Virus and Host Cell DNA Syntheses During Infection of *Acholeplasma laidlawii* by MVL3, a Nonlytic Cytocidal Mycoplasma virus

KLAUS HABERER† AND JACK MANIHOFF*

Department of Microbiology, University of Rochester Medical Center, Rochester, New York 14642

The replication of mycoplasma virus MVL3 in *Acholeplasma laidlawii* K2 host cells was studied by analysis of infected-cell lysates using sedimentation in sucrose gradients and DNA-DNA hybridization. Viral DNA replication was found to involve intermediates sedimenting faster than free viral DNA, which is a linear, double-stranded molecule of about 26×10^6 daltons. After the shutdown of cellular DNA synthesis, viral DNA synthesis continued for many hours. The fate of cellular and parental viral DNAs was examined.

Mycoplasma virus MVL3 is a nonlytic cytotoxic virus infecting *Acholeplasma laidlawii* cells. The structure and infectious cycle of this virus have been described by Haberer et al. (5). The virus particle has a polyhedral head, about 60 nm in diameter, with a short tail, about 10 nm in diameter and 20 nm long. The viral chromosome is a linear, double-stranded DNA molecule of about 26×10^6 daltons. Although MVL3 virus-infected cells are not viable, the infectious cycle is different from that of the lytic bacteriophages. Infected *A. laidlawii* cells begin to release progeny virus at about 90 min after infection and continue to release virus for 10 to 15 h. Intracellular progeny virus was demonstrated beginning 1 h after infection, by artificial lysis experiments (5). These data are in agreement with electron microscopic observations from other laboratories showing intracellular, virus-like particles in MVL3-infected cells (2, 4).

We report here the first studies on the biochemistry of MVL3 viral replication. These studies examined the fate of parental viral DNA, the synthesis of progeny viral DNA, and the fate of cellular DNA in infected cells.

MATERIALS AND METHODS

Medium and buffers. Tryptose broth prepared as described previously (5) was used for cultivating cells.

TES buffer (0.01 M Tris-hydrochloride, 0.001 M EDTA, 0.1 M NaCl, pH 8.0) was used to wash cells and as buffer for sucrose gradients. SSC (0.15 M NaCl, 0.015 M sodium citrate) was made up as a 10× stock and diluted as indicated below for hybridization experiments.

Cells and virus. The *A. laidlawii* K2 clone, the host cell used in these studies, and mycoplasma virus MVL3 were described previously (5). K2 cells were

assayed as colony-forming units, and MVL3 was assayed as PFU on lawns of K2 (5).

To prepare radioisotopically labeled virus, we added ^{32}P or [*methyl*- ^3H]thymidine to a culture at the start of infection to a final concentration of 5 mCi/liter. Virus was purified by polyethylene glycol precipitation and centrifugation to equilibrium in CsCl gradients as described previously (5). The specific activity of such purified MVL3 preparations was about 3×10^7 PFU/cpm for ^{32}P -labeled virus and 1×10^6 PFU/cpm for ^3H -labeled virus.

[^{14}C]thymidine (50 to 60 mCi/mmol), [*methyl*- ^3H]thymidine (40 to 60 Ci/mmol), and carrier-free ^{32}P were obtained from Amersham Corp. (Arlington Heights, Ill.).

Phenol extraction of DNA. Fractions from 5 to 20% sucrose gradients were made denser than phenol by the addition of half the fraction volume of 60% sucrose in TES buffer and then phenol extracted. TES buffer-saturated phenol (Mallinckrodt Inc., St. Louis, Mo.) was adjusted to a pH of about 7.5 by the addition of 5 M NaOH.

Whole-virus preparations were transferred into TES buffer by dialysis and then phenol extracted.

