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Replication of Bacteriophage M 13: Inhibition of Single-Strand DNA Synthesis by Rifampicin

(DNA replication/transcription/RNA polymerase/chloramphenicol/*E. coli*)

WALTER L. STAUDENBAUER AND PETER HANS HOFSCHNEIDER

Max-Planck-Institut für Biochemie, München, Germany

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ABSTRACT Synthesis of single-stranded DNA of bacteriophage M 13 is blocked by rifampicin. This inhibition is not observed in an *Escherichia coli* mutant possessing an RNA polymerase resistant to rifampicin. Since rifampicin stops single-strand synthesis faster than chloramphenicol, inhibition by rifampicin does not seem to result from a transcriptional block of protein synthesis, particularly in view of the long half-life of the messenger RNA for M 13. It is, therefore, concluded that the interaction of rifampicin with RNA polymerase directly affects single-strand DNA synthesis. Possible mechanisms are discussed.

The DNA of the filamentous phage M 13 replicates in three stages (1, 2). First, the infecting single-stranded DNA is converted by host enzymes into a double-stranded form (SS → RF). Early in the infection the replicative forms multiply to establish a pool of RF molecules (RF → RF). Later, double-strand synthesis ceases almost completely, and the RF molecules serve as precursors for single-strand synthesis (RF → SS). Single-stranded viral DNA is formed by an asymmetric replication process in which the viral strand of the RF is displaced as a new one is synthesized (3).

These three stages of phage DNA repliction show differences in their sensitivity towards antibiotics. Inhibition of protein synthesis by chloramphenicol has no effect on the SS → RF conversion (4). Ongoing RF replication is also rather insensitive to chloramphenicol, and continues at a slowly decreasing rate after all protein synthesis has stopped (5). If chloramphenicol is added late in the infection, an immediate switch back from single-strand synthesis to RF replication is observed (6). However, the conversion of single-stranded parental DNA into the replicative form, as well as the multiplication of the double-stranded forms, are rapidly inhibited by rifampicin (5), a specific inhibitor of RNA polymerase.

Since it has been shown in this lab that the specific messenger RNA for phage M 13 has an exceptionally long half-life (ref. 7 and unpublished data), we expected that blockage of transcription with rifampicin should have a considerably delayed effect on single-strand synthesis and phage maturation, as compared to chloramphenicol treatment. However, after we added rifampicin to M 13-infected cells late in infection, a rapid halt of phage release was observed (Fig. 1). Following up this observation, we found, as shown in this paper, that addition of rifampicin results in an immediate stop of single-strand synthesis. Since no inhibition was observed in an *Escherichia coli* mutant with an RNA polymerase resistant to rifampicin, we conclude that rifampicin stops M 13 single-strand synthesis by interaction with RNA polymerase.

Abbreviations: SS, single-stranded phage DNA; RF, double-stranded replicative form.

METHODS AND RESULTS

The effect of rifampicin and chloramphenicol on DNA synthesis in uninfected cells and in M 13-infected cells was measured 2 hr after infection, a time when single-strand synthesis is predominant, by exposure of aliquots of the treated and untreated cultures to [^{3}H]thymidine for 1 min. As shown in Fig. 2*A*, chloramphenicol had no immediate effect on DNA synthesis in uninfected cells, whereas rifampicin prevented a further increase in the rate of DNA synthesis. In the infected culture (Fig. 2*B*), an immediate drop in the incorporation rate was observed after the addition of rifampicin; thereafter, the incorporation continued at a constant rate during the time interval studied. In contrast, chloramphenicol led to a gradual decrease in the rate of DNA synthesis in the infected culture. To interprete these results, we assumed that rifampicin does not inhibit the ongoing replication of host DNA, and that the residual DNA synthesis in infected cells in the presence of rifampicin represents synthesis of host DNA.

