

Intracellular Replication of Mycoplasma virus MVL51

ALAN LISS¹ AND JACK MANILOFF

Departments of Microbiology and of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received for publication 25 November 1973

The intracellular replication of MVL51, a group L1 mycoplasma virus, was investigated. The single-stranded parental DNA was found to enter the cell and become converted to double-stranded DNA. This replicated to yield additional double-stranded DNA molecules. The parental viral DNA was found to leave the replication complex and become associated with large molecular weight DNA not involved with viral replication. Progeny viral DNA formed from the double-stranded DNA and an intracellular accumulation of virus chromosome size DNA was observed. The interpretation of this data and a suggested model for the viral replication are discussed and compared to viral DNA replication models for other single-stranded DNA viruses.

The replication of the single-stranded DNA of the *Escherichia coli* bacteriophage ϕ X174 has been extensively studied (10). As the single-stranded DNA penetrates the host cell, it is converted into a double-stranded replicative form (RF). Subsequently, progeny RF copies are made by using the parental RF for the template. The DNA molecule containing the infecting viral DNA is defined as the parental RF, and any other RF molecules are termed progeny RF. Single-stranded progeny viral DNA is synthesized on the RF molecules. The progeny DNA is assembled into mature ϕ X174 particles, which are then released from the infected cell.

The same general model seems to apply to the replication of the single-stranded DNA filamentous bacteriophage (8) and to a single-stranded DNA parvovirus (1).

The group L1 mycoplasma viruses are bullet-shaped (14 by 70 nm) DNA-containing particles (7). The DNA of MVL51, a group L1 isolate, has been shown to be a covalently closed single-stranded circle with a molecular weight of 2×10^6 (5), similar to that of the ϕ X174 and filamentous bacteriophage chromosomes. MVL51-infected cells continue to grow during virus production; virus assembly and maturation have been shown to occur at the cell membrane as the viral particles are extruded (6).

The studies described here suggest that MVL51 DNA replication follows a similar pat-

tern to that of other single-stranded DNA viruses.

MATERIALS AND METHODS

Cells and media. The mycoplasma strain used for virus propagation and as the indicator host was *Acholeplasma laidlawii* BN1-Nal^r (designated JA1). The isolation of this strain was described by Liss and Maniloff (3). Tryptose medium (containing 1% glucose and 1% serum) was used for propagation of cells and viruses. Eagle basal medium (MEM) was used for radioisotope incorporation studies as described by Quinlan et al. (9).

Virus. The virus for these studies was the group L1 mycoplasma virus MVL51. The isolation and characterization of this virus have been previously described (6). Radioactively labeled MVL51 was obtained by growing the virus on JA1 cells in MEM, containing either [³²P]phosphoric acid (carrier-free; New England Nuclear Corp., Boston, Mass.) or [*methyl*-³H]thymidine (10 Ci/mmol specific activity; New England Nuclear Corp.), and harvesting virus as described by Liss and Maniloff (4).

Viruses were assayed as PFU on JA1 lawns growing on tryptose agar plates (3).

Preparation of intracellular viral DNA. Extraction of viral DNA from infected cells followed a modification of the Hirt procedure (2) for preferential extraction of DNA of molecular weight less than about 5×10^6 . Unlabeled mycoplasma cells were infected with labeled virus; at the time of infection a different labeled DNA precursor was added to the growth medium to allow DNA products synthesized after infection to be followed. At specific time intervals, a sample was removed and the cells were centrifuged into a pellet (12,000 $\times g$ for 10 min). The pellet was washed in 0.1 M Tris-hydrochloride buffer (pH 8.0), containing 0.01 M EDTA, and suspended in 1 ml of buffer containing 1% sodium dodecyl sulfate (SDS).

¹ Present address: Department of Biology, York University, Downsview, Ontario M3J 1P3, Canada.