Infection and lysis of cells. Unlabeled or pre-labeled cells used for infection studies were prepared by diluting an overnight culture 1:100 into tryptose medium and incubating overnight (16 h) at 37°C. For prelabeling, the medium contained 1 μCi of [^{14}C]thymidine per ml. All overnight cultures were diluted 1:5 into fresh tryptose medium 2 h before the virus was added and incubated at 37°C. The overnight label was removed by washing the cells once in TES buffer before adding a fivefold volume of fresh tryptose broth. Cells were infected with MVL3 at various multiplicities of infection (MOI). To label newly synthesized DNA, we added [*methyl*- ^3H]thymidine to a concentration of 20 $\mu\text{Ci}/\text{ml}$. After designated times, infected cells were collected by centrifugation (10 min at 4,300 $\times g$) and washed once with TES buffer before lysis.

In general, lysis with sodium dodecyl sulfate (SDS) could not be used for the analysis of intracellular viral DNA intermediates because, using sedimentation velocity analysis, we have found that SDS lysis causes

† On leave from Abteilung Mikrobiologie, Universität Ulm, D7900 Ulm, Germany.

the fragmentation of the cellular chromosome into pieces similar in size to the viral DNA (data not shown). To prevent such fragmentation, we used a hot Triton X-100-SDS lysis procedure for all experiments involving labeled nascent DNA or prelabeled cellular DNA. The cell suspension was lysed by the addition of 0.1% (final concentration) Triton X-100. The lysate was then heated to 65°C for 20 min, 1% (final concentration) SDS was added, and the heating was continued for 15 min.

When the only label in an experiment was in parental viral DNA (hence, there could be no interference from cell DNA), cells were lysed by the addition of SDS to a 1% final concentration. This lysis method was also used in preparations for DNA-DNA hybridization studies where newly synthesized DNA had been labeled, because these experiments allowed the differentiation between viral and cellular DNA.

For examination of the fast-sedimenting viral complex, infected cells were treated for 10 min with 10 μ g of novobiocin per ml and lysed by the addition of Brij 58 (Atlas Chemicals, Wilmington, Del.) to a final concentration of 0.6%. This treatment has been shown empirically to keep *A. laidlawii* folded chromosomes condensed (J. A. Nowak, Ph.D. thesis, University of Rochester, Rochester, N.Y., 1978). The molecular basis of this unusual novobiocin effect on the *A. laidlawii* folded chromosome is not understood and is being investigated further in this laboratory.

Centrifugation. In all sedimentation studies, 5 to 20% linear sucrose gradients in TES buffer were formed over a shelf of 60% sucrose in TES buffer. These were either 34-ml gradients over a 3-ml shelf (long gradients) or 3.8-ml gradients over a 0.4-ml shelf (short gradients). To analyze cells lysed with Triton X-100-SDS or SDS, 1 ml of lysate was layered on a long gradient, and centrifugation was for 15 h at 14,000 rpm in an SW27 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 15°C. Brij lysates were either sedimented in long gradients in the SW27 rotor or on short gradients in the SW56.1 rotor. Centrifugation was for 45 min at 16,000 rpm at 5°C in both cases. Unlysed cells were sedimented in short gradients in the SW56.1 rotor under the same conditions.

Gradients were collected from the top with a gradient fractionator (ISCO, Lincoln, Nebr.). Long gradients were divided into 1.2-ml fractions; short gradients were divided into 0.2-ml fractions.

Radioactivity assay. Gradient fractions were precipitated by the addition of an excess of cold 5% trichloroacetic acid. Precipitates were collected on 0.45- μ m membrane filters (type HA; Millipore Corp., Bedford, Mass.), dried, and counted in a Beckman LS230 scintillation counter, using 8% concentrate I (Eastman Kodak Co., Rochester, N.Y.) in toluene as the scintillation fluid.