To investigate more closely the effect of rifampicin on single-strand synthesis, pulse–chase experiments were performed late in M 13 infection, and the distribution of radioactive label was analyzed by sucrose gradient centrifugation. As can be seen in Fig. 3*A*, most of the label incorporated during a 1-min pulse in untreated cells infected with M 13 is found in phage-specific RF molecules and in fast-sedimenting *E. coli* DNA. After 5 min in the presence of rifampicin, only a small portion of the incorporated radioactivity is found in the RF position, while most of the label sediments towards the bottom of the gradient (Fig. 3*B*). Chloramphenicol interferes only slightly with phage DNA synthesis (Fig. 3*C*); however, there is a decrease in the amount of label sedimenting as RF II.

After a 10-min chase, the difference in the labeling pattern between untreated and rifampicin-treated cells is even more striking. In untreated cells, most of the phage-specific label has been chased into single strands and, further, into extracellular phage particles (Fig. 4*A*). In the presence of rifampicin, however, practically no single strands have been formed. The small amount of radioactivity incorporated during the pulse stayed in the RF I position (Fig. 4*B*). Under the same conditions, single-strand synthesis is less inhibited in chloramphenicol-treated cells (Fig. 4*C*), although most of the label accumulates in the RF I position, as expected from previous results (6). It should be noted that the label incorporated in the untreated control was shifted rapidly from single strands into phage, without accumulation in a single-strand pool. This finding may explain why the rifampicin effect on single-

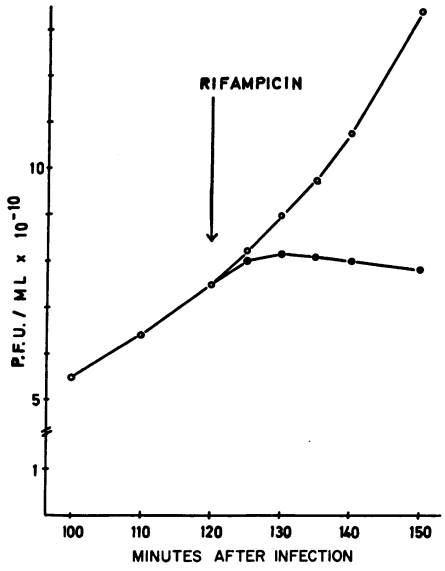

FIG. 1. Effect of rifampicin on phage production. *E. coli* AB 301 was grown in H medium (15) at 37° to 1 × 10^8/ml, and infected with M 13 at a multiplicity of 100. Aeration was continued for 90 min. The cells were then separated from the free phage by low-speed centrifugation, and resuspended in an equal volume of warmed medium. 120 min after infection, the culture was divided and one part was treated with rifampicin (200 μg/ml). Aliquots were removed at the times indicated and the phage was titrated. PFU-plaque forming units. O——O, control; ●——●, rifampicin treated.

strand synthesis is reflected in an immediate cessation of phage production (Fig. 1).

Although the most likely target for rifampicin is the RNA polymerase of the host (8), it is conceivable that some other protein involved in phage DNA synthesis might also be sensitive to this antibiotic. Therefore, the pulse–chase experiment was repeated with an *E. coli* mutant shown (D. Meier and P. H. Hofschneider, manuscript in preparation) to have a rifampicin-resistant RNA polymerase. As can be seen in Fig. 5, no inhibition of phage production by rifampicin could be demonstrated in this strain. Chloramphenicol blocked single-strand synthesis almost completely, but did not interfere with the incorporation of label into RF molecules.

DISCUSSION

The synthesis of M 13 DNA late in the infection, when only single-stranded viral DNA is being made, is completely inhibited by rifampicin. Since a mutant with an altered RNA polymerase resistant to rifampicin does not show this effect, it can be concluded that inhibition of synthesis of M 13 single-strand results from a specific interaction of rifampicin with RNA polymerase. There are three possible interpretations of this result: (*i*) transcription is needed for DNA replication, (*ii*) a block in transcription interferes with DNA synthesis indirectly by inhibition of the synthesis of specific proteins, and (*iii*) a rifampicin–RNA polymerase complex irreversibly attached to DNA inhibits DNA synthesis.

