *E. coli* gal⁻recA⁻ strain 152 (kindly supplied by A. Oppenheim).

Media and growth conditions. Cells were grown and infected in tryptone broth medium with the following composition: 0.8% tryptone, 0.8% NaCl, 0.1% yeast extract, 0.5% glucose, 0.002 M CaCl₂.

The cultures were incubated in a shaking bath with a shaking rate of 120 oscillations/min. Culture turbidity was measured in a Klett-Summerson photoelectric colorimeter at 420 nm.

Phage infection. If not stated otherwise, the procedure of phage infection was as follows. Exponentially growing cells at a density of 2×10^8 /ml were infected with phage at multiplicity of infection (MOI) of 1 at 37 or 30 C. After 5 min, the culture was diluted 10,000-fold into prewarmed tryptone medium and incubated further. At the indicated times, the extracellular phage yield in the cell-free supernatant fluids and the intracellular and total phage yields in lysed bacterial pellets and cultures, respectively, were determined as described (7). Maximum phage release was determined at 70 and 180 min after infection at 37 and 30 C, respectively.

Measurement of protein synthesis. Protein synthesis was followed by measuring incorporation of ³H-leucine into hot trichloroacetic acid-precipitable material. A 10- μ Ci amount of ³H-leucine (specific activity, 2.0 Ci/mmol) was added to exponentially growing cells in tryptone medium at a density of 2×10^8 /ml. Samples were withdrawn at intervals into cold trichloroacetic acid (final concentration 5%) and allowed to stand on ice for 30 min. They were then boiled for 10 min and again cooled on ice for 15 min.

The precipitates were collected by filtration through 0.45- μ m filters (Millipore Corp., Bedford, Mass.), washed, and counted as described (17).

DNA-dependent RNA polymerase. Exponentially growing cells were collected and ground with twice their weight of alumina and then centrifuged at 30,000 $\times g$ for 20 min at 4 C. The supernatant fluid was assayed for polymerase activity as described by Wehrli et al. (20).

Electron microscopy. The procedure was that of King (13) except for the following modifications: 400-mesh grids were covered with a thin film of collodion and stabilized with evaporated carbon. A drop of sample was placed on the grid and 3 min later was stained with a drop of 1% neutral ammonium molybdate. The preparations were air-dried, and the specimens were photographed at 60 kv in a Philips 300 electron microscope with a 50- μ m objective aperture.

Chemicals. ^3H -leucine (2.0 Ci/mmol) was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y. Rifampin, a gift of Ciba Co., Basel, was dissolved at 1 mg/ml in distilled water prior to use. Chloramphenicol was obtained from Sigma Chemical Co., St. Louis, Mo., dissolved at 2 mg/ml in distilled water, and stored in a refrigerator for several months.

RESULTS

MS2 phage release at 37 and 30 C. It was demonstrated (11) that the RNA-containing phage fr, which causes extensive bacterial disintegration when grown at 37 C, is released from bacteria at 31 C without cellular lysis. We therefore studied this temperature-dependent phenomenon for the RNA-containing phage MS2. *E. coli* cells (2×10^8 /ml) growing exponentially at 37 or 30 C were infected with MS2 phage at an MOI of 10 and were further incubated at the respective temperature. The cultures were assayed for total and extracellular phage yields, and cell lysis was followed by measuring the optical density. As shown (Fig. 1a), at 37 C phage were released from 50 to 180 min postinfection, and during this period the turbidity of the culture decreased drastically. At 30 C, on the other hand (Fig. 1b), liberation of progeny phage was accompanied by a continuous increase in the optical density. The fate of MS2-infected cells at 30 C was clearly demonstrated by electron microscopy. Figure 2 shows an *E. coli* culture 3 hr after infection at 30 C. The bacteria remained intact even though each cell had already released about 2,000 infective particles (Fig. 1b).