Hybridization studies. K2 cells infected with MVL3 at an MOI of 10 were labeled with exogenous [³H]thymidine (as described above) for 1-h periods at various times during infection. The cells were harvested and lysed with SDS. The lysate was sedimented through long sucrose gradients (as described above) and collected in 1.2-ml fractions. For each fraction, 0.2 ml was trichloroacetic acid precipitated and counted. From every peak fraction containing more than 10³

cpm/ml, 0.4 ml was phenol extracted and dialyzed overnight against 3 $\times$ SSC. The fractions were then heat denatured and, while still hot, added to scintillation vials. Each vial contained a filter which had been loaded with heat-denatured, phenol-extracted viral DNA as described by Denhardt (3). The vials were further incubated at 65°C for 16 h. The DNA samples were removed from the vials by suction. The filters were washed several times in 3 $\times$ SSC and then dried in the vials in an evacuated oven at 65°C for 6 h. Scintillation fluid was added, and the vials were counted in a liquid scintillation counter.

RESULTS

Parental virus. To determine the fate of parental virus, cultures of *A. laidlawii* K2 were infected with ³²P-labeled MVL3 virus at an MOI of 10. At various times of infection, samples were washed to remove unadsorbed virus and lysed with SDS. The intracellular parental viral DNA sedimented at the same position as phenol-extracted, ³H-labeled marker virus DNA, if the cells were lysed 30 min after adsorption (Fig. 1a). After another 1 or 2 h of incubation at 37°C, some of the parental label sedimented faster than free viral DNA, forming a shoulder on the heavy side of the peak (Fig. 1b and c). No distinct second peak could be resolved. The total intracellular parental viral DNA recovered in these experiments remained unchanged (data in legend to Fig. 1).

Incorporation of parental label into fast-sedimenting complex. To investigate the possible incorporation of parental viral DNA into a fast-sedimenting replicative complex, K2 cells were infected at various concentrations of ³²P-labeled virus. After a 50-min incubation at 37°C, 10 μ g of novobiocin per ml was added to stabilize the folded chromosome of the cells as described above. The culture was incubated for another 10 min at 37°C. The cells were then harvested by centrifugation and washed with TES buffer. A sample was filtered, and the filtrate was assayed for PFU. No significant number of PFU was found, indicating that the washed, infected-cell suspension contained few unadsorbed MVL3. The remainder of the washed, infected-cell suspension was lysed with Brij 58. The lysates were sedimented through 5 to 20% sucrose gradients, and three peaks of radioactivity were observed (Fig. 2): (i) a peak near the top of the gradient, which is the position of free viral DNA under these centrifugation conditions; (ii) a peak in the middle of the gradient, sedimenting at the position of whole virus (this was the only peak of infectious material [assayed as PFU] in the gradient [data not shown] and must represent either mature intracellular virus or adsorbed virus which had not injected its DNA); (iii) fast-sedimenting material was found on the 60% su-

FIG. 1. Velocity sedimentation analysis of intracellular parental viral DNA. Cells (15 ml) were infected with ^{32}P -labeled MVL3 (MOI of 10), and 5-ml samples were lysed with SDS at (a) 30 min, (b) 1 h, and (c) 2 h after infection. The total acid-precipitable ^{32}P counts per minute in these gradients were 1,500, 1,700, and 1,550, respectively. Symbols: $\circ$, ^{32}P -labeled DNA; $\bullet$, ^3H -labeled MVL3 marker DNA. Sedimentation is from left to right.

crose shelf. The relative amounts of parental viral DNA found in intracellular free viral DNA and virus particles increase with increasing MOI.

From the experiments shown in Fig. 2 and others at various virus concentrations, the distribution of parental viral DNA in intracellular intermediates was determined as a function of virus concentration. These data are plotted in Fig. 3. The amount of parental viral DNA in fast-sedimenting material reached a saturation value. The number of parental DNA molecules in this complex which sedimented with cellular folded chromosomes was calculated by multiplying the saturation value of about 220 cpm in the fast-sedimenting complex by the specific activity of the parental viral DNA, which was 5×10^7 PFU/cpm. This gives a saturation value of 1.1×10^{10} viral DNA molecules in the fast-sedimenting peak. Since these samples typically contained nearly 10^9 colony-forming units, there must be about 10 viral DNA molecules per infected cell in fast-sedimenting material.