(*i*) Brutlag *et al.* have shown (5) that the early stages of M 13 replication, i.e., the conversion of single-stranded DNA to the replicative form (SS → RF), and the multiplication of the double-stranded molecules (RF → RF), are inhibited by rifampicin. They suggested that the synthesis of RNA might be required for initiation of M 13 DNA replication. This RNA could fulfill two functions that are not mutually exclusive: (*a*) it might act as a primer for the synthesis of the complementary DNA strand, or (*b*) it might facilitate initiation of DNA synthesis by locally disrupting the helical structure of the DNA. At first glance, our data could suggest the same interpretation. However, since single-strand synthesis supposedly occurs by a "rolling circle" type mechanism (3, 9) with the parental viral strand as primer, an RNA primer is probably not needed. A structural role of RNA, i.e., facilitating single-strand synthesis by "melting" a segment of the double-stranded replicative form, is therefore more likely for single-strand synthesis (RF → SS). A similar mechanism has been suggested for the initiation of replication of bacteriophage λ (10) and of replication of *E. coli* DNA (11).

(*ii*) Rifampicin has a more immediate effect on M 13 single-strand synthesis than does chloramphenicol (Figs. 3 and 4). Thus, it seems unlikely that rifampicin interferes with single-strand synthesis by blocking the transcription of messenger RNA coding for proteins involved in phage replication. Only one phage-specified protein, the gene-5 protein, is required for single-strand synthesis (4). This protein plays a negative role, preventing the conversion of newly synthesized viral single strands into double-stranded RF molecules (12). If synthesis of gene-5 protein is inhibited by chloramphenicol, a rapid

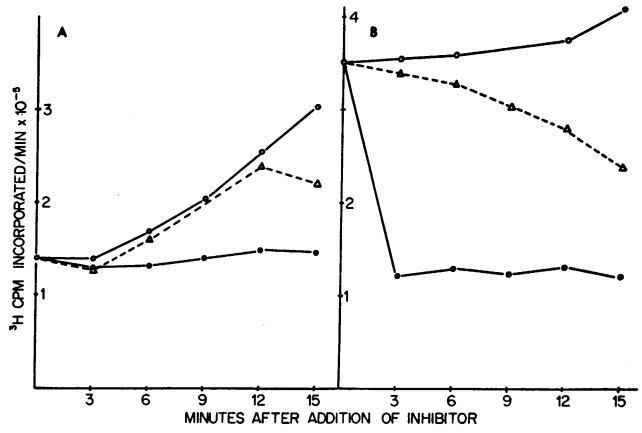

FIG. 2. Effect of rifampicin on DNA synthesis in uninfected and M 13-infected cells. *E. coli* HfrH 165, which requires thymidine, was grown in H medium supplemented with 2 μg/ml of thymidine at 34°, to 1 × 10^8 cells/ml. 30 ml Of the culture was infected with M 13 at a multiplicity of 100, another 30 ml was used as an uninfected control. Aeration was continued for 2 hr. Then the uninfected control (*A*) and the M 13-infected culture (*B*) were both divided into three parts: one part was left untreated, the other parts were treated either with rifampicin (200 μg/ml) or chloramphenicol (100 μg/ml). At 3-min intervals, 1-ml samples were removed and exposed to 10 μCi of [^{3}H]thymidine (23.3 Ci/mmol) at 34° for 1 min. Incorporation was stopped by freezing the samples in an acetone–dry ice bath. After addition of 20 μl of 2 M KCN, the samples were thawed and incubated for 15 min at 0° with 100 μg of lysozyme in 20 mM EDTA. 50 μl of 10% Sarkosyl and 50 μl of 6 N KOH were added, and the lysates were incubated for 1 hr at 45°C. After addition of 2.5 mg of unlabeled thymidine, the samples were assayed for Cl_3CCOOH-precipitable radioactivity by scintillation counting (16). O——O, control; ●——●, rifampicin treated, Δ– – –Δ, chloramphenicol treated.

