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Deoxyribonucleic Acid Replication and Expression of Early and Late Bacteriophage Functions in *Bacillus subtilis*

JACQUES J. PÈNE AND JULIUS MARMUR

Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, New York

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The role of deoxyribonucleic acid (DNA) replication in the control of the synthesis of deoxycytidylate (dCMP) deaminase and lysozyme in *Bacillus subtilis* infected with bacteriophage 2C has been studied. These phage-induced enzymes are synthesized at different times during the latent period. It was shown by actinomycin inhibition that the formation of the late enzyme (lysozyme) required messenger ribonucleic acid (mRNA) synthesized de novo after the initiation of translation of mRNA which specifies the early function (dCMP deaminase). The inhibition of phage DNA synthesis by mitomycin C prevented the synthesis of lysozyme only when added before the onset of phage DNA replication, but it did not affect the synthesis or action of dCMP deaminase when added at any time during the latent period. Treatment of infected cells with mitomycin C after phage DNA synthesis had reached 8 to 10% of its maximal rate resulted in the production of normal amounts of lysozyme. These observations suggest that mRNA specifying early enzymes can be transcribed from parental (and probably also from progeny) DNA, whereas late functional messengers can be transcribed only after the formation of progeny DNA.

The multiplication of certain bacteriophages requires the formation of viral specific enzymes which appear "early" or "late" in the infectious cycle (3, 10, 17; S. S. Cohen, Federation Proc. 20: 641, 1961). The synthesis of these enzymes appears to be under a stringent control mechanism which can be regarded as an example of phenotypic differentiation based on the temporal regulation of gene action.

The purpose of this communication is to present data on the temporal control of deoxycytidylate (dCMP) deaminase, an early enzyme, and of lysozyme in *Bacillus subtilis* infected with bacteriophage 2C. These data support the hypothesis advanced by Luria (11) who proposed that early phage functions are specified by unreplicated parental phage deoxyribonucleic acid (DNA), whereas late functions, such as lysozyme, are controlled by replicated progeny DNA. In agreement with Protass and Korn (14), our experiments suggest moreover that de novo messenger ribonucleic acid (mRNA) synthesis late in infection is necessary for the formation of lysozyme.

Phage 2C (J. J. Pène and J. Marmur, Federation Proc. 23:318, 1964) is a virulent bacteriophage whose DNA contains 5-hydroxymethyluracil instead of thymine. The presence of this unusual base in the viral DNA requires the formation in infected cells of a series of enzymes (e.g., dCMP deaminase) essential for the formation of this pyrimidine (F. Kahan, E. Kahan, and B. Riddle, Federation Proc. 23:318, 1964; M. Nishihara, A. Chrambach, H. V. Aposhian, Bacteriol. Proc., p. 126, 1966). The latent period of phage 2C under the conditions described in this paper is 45 min and the burst size is approximately 200 phages per bacterium.

MATERIALS AND METHODS

Enzyme assays. dCMP deaminase was assayed by the method of Maley and Maley (12) based on the chromatographic separation of dCMP from deoxyuridine monophosphate (dUMP) on Dowex 50 (H^+) columns. dCMP-*2*-C^{14} (14.2 mc/mM) was purchased from the New England Nuclear Corp., Boston, Mass. Enzyme activity was expressed as μmoles of dUMP formed per 5×10^8 infected bacteria per hr at 37 C.

Lysozyme activity was measured by the procedure of Pène (*in preparation*). The basis of the assay was the quantitation of cellular integrity of H^3-thymine labeled *B. subtilis* cells which were used as the enzyme substrate in the presence of excess deoxyribonuclease 1 and venom phosphodiesterase. Enzyme activity was measured by the radioactivity which was made

soluble in 5% trichloroacetic acid after 20 min of incubation at 37 C. Activity was expressed as units per 5×10^8 cells per 20 min in terms of the activity of known amounts of crystalline egg white lysozyme. One unit of phage lysozyme corresponds to the activity of 1 μg of egg white lysozyme.