Precursors of progeny virus DNA. Experiments were carried out to examine the roles of parental viral DNA and cellular DNA as precursors for progeny viral DNA synthesis. Cellular DNA was prelabeled by overnight growth in [^{14}C]thymidine. The cells were infected with [^3H]thymidine-labeled MVL3 (MOI of 10). Culture samples were removed at 30 min and 4, 8, and 16 h after infection and centrifuged in 5 to 20% sucrose gradients. These conditions sedimented intact cells to the bottom 60% sucrose shelf and free extracellular virus to about the middle of the gradient.

No significant amount of prelabeled cellular DNA was found in the virus peak at any time during infection (Fig. 4). At this MOI, parental viral DNA was incorporated into progeny virus, but not at a very high rate and only late in infection. At 8 h, about 10%, and at 16 h, about 20% of the parental viral DNA recovered were found in free extracellular virus (Fig. 4).

To measure the incorporation of exogenous precursors into viral DNA, we added [^3H]thymidine to a culture at the start of infection (MOI of 10). Culture samples were removed at 4, 8, and 16 h after infection and analyzed as described for Fig. 4. At all times, ^3H -labeled DNA was found in both cell and virus peaks (Fig. 5). The amount of label in the extracellular virus peak increased during infection. At 16 h, 30% of the total incorporated label was still in the cell peak, although the virus infectious cycle was essentially complete at this time.

Fate of cellular DNA in infected cells. As noted above, no significant amount of prelabeled cellular DNA could be found in progeny virus particles at any time during infection. To investigate changes in the cell chromosome during infection, cells were labeled overnight with [^{14}C]thymidine and infected with MVL3 (MOI of 10), and after 1 h for adsorption, [^3H]thymidine was added. A parallel control culture received no virus. After 1 h of incubation, cells were harvested, washed, lysed by the hot Triton X-100-SDS procedure, and analyzed by sedimentation velocity. Under these centrifugation conditions, chromosomes from control cells (^{14}C and ^3H labeled) sedimented to the shelf (Fig.

FIG. 2. Velocity sedimentation analysis of parental viral DNA in fast-sedimenting material in infected cells. Cells (5 ml) were infected with ^{32}P -labeled virus as described in the legend to Fig. 1, treated with novobiocin, and lysed with Brij 58. Sedimentation is from left to right. Infection was at an MOI of about (a) 5, (b) 20, or (c) 200. The position of whole, ^3H -labeled MVL3 virus in each gradient is indicated by an arrow.

6a). However, in infected cells the ^{14}C -labeled cell chromosomes sedimented slower and appeared more disperse (Fig. 6b). The ^3H -labeled

nascent DNA was in a peak sedimenting with the cell DNA and in a slower-sedimenting peak of viral DNA (Fig. 6b).

Less acid-precipitable, ^{14}C -labeled cell DNA (data in legend to Fig. 6) was recovered in the infected-cell sample (Fig. 6b) relative to the uninfected-cell sample (Fig. 6a). However, the absolute amounts of radioactivity in the two cell lysates cannot be compared, because infected and uninfected cells are not recovered by the harvesting and washing procedures with the same efficiency (data not shown). Hence, from this experiment, no statement can be made about possible virus-induced conversion of cellular DNA into acid-soluble material.

The slower sedimentation of cellular DNA in infected cells, relative to uninfected cells (Fig. 6), might be due to either (i) virus-induced degradation of the cell DNA in vivo or (ii) greater sensitivity of cell DNA from infected cells to degradation during lysis and sedimentation analysis. Therefore, the sedimentation of DNA was studied in cells treated with novobiocin and lysed with Brij 58, a procedure which stabilizes *A. laidlawii* folded chromosomes, as described above. A short (45-min) centrifugation can be used to sediment folded chromosomes to the bottom of sucrose gradients and partially unfolded chromosomes to the middle of gradients (Fig. 7a). Cell DNA fragments, which were observed in hot Triton X-100-SDS lysates in the middle of the gradient after a long (15-h) cen-

