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# Replication of Mycoplasma Virus L51

## VII. Effect of Chloramphenicol on the Synthesis of DNA Replicative Intermediates

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Chloramphenicol affects several steps in the DNA replication of mycoplasma virus L51, a noncytocidal, naked, bullet-shaped virion containing circular single-stranded (SS) DNA of $1.5 \times 10^6$ daltons (4.5 kilobases). In the presence of chloramphenicol, adsorption was normal and parental SS DNA was converted to double-stranded replicative forms (RF), but subsequent RF → RF replication was inhibited. Chloramphenicol added late in infection, when most viral nascent DNA is in progeny SS molecules, inhibited SS synthesis, but nascent RF molecules were formed. However, a chase experiment showed that these RF molecules could not be converted to SS DNA. Therefore, viral RF molecules made in the presence of chloramphenicol are not functional as SS DNA precursors.

Mycoplasma virus L51 is a naked, bullet-shaped virion (13 to 16 nm by 70 to 90 nm) containing circular single-stranded (SS) DNA of 4.5 kilobases or $1.5 \times 10^6$ daltons (6, 9). The virus host is *Acholeplasma laidlawii*, a cell that is bounded only by a 7-nm plasma membrane. L51 infection is noncytocidal: infected *A. laidlawii* cells continue to grow and divide while producing viruses (5).

The molecular events in L51 DNA replication have been reviewed by Maniloff et al. (7). Parental viral DNA is converted to the double-stranded replicative forms (RF) RFI, a supercoiled double-stranded derivative of the viral genome, and RFII, a relaxed (nicked) form of RFI. The conversion of parental DNA to RF appears to occur during penetration, and parental RF DNA is membrane associated. Semiconservative replication of parental RF produces membrane-associated progeny RF molecules. These progeny RF molecules are released into the cytoplasm and by asymmetric replication produce SS viral DNA. Virus assembly and release involve processes at the cell membrane and do not affect cell viability.

Little is known about the relative roles of viral and cellular gene products in L51 replication. Two virus-specific nonstructural proteins (molecular weights, 14,000 and 10,000) have been demonstrated in infected cells (4). Synthesis of the 14,000-dalton protein is maximal early in infection, when most viral DNA replication is RF → RF. The involvement of this protein in RF → RF replication is also indicated by the failure to detect synthesis of the 14,000-dalton protein in L51-infected $REP^-$ cells (4). The inability of *A. laidlawii* $REP^-$ cells to propagate SS DNA mycoplasma viruses is due to a block in the RF → RF replication step (8). The 10,000-dalton protein appears late in infection, when most viral DNA synthesis is in SS DNA formation, suggesting that this protein might have a role in SS DNA synthesis (4).

Rifampin blocks a step late in L51 infection, and the rifampin-sensitive step has been shown to be the assembly of SS DNA molecules into progeny virions (3). In contrast to the specificity of the rifampin effect, the production of progeny virions is sensitive to chloramphenicol at all times during the infection cycle (3).

We describe here studies on the effect of chloramphenicol on the synthesis of L51 viral DNA replicative intermediates, in an effort to elucidate further the roles of cellular and viral gene products in L51 replication.

### MATERIALS AND METHODS

**Organisms, media, and buffer.** *A. laidlawii* JA1 was used for mycoplasma virus L51 propagation and as the indicator host. JA1 cells were assayed as colony-forming units on tryptose agar plates, and L51 viruses were assayed as PFU on JA1 lawns. Cells and viruses were cultivated in tryptose broth or tryptose agar plates at 37°C as described previously (5).

Tris-EDTA-NaCl buffer (pH 8.0) was 0.01 M Tris-hydrochloride–0.001 M EDTA–0.1 M NaCl. High-salt buffer was Tris-EDTA-NaCl buffer containing 1 M NaCl.

**Preparation of labeled virus.** $^{32}P$-labeled L51 virions

and viral DNA were prepared as described previously (1).