In the experiments that will be described in the following sections, we followed MS2 phage release in diluted cultures. Exponentially growing *E. coli* cells were infected with an average of one infective particle per cell, incubated for 5 min, and diluted to prevent phage reinfection. The diluted cultures were further incubated, and at the indicated times samples were assayed for the

number of total and extracellular plaque-forming particles. As shown (Fig. 1c), at 37 C the number of intracellular phage begins to rise rapidly 30 min after infection, and the final plateau is obtained 20 min later. The synthesis of mature phage at 37 C precedes their extracellular appearance by approximately 10 min. At 30 C, phage is synthesized and released more slowly than at 37 C. The maximum rate of phage release was ca. 30 phage per min at 30 C and ca. 80 phage/min at 37 C.

Effect of rifampin on MS2 phage release at 37 and 30 C. Recently we reported that rifampin inhibits the release of progeny MS2 phages from *E. coli* cells at 37 C (7). It was also shown that this inhibitory effect can be completely separated from the effect of rifampin on phage synthesis by adding it 20 min or later after infection. We therefore studied the effect of rifampin (100 μ g/ml) on phage release at 37 C in greater detail (Fig. 3a). Inhibition of phage liberation was a function of the time of rifampin addition. Of the progeny phages, 14, 32, 54, and 84% were released when the drug was applied 20, 25, 30, and 35 min, respectively, after infection. Normal kinetics of phage release shown in the same figure reveal that the drug did not cause an immediate cessation of phage release but allowed it to proceed for about 23 min. For example, when rifampin was added 25 min postinfection, the percentage of maximum progeny phages released corresponds to those normally released at 48 min after infection.

We then studied the effect of the antibiotic on phage release at 30 C. As shown in Table 1, maximal phage yield was reduced to about 50% when the drug was added 60 to 75 min after infection. However, release was markedly inhibited, and the particles were found in the cells. The inhibitory effect of the drug on phage release was similar at 30 and 37 C (cf. Fig. 3a and 3b), but the release continued for a longer period (60 min) at 30 C.

Effect of rifampin on MS2 phage release from a rifampin-resistant mutant. The effects of rifampin on MS2 phage synthesis and release from a rifampin-resistant mutant were studied. This mutant grows normally on plates containing 100 μ g of the antibiotic per ml, and, as shown (Fig. 4), its DNA-dependent RNA polymerase activity is markedly more resistant to the drug than that of the wild type. In the rifampin-resistant mutant, neither phage synthesis nor phage release was affected by 100 μ g of the drug per ml (Table 2). This result indicates that the inhibitory effect of rifampin on MS2 phage release is a consequence of the inhibition of DNA-dependent RNA polymerase of the host.

FIG. 1. Growth and release of MS2 phage. (a) At 37°C in a dense culture. (b) At 30°C in a dense culture. (c) At 37 and 30°C in a diluted culture. Symbols: (▲) total phages and (●) extracellular phages at 37°C; (Δ) total phages and (○) extracellular phages at 30°C; (■) optical density.

FIG. 2. Electron micrographs of *E. coli* 3 hr after infection with MS2 phage at 30 C. $\times 16,000$.

FIG. 3. Effect of rifampicin on MS2 phage release. (a) At 37 C. (b) At 30 C. Symbols: ●, maximum extracellular phage yield in a culture treated at different times postinfection with 100 μ g of rifampicin per ml; ○, extracellular phage yield at different times postinfection. Maximum extracellular phage yield was determined 70 and 180 min after infection at 37 and 30 C, respectively.

TABLE 1. Extracellular and intracellular distribution of MS2 progeny phage after infection at 30 C in the presence of rifampin

Time (min) of addition of rifampin postinfection	Phage yield ^a					
	Total		Extracellular		Intracellular	
	PFU/ml	Per cent inhibition	PFU/ml	Per cent of total	PFU/ml	Per cent of total
60	1.7×10^{11}	50	2.2×10^{10}	13	1.5×10^{11}	90
75	1.5×10^{11}	45	6.0×10^{10}	40	9.4×10^{10}	63
90	3.4×10^{11}	4	2.4×10^{11}	72	9.6×10^{10}	29
No rifampin	3.5×10^{11}	0	3.5×10^{11}	100	1.4×10^{10}	4

^a Determined 180 min after infection.