FIG. 3. Distribution of intracellular parental viral DNA in free viral DNA (Δ), virus particles ($\circ$), and fast-sedimenting material ($\bullet$) as a function of virus concentration. At each value, 5 ml of cells (about 2×10^8 colony-forming units per ml) was infected with ^{32}P -labeled MVL3, and the intracellular intermediates were analyzed by sedimentation in sucrose gradients. Figure 2 shows the data from three such experiments. With reference to subsequent experiments, 2×10^9 PFU/ml corresponds to an MOI of about 10.

FIG. 4. Velocity sedimentation analysis of cells and virus particles in infected cultures. Cells were labeled overnight with [^{14}C]thymidine ($\circ$) and infected with [^3H]thymidine ($\bullet$)-labeled MVL3 (MOI of 10). At (a) 30 min, (b) 4 h, (c) 8 h, and (d) 16 h after infection, 0.5-ml samples were removed and centrifuged under conditions such that cells were found on the shelf, and free virus was found at about the middle of the gradient (arrows). Sedimentation was from left to right. The total acid-precipitable counts per minute recovered from each gradient were as follows: (a) 3,720 ^3H cpm and 2,050 ^{14}C cpm; (b) 3,220 ^3H cpm and 1,880 ^{14}C cpm; (c) 2,540 ^3H cpm and 1,820 ^{14}C cpm; and (d) 2,860 ^3H cpm and 1,435 ^{14}C cpm.

trifugation at a comparable centrifugal force (Fig. 6b), would be expected to remain at the top of a gradient after the short centrifugation of the novobiocin-Brij lysis procedure.

Cells labeled overnight with [^{14}C]thymidine were infected with MVL3 and incubated in medium containing [^3H]thymidine during 1 h of infection. After the novobiocin-Brij procedure, lysates of infected cells (Fig. 7b) had the same sedimentation pattern as lysates of uninfected cells (Fig. 7a). Hence, no change in cell chromosome organization was observed during 1 h of infection. It should be noted that the amount of ^{14}C -labeled DNA recovered in infected cells was less than that recovered in lysates of uninfected cells (data in legend to Fig. 7) as discussed above for Fig. 6. By comparison with the amount of

^{14}C -labeled DNA from uninfected cells, about 90% of the cell DNA from samples of infected cells was recovered. However, these lysates of infected cells contained only about one-third the amount of nascent DNA (labeled with [^3H]thy-

FIG. 5. Velocity sedimentation analysis of incorporation of exogenous thymidine in cells and virus particles in infected cultures. Cells were infected with MVL3 (MOI of 10), and [^3H]thymidine was added. At (a) 4 h, (b) 8 h, and (c) 16 h, 0.5-ml samples were removed and analyzed as described in the legend to Fig. 4. Cells were found on the shelf, and virus was found at the position shown by the arrow.

midine for 1 h) as did uninfected cells. Therefore, MVL3 infection significantly reduced total DNA synthesis during 1 h of infection.

MVL3-infected-cell samples were also incubated with [^3H]thymidine for 1 h during h 2 and 3 of infection. The amount of ^{14}C -labeled DNA recovered from cells after 1, 2, or 3 h of infection (data in legend to Fig. 7) was essentially the same. Therefore, during these infection times little measurable cell DNA was degraded to an acid-soluble form.

FIG. 6. Velocity sedimentation analysis of DNA from uninfected- and MVL3-infected-cell lysates. Cells were labeled overnight with [^{14}C]thymidine ($\circ$); (a) 5 ml was not infected, and (b) 5 ml was infected (MOI of 10). Uninfected cells were labeled with [^3H]thymidine for 1 h ($\bullet$). For the infected cells, after 1 h of adsorption, [^3H]thymidine ($\bullet$) was added for 1 h. Cells were harvested, washed, and lysed with hot Triton X-100-SDS. Centrifugation was for 15 h at $26,000 \times g$. Sedimentation was from left to right. The total acid-precipitable counts per minute recovered from these gradients were as follows: (a) 42,800 ^{14}C cpm and 280,750 ^3H cpm; (b) 20,500 ^{14}C cpm and 26,100 ^3H cpm.