**Preparation of virus-infected cell lysates.** A log-phase culture of *A. laidlawii* JA1 was infected with either $^{32}P$-labeled or unlabeled virus. After 5 min, cells were centrifuged (10,000 × *g* for 10 min at 5°C) to remove unadsorbed virus, and the pellet was washed once with Tris-EDTA-NaCl buffer and resuspended in prewarmed (37°C) tryptose broth. At different times after infection, chloramphenicol was added to the infected culture. At various times [$^3H$]deoxythymidine (final activity, 40 μCi/ml) was added to label nascent DNA. [$^3H$]deoxythymidine (specific activity, 50 to 80 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). Labeling was terminated by adding an equal volume of ice-cold Tris-EDTA-NaCl buffer containing 10 mM NaCN, and the culture was centrifuged (10,000 × *g* for 10 min at 5°C). The cell pellet was washed and lysed by 0.4% (final concentration) Sarkosyl NL97 (Geigy Industrial Chemicals, Ardsley, N.Y.) as described previously (1).

For isolating membrane-associated viral DNA, the infected-cell suspension was gently lysed by three cycles of freezing in liquid nitrogen and thawing, as described previously (2).

**Sucrose gradient centrifugation.** Infected cell lysate (1 ml) was layered on top of 38 ml of a 5-to-20% (wt/vol) sucrose gradient in high-salt buffer and centrifuged for 16 h at 5°C at 24,000 rpm in an SW27 rotor as described previously (1). For isolating membrane-associated viral DNA, the 5-to-20% high-salt sucrose gradient was formed over a CsCl shelf (2), and 1 ml of cell lysate was layered on top of the gradient and centrifuged (3 h at 5°C at 24,000 rpm) in an SW27 rotor. Fractions (1.2 ml) were then collected and analyzed for radioactivity.

**Alkaline equilibrium gradient centrifugation.** For equilibrium sedimentation in alkaline CsCl, 2.3 g of CsCl was added to a mixture of 1.2 ml of sample, 0.4 ml of 100 mM EDTA, and 0.4 ml of 1 M NaOH. The remainder of the 5-ml tube was filled with mineral oil. The solution was centrifuged (48 h at 20°C at 35,000 rpm) in an SW50.1 rotor. Fractions (5 drops) were then collected and analyzed for radioactivity.

**Radioactivity assay.** Gradient fractions were precipitated by adding an equal volume of ice-cold 10% trichloroacetic acid, with 100 μg of bovine serum albumin per fraction as the carrier. The precipitated samples were filtered, washed, dried, and assayed for radioactivity as described previously (1).

## RESULTS

**Penetration and parental RF synthesis.** To inhibit protein synthesis during adsorption and penetration, 100 μg of chloramphenicol per ml (final concentration) was added to cells 30 min before infection. The cells were then infected with $^{32}P$-labeled virus at a multiplicity of infection (MOI) of 10. At the time of infection, 40 μCi of [$^3H$]deoxythymidine per ml (final activity) was added to the culture to label nascent DNA. After 5 min, the cells were washed to remove unadsorbed virus and gently lysed by freezing and thawing. The lysate was centrifuged to separate fast (membrane-associated)- and free-sedimenting material, as described above.

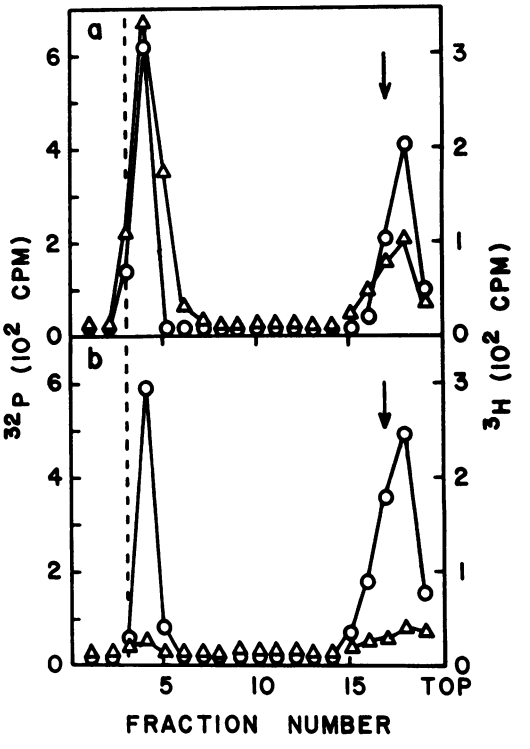


FIG. 1. Distribution of viral DNA in fast- and free-sedimenting components in untreated and chloramphenicol-treated cells. (a) A logarithmic-phase culture was mixed with $^{32}P$-labeled L51 at an MOI of 10. At the same time, 40 μCi of [$^3H$]deoxythymidine per ml was added to label nascent DNA. After 5 min, the cells were washed, lysed, and analyzed on a high-salt sucrose gradient over a CsCl shelf. (b) The same procedure as in (a) was used except that 30 min before infection the cells were mixed with 100 μg of chloramphenicol per ml. The dotted line indicates the position of the CsCl-sucrose boundary, and the arrow indicates the position of added marker L51 DNA. Symbols: ○, $^{32}P$; △, $^3H$.