Propagation and purification of phage 2C. Phage 2C was propagated on *B. subtilis* 168-2, a diauxotroph requiring indole and leucine for growth. Bacteria growing in L broth (Difco tryptone, 10 g; Difco yeast extract, 10 g; NaCl, 5 g; $MnCl_2$, 10^{-5} M; water, 1 liter) were infected when the cell concentration reached 7×10^7 per milliliter with phage 2C at a multiplicity of five phages per bacterium. Lysates were centrifuged at 6,000 rev/min and 4 C for 10 min in a Sorvall centrifuge to eliminate bacterial debris. The supernatant broth was then centrifuged in a 19 rotor of a Spinco model L2 ultracentrifuge at 15,000 rev/min for 40 min. Phage pellets were gradually resuspended by gentle agitation in Spizizen minimal salts medium (15) overnight at 4 C, were centrifuged at 5,000 rev/min for 10 min at 4 C, and were sterilized by passing the opalescent solution through a membrane filter, type HA (Millipore Filter Corp., Bedford, Mass.). Phages were assayed according to the procedures outlined by Adams (1) and stored at 4 C.

Preparation of extracts. B. subtilis A26, a transformable, uracil-requiring auxotrophic derivative of strain 168 (15), growing in the logarithmic phase in L broth, was infected with phage 2C at a multiplicity of five phages per bacterium. Samples of the infected culture were removed at various times and poured over ice containing 0.01 M sodium azide. The cells were collected by centrifugation and crushed in a French pressure cell. Extracts were made in 0.01 M tris(hydroxymethyl)aminomethane-0.01 M $MgCl_2$-0.01 M mercaptoethanol (*p*H 9.0). The extracts were centrifuged at 4 C for 10 min at 5,000 rev/min in a Sorvall centrifuge and the supernatant solution was assayed immediately for dCMP deaminase and lysozyme activity. Under the conditions of the assays, the amounts of dCMP deaminase and lysozyme found in uninfected bacteria were less than 1% of the activity of extracts prepared from phage-infected cells 25 min after phage addition.

Antibiotics used. Mitomycin C was purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. Actinomycin D was a gift of the Cancer Chemotherapy Division of the National Institutes of Health.

Measurement of phage DNA synthesis. Phage DNA synthesis was measured by the incorporation of 18 c/mM of H^3-uridine (Nuclear-Chicago Corp., Des Plaines, Ill.) into KOH-resistant, trichloroacetic acid-precipitable material. Infected bacteria (3 ml) were pulsed in L broth with 50 μc of H^3-uridine for 3 min at various times during the latent period. Uptake of radioactivity was terminated by addition of 3 ml of 20% trichloroacetic acid containing 2 mg/ml of uridine, and the sample was chilled rapidly. Cells were collected by centrifugation and were washed once with 5% trichloroacetic acid; the pellet was resuspended in 1 ml of 1 N KOH. The material was incubated for 18 hr at 37 C, was neutralized with 1 N HCl, was chilled at 0 C, and was precipitated with 10% trichloroacetic acid (final concentration). The samples were passed through membrane filters, type HA (Millipore Filter Corp., Bedford, Mass.), and washed with 30 to 40 ml of 5% trichloroacetic acid. The filters were dried and counted in a toluene scintillator (Liquifluor, Pilot Chemicals, Inc., Watertown, Mass.) in an Ansitron liquid scintillation counter.

RESULTS

The formation of dCMP deaminase and lysozyme in 2C-infected *B. subtilis* A26 is presented in Fig. 1. An early functioning enzyme, dCMP deaminase was detected 3 to 5 min after phage addition; synthesis continued until the end of the latent period. In most experiments, a change in the rate of synthesis was observed 20 min after infection. This is in contrast to early functions in *Escherichia coli* infected with T-even bacteriophages. Synthesis of these early enzymes stops about 15 min after the initiation of infection (3, 17). In 2C-infected *B. subtilis*, lysozyme, a late function, could not be detected prior to 15 min after phage addition although the enzyme assay used in these experiments could detect an amount of lysozyme corresponding to 5×10^3 infected