Effect of chloramphenicol on MS2 phage release at 37 and 30 C. Host DNA-dependent RNA polymerase might be indirectly involved in the release of RNA phage if host protein synthesis is required for this process. To determine whether protein synthesis was necessary, the effect of chloramphenicol on the process was studied. Chloramphenicol (100 µg/ml) promptly and completely stopped protein synthesis in *E. coli* cells both at 37 and 30 C (Fig. 5). The effect of chloramphenicol on phage synthesis and on phage release was studied by the addition of 100 µg of the drug per ml at 37 and 30 C. The results of this experiment are summarized in Fig. 6 and Table 3. As expected, when chloramphenicol was added, phage synthesis was immediately arrested (Fig. 6a); the total phage yield obtained represents the phage particles synthesized up to the time of drug application. Furthermore, it was found that chloramphenicol also prevents the release of preformed phage which accumulated in the cells (Table 3). In contrast to the abrupt inhibition of phage synthesis, phage release was not inhibited immediately but continued for about 17 min at 37 C and 35 min at 30 C after the addition of the drug (Fig. 6b). It should be noted that the difference in the time required for chloramphenicol and rifampin to stop phage release can be accounted for by the additional period required by rifampin to inhibit protein synthesis in host cells (Fig. 5).

DISCUSSION

We have shown that rifampin prevents the release of progeny MS2 particles from *E. coli* cells. Phage release was unaffected when the host possesses a rifampin-resistant DNA-dependent RNA polymerase. This host enzyme might be indirectly involved in RNA phage release if host-controlled protein synthesis is required for this process. This is supported by the finding that MS2 phage release was also arrested when protein synthesis was directly inhibited by chloram-

FIG. 4. Effect of rifampin on the activity of DNA-dependent RNA polymerase from rifampin-resistant mutant and the wild-type *E. coli*.

FIG. 5. Rate of protein synthesis in the presence of chloramphenicol and rifampin. In the presence of 100 µg of chloramphenicol per ml: at 37 C (Δ), at 30 C (○). In the presence of 100 µg of rifampin per ml: at 37 C (▲), at 30 C (●).

TABLE 2. Comparative effect of rifampin on MS2 phage synthesis and release in a rifampin-resistant mutant and in wild-type *E. coli*

Cells ^a	Phage yield			
	Total		Extracellular	
	PFU/ml	Per cent of control	PFU/ml	Per cent of control
Rifampin-resistant cells				
Control	3.0×10^{11}		3.1×10^{11}	
With rifampin	3.1×10^{11}	100	3.0×10^{11}	100
Rifampin-sensitive cells				
Control	3.0×10^{11}		3.2×10^{11}	
With rifampin	3.3×10^{10}	11	3.1×10^9	1

^a Exponentially growing rifampin-resistant and -sensitive cells were infected with MS2 at 37 C at a multiplicity of infection of 10. Rifampin (100 µg/ml) was added at the time of infection. The total and extracellular phage yields were determined 150 min postinfection.

FIG. 6. Effect of chloramphenicol on phage synthesis at 37 and 30 C. (a) Phage synthesis. Maximum phage yield in a culture treated at different times postinfection with 100 µg of chloramphenicol per ml at 37 C (▲) and at 30 C (■). Phage yield at different times after infection at 37 C (Δ) and at 30 C (□). (b) Phage release. Maximum extracellular phage yield in a culture treated at different times postinfection with 100 µg of chloramphenicol per ml at 37 C (●) and at 30 C (×, solid line). Extracellular phage yield at different times postinfection at 37 C (○) and at 30 C (×, dashed line.)