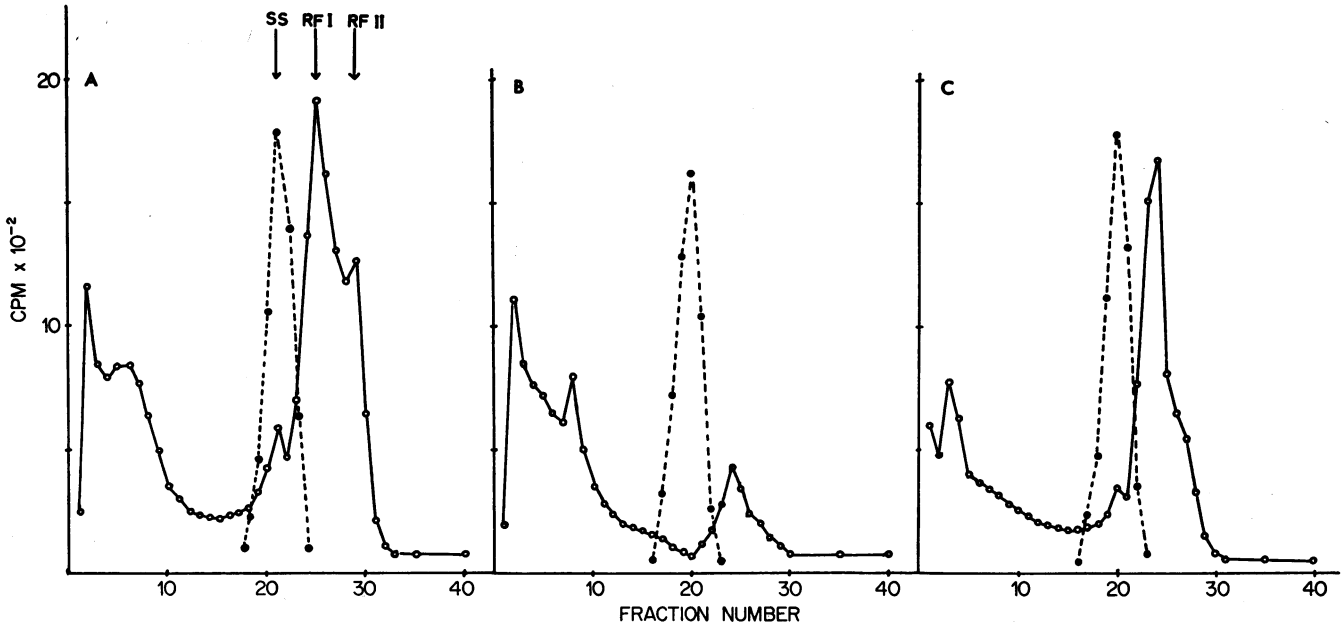

FIG. 3. Effect of rifampicin on the sedimentation patterns of pulse-labeled DNA from M 13-infected cells. *E. coli* AB 301 was grown to 1 × 10^8/ml at 37° and infected with M 13 at a multiplicity of 100. 120 min after infection, 1-ml samples were treated either with rifampicin (200 μg/ml), or chloramphenicol (100 μg/ml), or left untreated. 5 min after addition of the inhibitor, the samples were pulse-labeled for 1 min with 10 μCi of [^{3}H]thymidine. Incorporation was stopped by freezing the samples in acetone–dry ice. The samples were thawed in the presence of 20 mM KCN and further analyzed by a modification of the procedure of Ray and Schekman (17): 0.5-ml aliquots were incubated with 100 μg of lysozyme in 20 mM EDTA for 15 min at 37°, 50 μl of 5% Sarkosyl was then added, and the incubation was continued for 5 min. The lysed samples were layered directly on 5–20% (w/w) sucrose gradients in buffer (0.05 M Tris·HCl, pH 7.5–5 mM EDTA–1 M NaCl). Centrifugation was in a Spinco SW40 rotor at 25,000 rpm for 13 hr at 4°. 0.3-ml Fractions were collected from the top of the gradient by pumping 50% (w/w) sucrose into the bottom of the centrifuge tube, and assayed for acid-precipitable radioactivity. ^{32}P-labeled M 13 single strands were used as a sedimentation marker. Sedimentation is from *right* to *left*. The positions of RF I and RF II were calculated from the position of the ssDNA marker. (*A*) untreated control; (*B*) rifampicin treated; (*C*) chloramphenicol treated. O——O, pulse-labeled [^{3}H] DNA; ●——●, ^{32}P-labeled M 13 DNA.