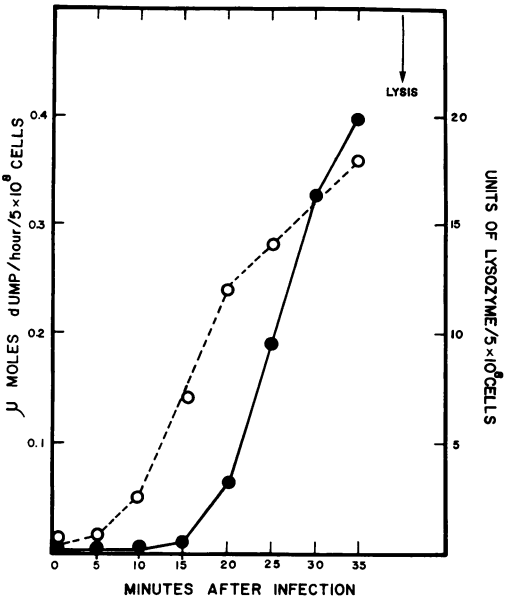

FIG. 1. *Formation of dCMP deaminase and lysozyme in 2C-infected Bacillus subtilis A26. Bacteria growing in L broth were infected with phage 2C (five phages per bacterium). Samples of 100 ml were removed at various times and processed as described in Materials and Methods. dCMP deaminase* (○) *and lysozyme* (●) *activities were determined immediately after the preparation of the extracts.*

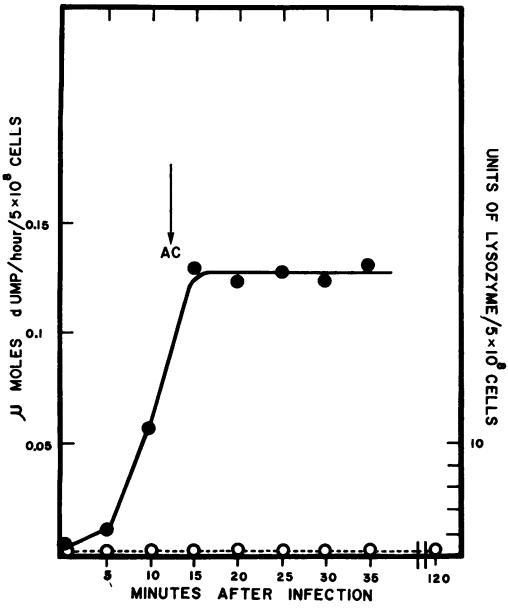

FIG. 2. *Effect of actinomycin D on the formation of dCMP deaminase and lysozyme in 2C-infected Bacillus subtilis A26. Actinomycin D (10 μg/ml) was added 13 min after infection of B. subtilis A26 with phage 2C (five phages per bacterium). dCMP deaminase (●) and lysozyme (○) were assayed in extracts prepared from 100 ml of infected bacteria as described in Materials and Methods.*

centers. The synthesis of both dCMP deaminase and lysozyme was prevented by chloramphenicol (100 μg/ml) added with the phage.

When actinomycin D (10 μg/ml) was added 13 min after infection (Fig. 2), dCMP deaminase synthesis stopped within 2 to 3 min, and lysozyme did not appear. For up to 2 hr after actinomycin addition, lysozyme could not be detected. This experiment indicates that early dCMP mRNA is not stable since dCMP deaminase synthesis is inhibited by actinomycin D and that a new messenger is necessary for the production of lysozyme.

It is not possible to say whether this mRNA, synthesized 13 min after infection, specifies the structure of lysozyme per se or operates in the translational control of preformed but inactive lysozyme mRNA. In any case, the experiment shows that the formation of lysozyme requires the de novo synthesis of a functional mRNA produced at a different time than the messenger which specifies dCMP deaminase.

When a high concentration (25 μg/ml) of mitomycin C was added to phage-infected bacteria, the synthesis of phage DNA stopped abruptly (Fig. 3). An 80% decrease in the rate of DNA synthesis was observed 4 min after addition of the antibiotic. Inhibition of DNA synthesis by mitomycin C is thought to be due to the cross linking of the complementary DNA strands (9).

Mitomycin C (25 μg/ml) added along with phage 2C (Fig. 4) prevented the lysis of the infected culture for more than 3 hr. Under these conditions, control 2C-infected bacteria began to lyse 45 min after phage addition.

Treatment with mitomycin C immediately after infection did not prevent the normal function of dCMP deaminase. Under these conditions, however, lysozyme was not formed for up to 2 hr (Fig. 5).