phenicol. As shown, chloramphenicol does not cause an immediate cessation of phage release which continued for about 17 min at 37 C and about 35 min at 30 C after complete inhibition of protein synthesis. In the case of rifampin, a longer period (about 23 min at 37 C and about 60 min at 30 C) is required to exert an inhibitory effect on the release process. The difference in the time

required for chloramphenicol and for rifampin to stop phage release can be accounted for by the additional period needed for rifampin to inhibit protein synthesis (Fig. 5). This supports the idea that both drugs inhibit RNA phage release by a common mechanism, i.e., inhibition of bacterial protein synthesis. The fact that phage release is not immediately inhibited after inhibition of host

TABLE 3. *Effect of chloramphenicol on the intracellular and extracellular distribution of MS2 phage after infection at 37 and 30 C*

Temp (C)	Time (min) of addition of chloramphenicol postinfection	Phage yield ^a				
		Total (PFU/ml)	Extracellular		Intracellular	
			PFU/ml	Per cent of total	PFU/ml	Per cent of total
37	25	4.5×10^9	1.2×10^9	26	3.5×10^9	77
	30	2.5×10^{10}	1.1×10^{10}	44	1.5×10^{10}	60
	35	6.2×10^{10}	4.0×10^{10}	65	2.0×10^{10}	32
	No chloramphenicol	2.0×10^{11}	2.0×10^{11}	100	1.5×10^9	0.7
30	90	5.5×10^{10}	1.2×10^{10}	22	4.4×10^{10}	80
	105	1.5×10^{11}	7.6×10^{10}	51	8.1×10^{10}	54
	120	1.7×10^{11}	1.3×10^{11}	78	4.2×10^{10}	25
	No chloramphenicol	3.7×10^{11}	3.7×10^{11}	100	1.7×10^{10}	5

^a Phage yields were determined 70 and 180 min postinfection at 37 and 30 C, respectively.

protein synthesis requires comment. We interpret this delay as indicating that a short-lived host-controlled protein(s) participates in the process of RNA phage release and that this protein(s) disappears after the arrest of protein synthesis. The possibility has also to be considered that the time interval between inhibition of protein synthesis and the arrest of phage release may reflect, in addition to the lifetime of the active protein, the time required for the conversion of a pre-existing inactive protein into the active form. Such a conversion has been found for histidase of *Bacillus subtilis* (10) and tryptophanase of *E. coli* (1).

RNA phage release at 37 C is accompanied by the destruction of host cells (15). The lysis does not appear to be due to the action of lysozyme-like enzyme. Coat protein of RNA phage is required for this lysis although lytic activity of the coat protein has not been directly demonstrated (21). On the other hand, at 30 C RNA phage release is not accompanied by cellular lysis. Hoffmann-Berling and Mazé (11) followed the release of the RNA-containing phage fr from individually infected bacteria and showed that at 31 C phage are released from intact cells which continue to grow and to divide. We confirmed this observation by electron microscopy and demonstrated that host cells remain intact at 30 C after releasing, on the average, 2,000 infective MS2 phage per cell. Our experiments point to a common release mechanism at 37 and at 30 C which may involve a requirement for a short-lived host-controlled protein(s). Cell lysis which accompanies phage release at 37 C may only reflect a secondary effect that occurs at this temperature.

The fundamental and intriguing aspect of RNA

phage release is the ability of macromolecules to cross the wall-membrane barrier without lysing the cell. Such a process was also described for the DNA-containing male-specific phages (11, 12). The exceptional ability of both types of male-specific phages, which differ considerably in size, shape, and chemical composition, to pass out of the intact cell suggests that the host contributes to such a mechanism. The presence of sex factor F in male *E. coli* cells results in the production of F pili which are short-lived protein appendages implicated in the transport of nucleic acid during bacterial conjugation (2, 3) and male-specific phage infection (4, 5, 19). Without excluding other possibilities, it is tempting to speculate that these protein organelles are required for the release of RNA phage.

ACKNOWLEDGMENTS

We thank A. Friedmann for taking electron micrographs and M. Staehelin (Ciba AG, Basel) for supplying the rifampin.

This investigation was supported by a grant from The Joint Research Fund of the Hebrew University and Hadassah.