In both untreated control cells and chloramphenicol-treated cells (Fig. 1), about 60% of the infecting parental viral [$^{32}P$]DNA was found as fast-sedimenting material. This implies that L51 adsorption and penetration are the same in untreated and chloramphenicol-treated cells. The amount of membrane-associated parental DNA in untreated cells (Fig. 1a) is in agreement with previously reported data (2).

As previously reported (2), most nascent DNA is in fast-sedimenting material (Fig. 1a). However, little new synthesis was detected in the fast-sedimenting material of chloramphenicol-treated cells (Fig. 1b).

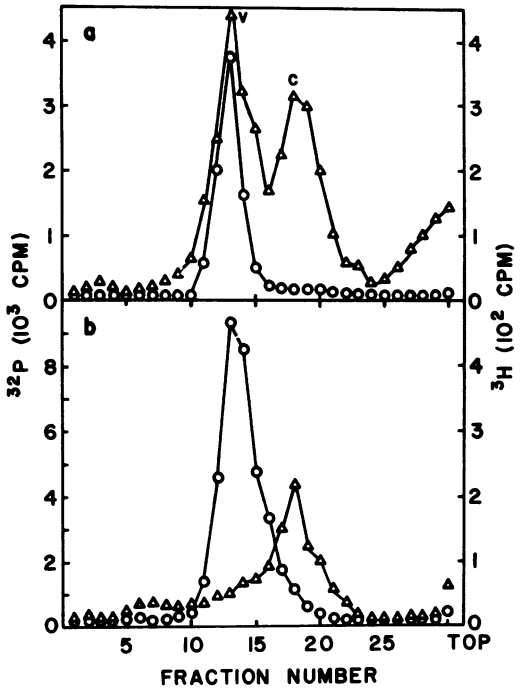


FIG. 2. Alkaline CsCl equilibrium centrifugation of DNA of fast-sedimenting material from the gradients in Fig. 1. (a) Untreated cells. Material from two gradients (Fig. 1a) was pooled for analysis. (b) Chloramphenicol-treated cells. Material from six gradients (Fig. 1b) was pooled for analysis. Parental DNA was labeled with $^{32}$P (○), and nascent DNA was labeled with $^3$H (△). V and C indicate the positions of viral and complementary strands. Deproteinization of the fast-sedimenting material was done as described previously (8).

To further analyze the nascent DNA, fast-sedimenting material from both untreated and chloramphenicol-treated cells was deproteinized and analyzed by equilibrium centrifugation in alkaline CsCl gradients. This procedure separates viral and complementary strands (2), with the [$^{32}$P]DNA of the infecting virus marking the position of the viral strand. In the gradient of fast-sedimenting-material DNA from untreated cells, the $^3$H label was in both nascent viral and complementary strands (Fig. 2a). However, in chloramphenicol-treated cells all nascent material was only in complementary strands (Fig. 2b). Therefore, in the absence of protein synthesis, parental SS DNA can be converted to double-stranded RF, but further semiconservative replication is blocked.

**RF → RF synthesis.** To examine the effect of chloramphenicol on RF → RF synthesis, cells were infected with L51 at an MOI of 10. After 5 min, the cells were washed to remove unadsorbed virus and suspended in medium containing 100 μg of chloramphenicol per ml. After 25 min of incubation with chloramphenicol, 40 μCi of [$^3$H]deoxythymidine per ml was added to label new DNA synthesis after this treatment. Labeling was terminated 10 min later. Cells were lysed with 0.4% Sarkosyl NL97, and viral DNA intermediates were analyzed by velocity sedimentation in high-salt sucrose gradients.