During h 2 (Fig. 7c) and h 3 (Fig. 7d) after infection, there was a progressive loss of ^{14}C -labeled DNA from the fast-sedimenting peak and appearance of ^{14}C -labeled DNA in slow-sedimenting material at the top of the gradients. Since there was no loss of acid-insoluble ^{14}C -labeled DNA during these times, this change in sedimentation must have reflected a progressive unfolding and fragmentation of the cell chromosome. At these infection times, nascent (^3H -labeled) DNA was found at the positions of cell DNA (at the top and bottom of the gradients) and at a position in the gradient (fraction 11) where intact virus (which was not solubilized by Brij lysis) sedimented.

Viral replicative intermediates. Experiments to label nascent DNA during early infection (e.g., Fig. 6b) and analyze such infected-cell lysates by sedimentation velocity in sucrose gradients did not allow resolution of viral replicative intermediates because the gradient region between viral chromosomes and the bottom of the gradient contained nascent cell DNA. However, there was an indication that this gradient region did contain viral DNA replicative intermediates. After 2 to 3 h of infection and 1 h of incubation in medium containing [^3H]thymidine, sedimentation analysis showed label incorporation mainly into free viral DNA with a shoulder of faster-sedimenting DNA (data not shown). This shoulder was not found after a 1-h chase. Therefore, to identify nascent cell and viral DNA in infected-cell lysates after sedimentation in sucrose gradients, we performed DNA-DNA hybridization experiments.

Due to the large excess of unlabeled cell DNA, it was not possible to measure relatively small amounts of [^3H]thymidine incorporation into cell DNA by hybridization to filter-bound cellular DNA (data not shown).

Hybridization to filter-bound viral DNA was standardized with [^3H]thymidine-labeled MVL3. After phenol extraction of labeled MVL3, the recovery of ^3H counts hybridized to membrane filter-bound viral DNA was about 50% of the input ^3H counts. Similar yields for this procedure have been described by Kelly and Thomas for T7 DNA (7).

K2 cells were infected with MVL3 at an MOI of 10. At several times during infection, samples were removed and labeled with [^3H]thymidine for 1 h. Cells were harvested, washed, and lysed with SDS. The lysates were sedimented through 5 to 20% sucrose gradients. Of each 1.2-ml fraction, 0.2 ml was removed, acid precipitated, and assayed for radioactivity. Samples (0.4 ml each) of peak fractions containing more than 10^3 cpm/ml were phenol extracted and used in hybridization assays against filter-bound viral DNA.

FIG. 7. Velocity sedimentation of infected-cell lysates by folded chromosome protocol. The labeling and infection procedure was as described in the legend to Fig. 6. Cell DNA was prelabeled with [^{14}C]thymidine ($\circ$), and nascent DNA was labeled with [^3H]thymidine ($\bullet$). (a) Uninfected cells. Infected cells were labeled with [^3H]thymidine at (b) 0 to 1 h, (c) 1 to 2 h, and (d) 2 to 3 h after infection. The cells were treated with novobiocin before harvesting; lysis was with Brij 58, and centrifugation was for 45 min at $24,000 \times g$. Sedimentation was from left to right. The total acid-precipitable counts per minute recovered from each gradient were as follows: (a) 14,200 ^{14}C cpm and 46,960 ^3H cpm; (b) 12,720 ^{14}C cpm and 15,710 ^3H cpm; (c) 12,010 ^{14}C cpm and 15,650 ^3H cpm; (d) 11,820 ^{14}C cpm and 15,580 ^3H cpm.