LITERATURE CITED

1. Bilezikian, J. P., R. O. R. Kaempfer, and B. Magasanik. 1967. Mechanism of tryptophanase induction in *E. coli*. *J. Mol. Biol.* 27:495-506.
2. Brinton, C. C., Jr. 1965. The structure, function, synthesis, and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. *Trans. N.Y. Acad. Sci.* 27:1003-1054.
3. Brinton, C. C., Jr., P. Gemski, Jr., and J. Carnahan. 1964. A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K12, and its role in chromosome transfer. *Proc. Nat. Acad. Sci. U.S.A.* 52:776-783.
4. Caro, L. G., and M. Schnos. 1966. The attachment of male specific bacteriophage f1 to sensitive strains of *E. coli*. *Proc. Nat. Acad. Sci. U.S.A.* 56:126-132.
5. Crawford, E. M., and R. F. Gesteland. 1964. The adsorption of bacteriophage R17. *Virology* 22:165-167.

6. Di Mauro, E., L. Snyder, P. Marino, A. Lamberti, A. Coppo, and G. P. Tocchini-Valentini. 1969. Rifampicin sensitivity of components of DNA-dependent RNA polymerase. *Nature (London)* 222:533-537.
7. Engelberg, H., and E. Soudry. 1971. Inhibition of ribonucleic acid bacteriophage release from its host by rifampin. *J. Virol.* 7:897-898.
8. Fromageot, H. P. M., and N. D. Zinder. 1968. Growth of bacteriophage f2 in *E. coli* treated with rifampicin. *Proc. Nat. Acad. Sci. U.S.A.* 61:184-191.
9. Hartmann, G., K. O. Honikel, F. Knüsel, and J. Nüesch. 1967. The specific inhibition of DNA-directed RNA synthesis by rifamycin. *Biochim. Biophys. Acta* 145:843-844.
10. Hartwell, L. H., and B. Magasanik. 1963. The molecular basis of histidase induction in *B. subtilis*. *J. Mol. Biol.* 7:401-420.
11. Hoffmann-Berling, H., and R. Mazé. 1964. Release of male-specific bacteriophage from surviving host bacteria. *Virology* 22:305-313.
12. Hofschneider, P. H., and A. Preuss. 1963. M13 bacteriophage liberation from intact bacteria as revealed by electron microscopy. *J. Mol. Biol.* 7:450-451.
13. King, J. 1968. Assembly of the tail of bacteriophage T4. *J. Mol. Biol.* 32:231-262.
14. Lancini, G. C., and G. Sartori. 1968. Rifampicin. LXI. *In vivo* inhibition of RNA synthesis by rifampicins. *Experientia* 24:1105-1106.
15. Loeb, T., and N. D. Zinder. 1961. A bacteriophage containing RNA. *Proc. Nat. Acad. Sci. U.S.A.* 47:282-289.
16. Mario, P., M. I. Baldi, and G. P. Tocchini-Valentini. 1968. Effect of rifampicin on DNA-dependent RNA polymerase and RNA phage growth. *Cold Spring Harbor Symp. Quant. Biol.* 32:125-127.
17. Reid, P., and J. Speyer. 1970. Rifampicin inhibition of ribonucleic acid and protein synthesis in normal and ethylenediaminetetraacetic acid-treated *E. coli*. *J. Bacteriol.* 104:376-389.
18. Sippel, A., and G. Hartman. 1968. Mode of action of rifamycin on RNA polymerase reaction. *Biochim. Biophys. Acta* 157:218-219.
19. Valentine, R. C., and M. Strand. 1965. Complexes of F pili and RNA bacteriophage. *Science* 148:511-513.
20. Wehrli, W., F. Knüsel, K. Schmid, and M. Staehelin. 1968. Interaction of rifamycin with bacterial RNA polymerase. *Proc. Nat. Acad. Sci. U.S.A.* 61:667-673.
21. Zinder, N. D., and L. B. Lyons. 1968. Cell lysis: another function of coat protein of the bacteriophage f2. *Science* 159:84-85.