When mitomycin was added 20 min after infection, phage DNA synthesis stopped abruptly (Fig. 6). Under these conditions, the mitomycin-treated infected culture lysed at the same time as infected cells not treated with mitomycin, but no viable phages were released. Normal levels of lysozyme at 30 min after infection were found in

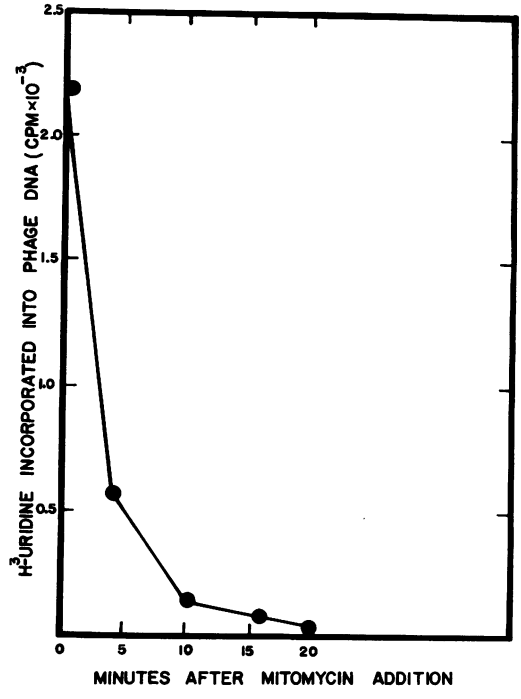

FIG. 3. *Rate of phage DNA synthesis in 2C-infected Bacillus subtilis A26 after mitomycin C addition. Mitomycin C (25 μg/ml) was added to phage-infected bacteria growing in L broth (five phages per bacterium) 20 min after infection. The rate of phage DNA synthesis was determined by pulse-labeling samples of the culture with H³-uridine and measuring the radioactivity incorporated into trichloroacetic acid-precipitable and KOH-resistant material retained on membrane filters.*

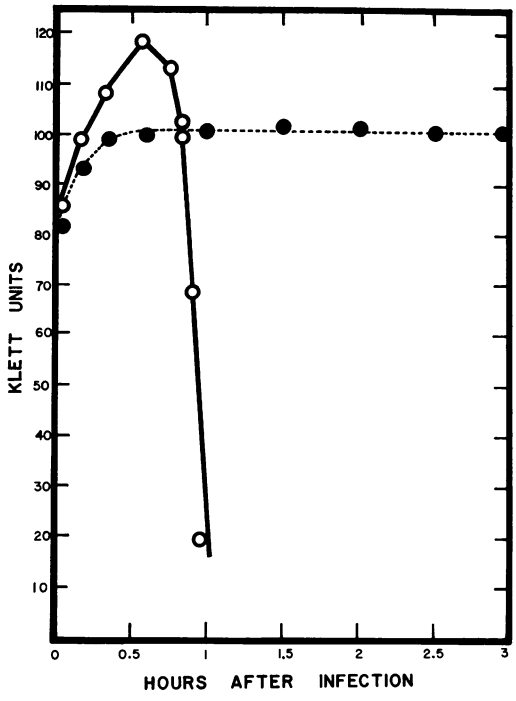

FIG. 4. *Effect of mitomycin C on the turbidity of 2C-infected Bacillus subtilis A26. Mitomycin C (25 μg/ml) was added to B. subtilis A26 immediately after infection. The turbidity of the control (○) and treated (●) cultures was measured with a Klett-Summerson colorimeter fitted with a 620 mμ filter.*