In untreated control cells (Fig. 3), nascent viral DNA is in RFI and RFII (cf. reference 1). However, in chloramphenicol-treated cells, almost no nascent viral DNA could be detected. This is in agreement with the conclusion from Fig. 2 that chloramphenicol inhibits RF → RF replication.

**Synthesis of progeny SS DNA.** To examine the effect of chloramphenicol on the synthesis of progeny SS DNA from RF, we made use of the observation that progeny SS DNA is the predominant DNA species synthesized after about 70 min of infection (9). Therefore, cells were infected with L51 at an MOI of 10 and incubated for 70 min. Chloramphenicol (100 μg/ml) was then added. After 25 min, 40 μCi of [$^3$H]deoxythymidine per ml was added. Labeling was terminated 10 min later. The cells were lysed with Sarkosyl and analyzed by sedimentation in high-salt sucrose gradients.

In untreated control cells (Fig. 4), nascent viral DNA was in double-stranded (RFI and RFII) and SS DNAs. However, in chloramphen-

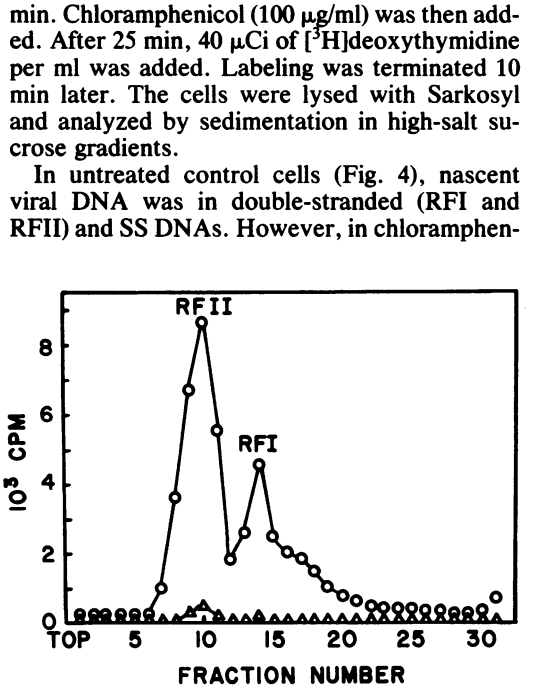


FIG. 3. Velocity sedimentation analysis of viral DNA intermediates in cells treated with chloramphenicol early in infection. Logarithmic-phase cultures were infected with L51 at an MOI of 10. After 5 min, the cells were washed and suspended in either fresh medium (○) or fresh medium containing 100 μg of chloramphenicol per ml (△). After 25 min, 40 μCi of [$^3$H]deoxythymidine per ml was added. Labeling was terminated after 10 min, and cells were lysed and analyzed by sedimentation in high-salt sucrose gradients. Under these conditions, the positions of RFI, RFII, and SS viral molecules have been identified by Das and Maniloff (9).

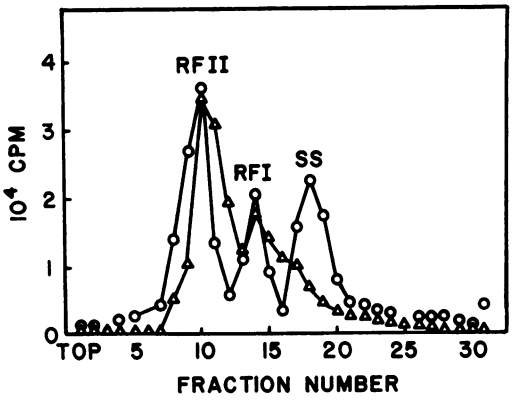


FIG. 4. Velocity sedimentation analysis of viral DNA intermediates in cells treated with chloramphenicol late in infection. Logarithmic-phase cultures were infected with L51 at an MOI of 10. After 5 min, the cells were washed and resuspended in fresh medium. After 70 min of incubation, the infected culture was divided into two parts. One part was untreated (○), and the other part was mixed with 100 μg of chloramphenicol per ml (△). After 25 min, both cultures were labeled with [$^3$H]deoxythymidine for 10 min and processed as described in the legend to Fig. 3.

icol-treated cells, nascent DNA was observed at the RFI and RFII positions, but not at the SS position.