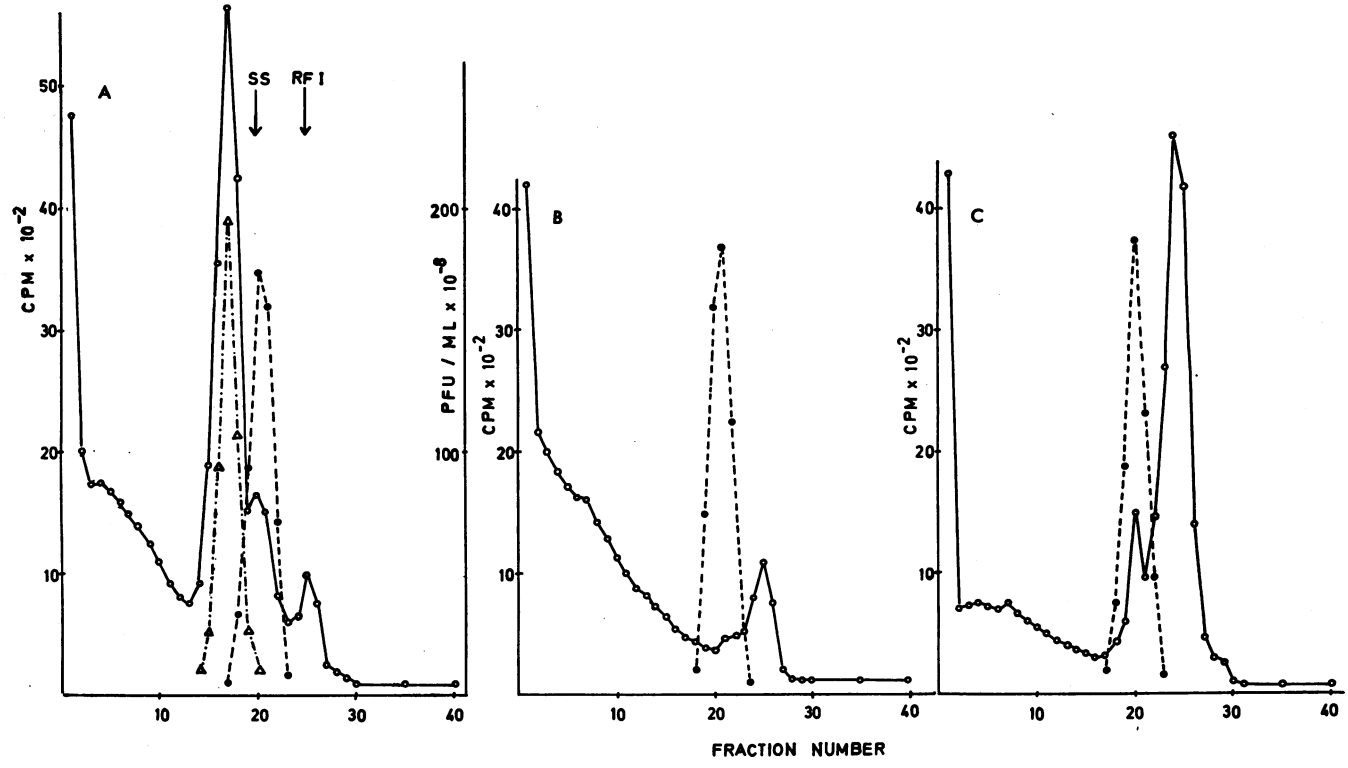

FIG. 4. Sedimentation patterns of a pulse–chase experiment. The experiment was performed as described in Fig. 3, except that the 1-min pulse was followed by a 10-min chase in the presence of 5 mg/ml of unlabeled thymidine. In addition to the radioactivity assays, the fractions were assayed for infective titer. (*A*) untreated control; (*B*) rifampicin treated; (*C*) chloramphenicol treated. O——O, pulse-labeled [^{3}H]DNA: ●– – –●, ^{32}P-labeled M 13 DNA; Δ–·–·–Δ, M 13 plaque forming units/ml.