Since in this procedure the maximum hybridization was about 50% of the input DNA, the maximum ratio (bound counts per minute to total counts per minute) was 1. Hence, the ratio of hybridizable counts per minute to total acid-precipitable counts per minute was approximately the fraction of ^3H -labeled DNA that was viral. In uninfected control cells (Fig. 8a) with these lysis conditions, cell DNA sedimented as a broad peak (fractions 5 through 18). Less than 5% of the total ^3H counts per minute remained on filters containing bound viral DNA. Throughout infection, most nascent DNA continued to sediment in fractions 5 through 18. During h 1 and 2 after infection (Fig. 8b and c), there was a progressive increase in the percentage of nascent DNA in fractions 5 through 18 which hybridized to viral DNA. By 2 to 3 h after infection (Fig. 8d), essentially all nascent DNA was viral. The

amount of nascent viral DNA made 7 to 8 h after infection was similar to that made after 2 to 3 h (cf. Fig. 8d and e).

During the infection times examined (Fig. 8b through e), nascent viral DNA appeared to sediment at the position of free viral DNA, plus one or two faster-sedimenting peaks. Experiments are in progress to determine whether these faster peaks are distinct viral DNA replicative intermediates.

DISCUSSION

In these experiments, we have examined some aspects of DNA structure and replication in *A. laidlawii* cells infected by MVL3. The roles of parental viral DNA, cellular DNA, and exogenous nucleosides as precursors for progeny viral DNA were investigated.

By a gentle lysis procedure (empirically found

FIG. 8. Velocity sedimentation and DNA-DNA hybridization analysis of DNA from infected-cell lysates. K2 cells were labeled with [^3H]thymidine for 1 h at various times after infection: (a) uninfected control cells; infected cells with label added at (b) 0 h, (c) 1 h, (d) 2 h, or (e) 7 h of infection. Cells (5 ml for each sample) were harvested, washed, and lysed with SDS. The lysates were sedimented through 5 to 20% sucrose gradients. Of each 1.2-ml fraction, 0.2 ml was removed, acid precipitated, and assayed for radioactivity (●). Samples (0.4 ml each) of peak fractions were phenol extracted and hybridized to membrane filters loaded with denatured MVL3 DNA. The filters were washed in $3\times$ SSC and assayed for radioactivity (○). The position of marker viral DNA in parallel gradients is indicated by arrows.

to stabilize the cell folded chromosome as described above) on MVL3-infected cells, the intracellular parental viral DNA was separated by sedimentation in three distinct forms: fast-sedimenting material, virus particles, and free viral DNA (Fig. 2). The distribution of parental viral DNA in these three forms was a function of virus concentration (Fig. 3), but the amount in fast-sedimenting material saturated at about 10 parental viral DNA molecules per cell. The appearance of labeled parental viral DNA in progeny virus particles was most pronounced late in infection. The fact that most of the labeled parental DNA was recovered during infection as acid-precipitable material (Fig. 1) indicated that parental DNA went into progeny DNA molecules as either large fragments or full-size molecules, rather than being degraded to acid-soluble material and reutilized.

Labeled cell DNA was not recovered in progeny MVL3 virus particles (Fig. 4). However, during MVL3 infection, the *A. laidlawii* folded chromosome was progressively unfolded and

possibly fragmented, without the release of a significant amount of acid-soluble material (Fig. 4 and 7).

When infected cells were lysed (with hot Triton X-100-SDS) and sedimented in sucrose gradients, the change in infected cellular DNA structure caused a major fraction of the cell DNA to sediment between the positions of free viral DNA and intact cell chromosomes. Hence, cell DNA contaminated those fractions where viral DNA replicative intermediates were expected to be found. Using DNA-DNA hybridization (Fig. 8), we demonstrated that viral DNA replicative intermediates sedimented faster than free viral DNA.

There was a significant decrease in DNA synthesis in *A. laidlawii* cells during h 1 after MVL3 infection (Fig. 7 and 8). During h 1, about 40% of the nascent DNA was viral (Fig. 8b). In h 2 of infection, 60 to 80% of the nascent DNA was viral (Fig. 8c), and by h 3, essentially all nascent DNA was viral (Fig. 8d).