These RF molecules (made in the presence of chloramphenicol) were studied to see whether they could be chased into SS molecules. For this experiment, the protocol for Fig. 4 was repeated (after 70 min of infection, 25 min of chloramphenicol treatment, and 10 min of labeling). The cells were then washed to remove chloramphenicol and [$^3$H]deoxythymidine, resuspended in fresh medium, and incubated for 30 min. The cells were then lysed and analyzed by sedimentation in high-salt sucrose gradients.

In untreated control cells (Fig. 5), during the 30-min chase most of the RF molecules were converted to SS molecules, and a small peak of (Sarkosyl-resistant) viral particles (Fig. 5, fraction 23) was detected. However, in the chloramphenicol-treated cells, there was little change in the gradient profile (cf. Fig. 4 and 5). Therefore, the RF molecules made in the presence of chloramphenicol are not functional; i.e., they cannot be converted into SS viral DNA.

## DISCUSSION

The results presented here show that chloramphenicol has no effect on the adsorption and penetration of the viral DNA into the indicator host. The SS parental DNA that enters the cell is found in membrane-associated double-stranded forms. Hence, conversion of parental DNA to parental RF molecules does not require a virus-coded protein or new synthesis of a cell protein.

However, replication of RF molecules is completely inhibited by chloramphenicol, indicating that virus-specific protein synthesis is required for RF → RF synthesis. This is in agreement with the observation that a cell gene product is required for L51 replication: *A. laidlawii* cells having a REP$^-$ phenotype allow the conversion of parental L51 SS DNA to double-stranded RF molecules, but these RF molecules are not replicated further (8). The REP gene product may be a 14,000-dalton protein, since in vivo synthesis of this protein is maximal in wild-type L51-infected cells at the time in infection when most replication involves RF → RF formation, but synthesis of this protein is not observed in infected REP$^-$ cells (4).

Chloramphenicol also inhibits the synthesis of progeny SS viral DNA. This is consistent with the observation that in L51-infected cells the synthesis of a 10,000-dalton protein is maximal when most viral DNA replication is in progeny SS synthesis (4).

However, it is interesting that, although chloramphenicol inhibits the synthesis of RF molecules when added early during infection, when it was added late during infection, RF replication occurred (Fig. 4). This indicates that the 14,000-dalton protein and other proteins synthesized early in infection are sufficient to maintain RF → RF replication throughout the infectious cycle. The failure of RF molecules synthesized in the presence of chloramphenicol to be chased into SS molecules (Fig. 5) shows that these RF molecules cannot serve as precursors for progeny SS viral DNA.

The chloramphenicol inhibition of progeny SS synthesis but not of RF replication has previously been reported for filamentous phage M13 infection (10); however, for M13 the RF molecules made in the presence of chloramphenicol are functional and can be chased into progeny

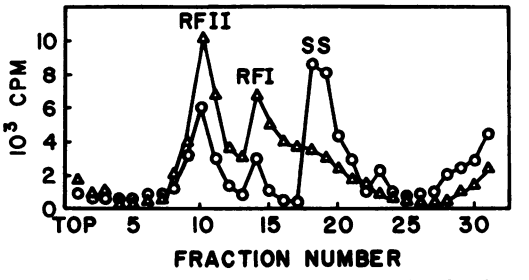


FIG. 5. Velocity sedimentation analysis of pulse-chase of viral DNA intermediates in cells treated with chloramphenicol late in infection. The protocol was as described for Fig. 4. Both untreated (○) and chloramphenicol-treated (△) cells were then washed and resuspended in fresh medium. After 30 min of incubation, the cells were lysed and analyzed as described for Fig. 3.

SS. In M13 the chloramphenicol effect is explained by RF → SS replication being regulated by the availability of the phage gene 5 protein (reviewed in reference 11). This DNA-binding protein is believed to regulate SS DNA synthesis by binding the nascent viral strands and inhibiting complementary strand synthesis. However, even in the case of M13, RF → SS replication after chloramphenicol removal is abnormal, since under these conditions about one-third of the molecules in the progeny pool of circular SS DNA are complementary strands which are not packaged into phage particles (10).

Since RF → SS synthesis is coupled to viral assembly and release, the difference of the effect of chloramphenicol on this process in a mycoplasma virus and a coliphage must reflect alternative maturation mechanisms of SS DNA viruses in the two cellular systems.

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