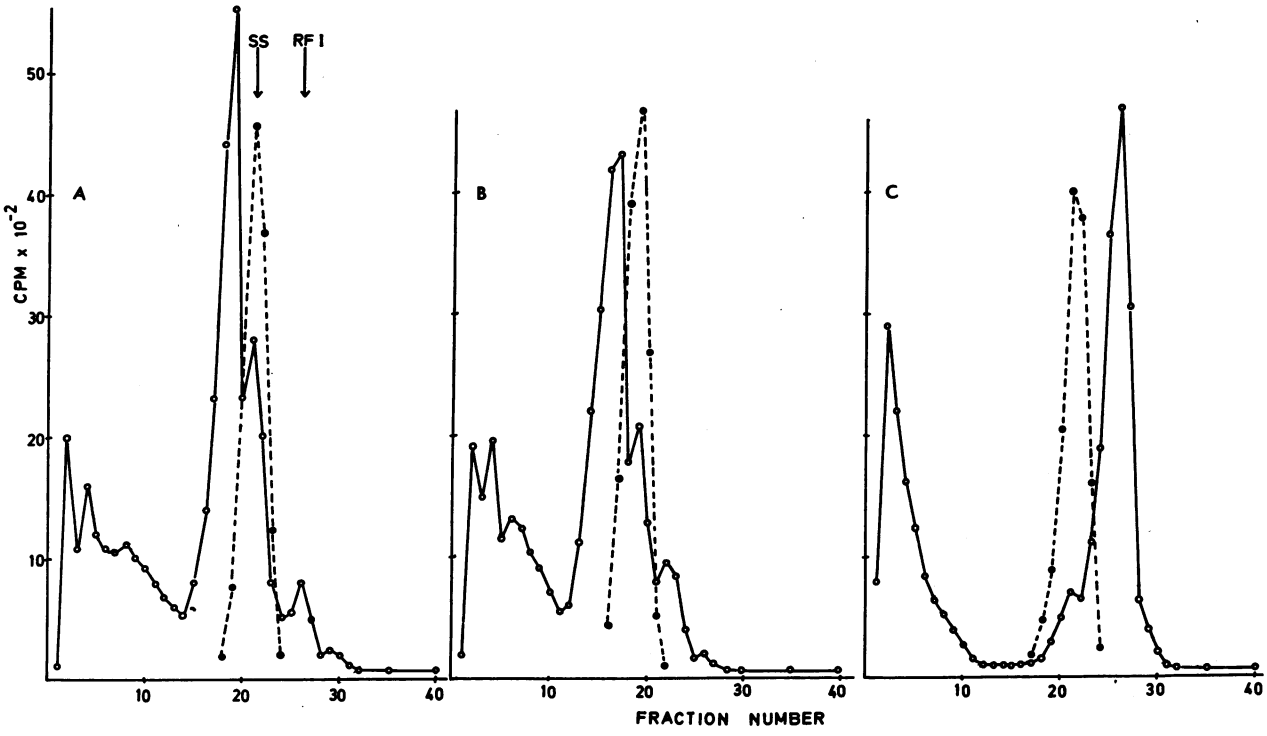

FIG. 5. Sedimentation patterns of a pulse–chase experiment on a host with a rifampicin-resistant RNA polymerase. A pulse–chase experiment was performed as described in Fig. 4, except that *E. coli* 2340, a rifampicin-resistant mutant (G. Hartmann, Würzburg) was used. (*A*) Untreated control; (*B*) rifampicin treated; (*C*) chloramphenicol treated. O——O, pulse-labeled [^{3}H]DNA; ●- - -●, ^{32}P-labeled M 13 DNA.

switch back to RF replication is observed (6). If the stability of M 13 mRNA is considered, rifampicin should have a delayed effect on gene-5 protein synthesis, as compared to chloramphenicol. It could even be argued that if the current model of M 13 replication (13) is correct, single-strand synthesis should continue in the presence of rifampicin even without gene-5 protein, since rifampicin would inhibit the conversion of single strands into double strands (5).

(*iii*) Inhibition of DNA synthesis by rifampicin does not necessarily mean that transcription is actually required for DNA synthesis. It is known that RNA polymerase can bind to DNA in the presence of rifampicin, even though it is unable to initiate RNA synthesis (14). Conceivably, a rifampicin–RNA polymerase complex attached to a replicating DNA molecule might block further replication beyond the point of attachment. It should be noted, however, that rifampicin does not interfere with an ongoing round of replication of *E. coli* DNA (see Fig. 2). This result still leaves the possibility that RNA polymerase and DNA "replicase" use the same initiation site. The rifampicin-inhibited RNA polymerase would then remain at the site and block the attachment of the replication enzyme.