After 30 min at room temperature (about 20 C) the solution was adjusted to 1 M NaCl with 5 M NaCl. The solution was mixed slowly and stored overnight at 4 C. It was then centrifuged at low speed to remove the SDS, protein, and large molecular weight ($> 5 \times 10^6$) DNA. The resulting supernatant was either left untreated or was phenol extracted as previously described (4). This final DNA solution was then dialyzed overnight in 0.01 M Tris-hydrochloride buffer (pH 8.0), containing 10^{-4} M EDTA, and then analyzed by CsCl band sedimentation.

Band sedimentation analysis. Band sedimentation analysis in CsCl density gradients was used as described by Vinograd et al. (12). In these experiments, 2 ml of the DNA solution was slowly layered on top of a CsCl solution containing 0.43 g of CsCl per ml ($\rho = 1.5 \text{ g/cm}^3$; Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), in a buffer of 0.01 M Tris-hydrochloride- 10^{-4} M EDTA (pH 7.2). The tube was then filled to the top with light oil (about 4 ml) and centrifuged for 3 h at 40,000 rpm in a Beckman 42.1 fixed-angle rotor. The polycarbonate centrifuge tubes were previously boiled for 10 min in 0.1 M EDTA to remove any DNase activity associated with them. Samples were taken from the bottom of the tube and were treated overnight with 1 N KOH at room temperature. After this, 100 μg of bovine serum albumin (Calbiochem, Los Angeles, Calif.) per ml was added to each sample as a carrier, and the DNA was precipitated by the addition of an equal volume of cold 10% trichloroacetic acid. Each precipitate was collected on a membrane filter (0.22 μm pore size, HAWP; Millipore Corp.) and washed with distilled water and 95% ethanol. The filters were added directly to a solution of 9 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) plus 1 ml of distilled water. The radioactivity was measured on a Beckman LS-230 liquid scintillation counter. Quenching was corrected by the channels-ratio method.

Analysis of whole cells. For examination of the isotope label in whole cells, a culture was centrifuged as above. The cell pellet was resuspended in 1 ml of 0.1 M Tris-hydrochloride (pH 8.0) and collected on membrane filters (HAWP, Millipore Corp.), and the DNA was precipitated with trichloroacetic acid, washed, and counted as described above.

Equilibrium sedimentation analysis. Alkaline CsCl isopycnic sedimentation was carried out as described by Liss and Maniloff (5): 4.0 g of CsCl and 2.9 g of sample, adjusted to pH 12.5, were spun for 48 h at 17 C in an SW50.1 rotor at 27,000 rpm. The density of each gradient fraction was determined by using a Bausch and Lomb precision refractometer.

RESULTS AND DISCUSSION

The fate of the DNA of infecting MVL51 virus was investigated. Preliminary experiments were carried out to see whether or not the DNA from the infecting particles (the parental DNA) remained in the cell during virus production or was released in the progeny viruses. For these studies, ^{32}P - or ^3H -labeled MVL51 was added to unlabeled JA1 cells at a multiplicity of infection

(MOI) of about 1; the specific activity of the infecting viral DNA was about 2×10^{-6} counts/min per PFU. The mixture was incubated at 37 C and, at various times, samples were removed and the cells were harvested and assayed for the presence of the labeled parental DNA. Progeny viruses are not collected by this procedure, hence the cell pellet counts per minute measure only intracellular viral DNA. As shown in Table 1, there is no apparent migration of parental DNA out of the infected cells, although by the end of this experiment each infected cell should have released over 50 PFU (Fig. 1).

It should be noted that the latent and rise periods for MVL51 growth in cells grown in MEM are not the same as the times reported for infected cells growing in tryptose broth (6). Figure 1 shows a one-step growth curve of MVL51 in JA1 cells growing in MEM. There is a latent period of about 25 min during which no virus release was observed; the reported latent

TABLE 1. Fate of parental virus DNA

Time after injection (min)	Counts per min per cell pellet ($\times 10^{-4}$) ^a
0	2.40
30	2.24
60	2.45
120	2.55
240	2.45

^a No counts were found in the supernatant, except for the counts per minute equivalent to the number of free unadsorbed parental virus (about 200 counts per min per ml or 7×10^7 PFU/ml).