There are a number of apparent analogies

between mycoplasma virus MVL3 and coliphage T7. Both viruses have similar morphologies and chromosome sizes (5, 8, 12).

The sedimentation profiles of nascent MVL3 DNA (Fig. 8) resemble those reported for coliphage T7 replicative intermediates (6, 7). The material sedimenting faster than free T7 DNA has been identified as concatemers by electron microscopy (7). Electron microscopic studies of MVL3 replicative intermediates were not possible, since (as discussed above) viral DNA could not be separated from cellular DNA. A fast-sedimenting complex of parental viral DNA, as shown for MVL3 (Fig. 2), has been reported for other bacteriophages, e.g., T7 (1, 9, 11).

There are, however, several important differences between the infectious cycles of T7 and MVL3. In T7 infection, host cell DNA is broken down and reused (10). No breakdown and reutilization of *A. laidlawii* cellular DNA has been found in MVL3-infected cells. T7 is a lytic bacteriophage, with progeny virus release in a burst at about 25 min after infection at 37°C (8, 12). MVL3 is a nonlytic cytocidal virus, with a gradual virus release continuing for 10 to 15 h after infection (5). From the results presented here, it is clear that the long period of MVL3 virus production reflects the continued production of virus by the biosynthetic apparatus of the infected cell, and not merely passive release of virus particles synthesized during the first hours of infection.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service research grant AI 10605 from the National Institute of Allergy and Infectious Diseases (to J.M.) and by research fellowship

HA 1012/2,3 from Deutsche Forschungsgemeinschaft (to K.H.).

The technical assistance of David Gerling is acknowledged.

LITERATURE CITED

- Center, M. S. 1973. Bacteriophage T7 DNA synthesis in isolated DNA-membrane complexes. *J. Virol.* 12:847-854.
- Cole, R. M. 1979. Mycoplasma and spiroplasma viruses: ultrastructure, p. 385-410. In M. F. Barile and S. Razin (ed.), *The mycoplasmas*, vol. 1. Academic Press Inc., New York.
- Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.
- Gourlay, R. N., S. G. Wyld, and A. P. Bland. 1979. Demonstration by electron microscopy of intracellular virus in *Acholeplasma laidlawii* infected with either MVL3 or a similar but serologically distinct virus (BN 1 virus). *J. Gen. Virol.* 42:315-322.
- Haberer, K., G. Klotz, J. Maniloff, and A. K. Kleinschmidt. 1979. Structural and biological properties of mycoplasma virus MVL3: an unusual virus-procaryote interaction. *J. Virol.* 32:268-275.
- Hausmann, R. 1968. Sedimentation analysis of phage T7-directed DNA synthesized in the presence of a dominant conditional lethal gene. *Biochem. Biophys. Res. Commun.* 31:609-615.
- Kelly, T. J., Jr., and C. A. Thomas, Jr. 1969. An intermediate in the replication of bacteriophage T7 DNA molecules. *J. Mol. Biol.* 44:459-475.
- McCorquodale, D. J. 1975. The T-odd bacteriophages. *Crit. Rev. Microbiol.* 4:101-159.
- Pacumbaba, R. P., and M. S. Center. 1973. Association of replicating bacteriophage T7 DNA with bacterial membranes. *J. Virol.* 12:855-861.
- Putnam, T. W., D. Miller, L. Palm, and E. A. Evans, Jr. 1952. Biochemical studies of virus reproduction. X. Precursors of bacteriophage T7. *J. Biol. Chem.* 199:177-191.
- Serwer, P. 1974. Fast sedimenting bacteriophage T7 DNA from T7 infected *Escherichia coli*. *J. Mol. Biol.* 68:319-345.
- Studier, W. F. 1972. Bacteriophage T7. *Science* 176:367-376.