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1. Marvin, D. A. & Hohn, B. (1969) *Bacteriol. Rev.* **33**, 172–209.
2. Pratt, D. (1969) *Annu. Rev. Genet.* **3**, 343–362.
3. Ray, D. S. (1969) *J. Mol. Biol.* **43**, 631–643.
4. Pratt, D. & Erdahl, W. S. (1968) *J. Mol. Biol.* **37**, 181–200.
5. Brutlag, D., Schekman, R. & Kornberg, A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2826–2829.
6. Ray, D. S. (1970) *J. Mol. Biol.* **53**, 239–250.
7. Jacob, E., Jaenisch, R. & Hofschneider, P. H. (1970) *Nature* **227**, 59–60.
8. Wehrli, W. & Staehelin, M. (1971) *Bacteriol. Rev.* **35**, 290.
9. Forsheit, A. B. & Ray, D. S. (1971) *Virology* **43**, 647–664.
10. Dove, W. F., Hargrove, E., Ohashi, M., Haugli, F. & Guha, A. (1969) *Jap. J. Genet.* **44**, Suppl. 1, 11–21.
11. Messer, W., *Mol. Gen. Genet.*, in press.
12. Salstrom, J. S. & Pratt, D. (1971) *J. Mol. Biol.* **61**, 489–501.
13. Hohn, B., Lechner, H. & Marvin, D. A. (1971) *J. Mol. Biol.* **56**, 143–154.
14. Burgess, R. R. (1971) *Annu. Rev. Biochem.* **40**, 711–740.
15. Maaløe, O. & Hanawalt, P. C. (1961) *J. Mol. Biol.* **3**, 144–155.
16. Wirtz, A. & Hofschneider, P. H. (1970) *Eur. J. Biochem.* **17**, 141–150.
17. Ray, D. S. & Schekman, R. W. (1969) *Biochim. Biophys. Acta* **179**, 398–407.