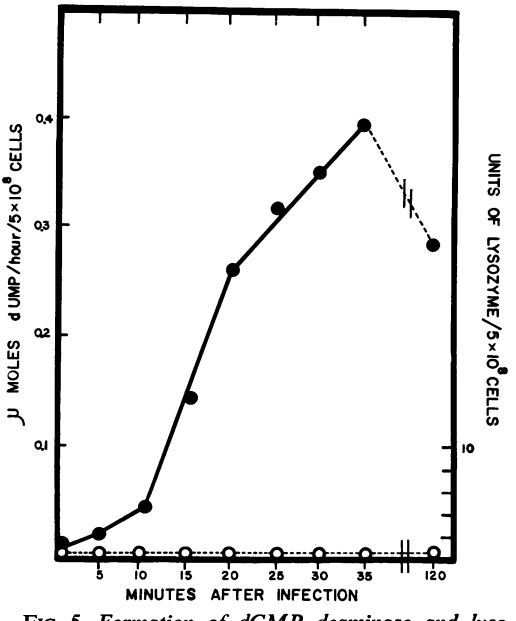

FIG. 5. *Formation of dCMP deaminase and lysozyme in mitomycin C-treated Bacillus subtilis A26 infected with phage 2C. Mitomycin C (25 μg/ml) was added to B. subtilis A26 immediately after phage addition (five phages per bacterium). At various times, 100-ml samples were collected. dCMP deaminase (●) and lysozyme (○) were assayed as described in Materials and Methods.*

infected cells treated with mitomycin 20 min after phage addition.

These experiments suggest that mitomycin (i) does not greatly affect the function of early or late mRNA specifying dCMP deaminase and lysozyme, (ii) does not prevent the synthesis of the messenger specifying dCMP deaminase, and (iii) appears to prevent the formation of mRNA involved in the synthesis of lysozyme when added before the onset of phage DNA replication.

Table 1 summarizes the effects of mitomycin added at various times during the latent period of phage 2C. Treatment with mitomycin 5 min before infection and 0 or 5 min after phage addition resulted in complete prevention of the synthesis of lysozyme. Infected bacteria treated with mitomycin 10 min after phage addition lysed with a 10- to 12-min delay. Infected bacteria treated with mitomycin after 10 min lysed normally and produced normal levels of lysozyme. The rate of phage DNA synthesis between 5 and 10 min after infection was 2 to 5% of the maximal rate which occurred just before lysis.

DISCUSSION

The experiments presented above indicate that the formation of lysozyme requires de novo synthesis of mRNA during a time segment of the latent period which differs from the period during which functional mRNA specifying early functions are produced. The possibility still remains that unstable and nonfunctional messengers specifying late enzymes are transcribed from parental phage DNA. In T4-infected *Escherichia coli*, the recent experiments of Bautz et al. suggest that this is the case (2). Although the experiments described above cannot eliminate this possibility, they indicate, however, that functional de novo mRNA synthesis is necessary late in infection for the formation of lysozyme. Moreover, the synthesis of functional mRNA specifying lysozyme can occur only after the appearance of some progeny DNA. It has been shown that mitomycin does not interfere with the action of dCMP deaminase or lysozyme and does not prevent at any time the synthesis of dCMP deaminase. Total inhibition of the synthesis of the late function can occur only if mitomycin C is added when the rate of phage DNA synthesis is less than 5% of its maximal value. It seems likely, therefore, that

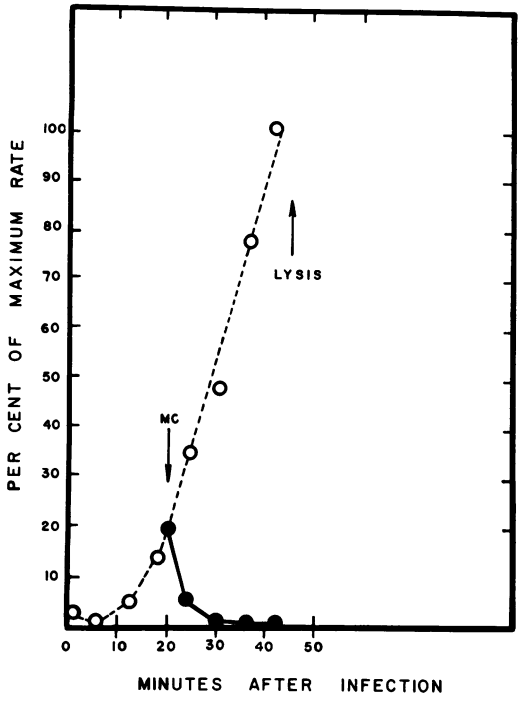

FIG. 6. *Rate of phage DNA synthesis in 2C-infected Bacillus subtilis A26. The rate of phage DNA synthesis was determined as described in the text by pulse-labeling samples of infected bacteria with H^3-uridine and quantitating the amount of radioactivity incorporated into trichloroacetic acid-precipitable, KOH-resistant material on membrane filters. The symbol ○ represents the rate of phage DNA synthesis in control infected bacteria, and the symbol ● represents the rate of phage DNA synthesis after mitomycin C addition (25 μg/ml) to a sample of the same culture, 20 min after phage infection.*

inhibition of the first (or part of the first) round of phage DNA replication is sufficient to prevent the synthesis of lysozyme.

Since inhibition of DNA synthesis after the 10th min of infection (5% of the maximal rate) interferes but slightly with the normal production of lysozyme, it seems unlikely that the gene-dosage model proposed by Edlin (5) operates in the control of lysozyme in 2C-infected *B. subtilis*.

Viable phages are not released when mitomycin C is added at any time before the 20th min after infection. Morphologically complete virus particles may not be formed under these conditions. Presumably, if such particles were released from cells treated with mitomycin C, they would contain cross-linked DNA which might be incapable of initiating subsequent DNA synthesis. It is unlikely that the induction of defective prophages can modify the interpretation of these experiments since lysis cannot be detected for 3 hr after addition of high concentrations of mitomycin early in the infectious cycle.

The physical or topological differentiation of parental and progeny DNA seems to be a prerequisite for the formation of functional mRNA such as the one specifying lysozyme. Parental phage DNA must be initially transcribed by the host RNA polymerase. Since inhibition of phage DNA replication does not lead to the formation of functional late mRNA, this suggests that the host RNA polymerase can transcribe only part of the parental DNA in vivo. In agreement with this, recent work by Geiduschek and his associates (7) indicates that the in vitro transcription of T2 and T4 native DNA by *E. coli* RNA polymerase results in the selective synthesis of RNA complementary only to DNA segments specifying early functions.

The nature of the mechanism by which the transition of parental to progeny DNA allows the formation of functional late mRNA is not known. Among many possible mechanisms, two can be suggested. Firstly, there may be a transcription of a segment of a parental strand to produce early mRNA, followed by DNA replication and

TABLE 1. *Formation of lysozyme in phage 2C-infected Bacillus subtilis A26 treated with mitomycin C*

Time after infection of mitomycin C addition[a]	Percentage of maximal rate of phage DNA synthesis in control culture[b]	Lysis of treated culture[c]	Lysozyme activity[d]
min			
−5	No data	−	0.05
0	No data	−	0.07
5	2	−	0.06
10	5	+ (Delayed)	19
15	10	+	27
20	25	+	25

[a] Mitomycin C (25 μg/ml) was added at various times after infection of *B. subtilis* A26 with phage 2C (five phages per bacterium).

[b] Phage DNA synthesis in a control parallel culture was determined by incorporation of H^3-uridine into trichloroacetic acid-precipitable, KOH-resistant material.

[c] The lysis of the treated cultures (+, lysis beginning 45 min after phage addition; −, no lysis within 3 hr) was determined turbidimetrically in a Klett-Summerson colorimeter at 620 mμ.

[d] Lysozyme activity was measured as described in the text in extracts prepared from 25 ml of bacteria; figures represent units of lysozyme per 5×10^8 cells at 30 min.

partial or complete copy of either the same progeny strand or the complementary progeny strand. The specificity of host RNA polymerase initiation could be controlled by structural features of the progeny strands (e.g., methylation) or by topological features of the replicating DNA such as circularization (16) or concatonate forms (6). Several examples of genetic transcription restricted to one of the complementary strands of DNA have been described (8, 13).

Secondly, late functional messengers could also be produced after DNA replication by a phage specific RNA polymerase or through the action of an initiator factor allowing the host RNA polymerase to carry out transcription of the late function region.

The recent experiments of Dove (4) with bacteriophage λ suggest that in that system a gene product is required in addition to DNA replication for the formation of late functions.

Experiments are being conducted with native, sheared, and replicating phage DNA added to transformable *B. subtilis* cells under various conditions to study further the control mechanisms involved in the formation of early and late enzymes in vivo.

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