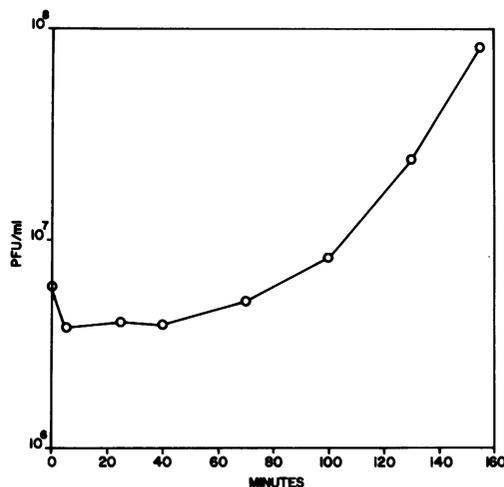


FIG. 1. One-step growth curve of MVL51 virus in JA1 cells in MEM. The protocol for these studies with mycoplasma viruses has been described by Liss and Maniloff (6).

period in tryptose medium is 10 min. At about 25 min (Fig. 1), gradual virus release began; the rate was slower than that reported for virus growth in cells in tryptose medium.

As previously noted (6), infected cells continue to grow and divide during virus growth and release. Therefore, for an analysis of the intracellular fate of the parental viral DNA and the synthesis of progeny viral DNA, it was necessary that these experiments include the Hirt extraction procedure (2) to specifically exclude the high molecular weight cell DNA and allow the examination of the lower molecular weight viral DNA products. Table 2 shows the distribution of label when unlabeled cells were infected by ³²P-labeled MVL51 and [methyl-³H]thymidine was added to label newly synthesized DNA. The gradient analysis of the soluble low molecular weight DNA (Hirt supernatant) at each time is shown in Fig. 2.

Twenty-five minutes after start of the infection, parental DNA is found in two peaks (fractions 6 and 9 of Fig. 2A). Material banding in fraction 9 will be called "P-DNA" and material in fraction 6 will be designated "R-DNA". As the infection continued, more parental virus DNA appeared as R-DNA (Fig. 2B and C), and an increasing amount of newly synthesized DNA was seen to band as both P- and R-DNA (Fig. 2D and E). By 100 min after infection (Fig. 2D) parental DNA was found to band again in the P-DNA region, but not as a sharp peak. In alkaline CsCl isopycnic sedimentation analysis of this late parental P-DNA, no sharp bands were found, but instead the DNA was distributed throughout the gradient (data not shown). By 160 min (Fig. 2F), no parental DNA could be

isolated any longer by the Hirt extraction technique.

Figure 3 represents a parallel experiment where the Hirt-extracted DNA was treated with phenol prior to CsCl band sedimentation analy-

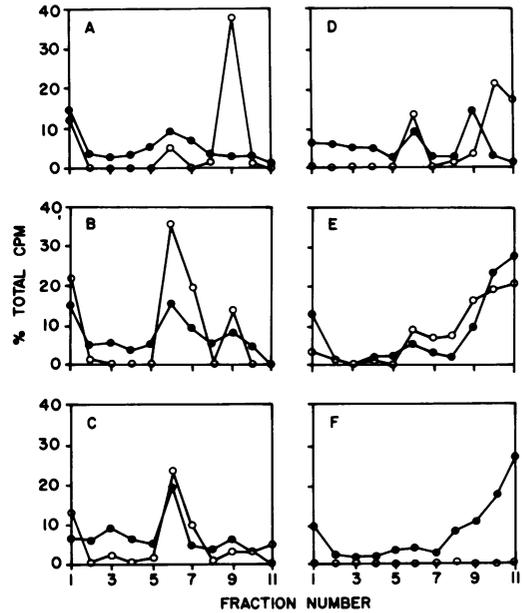


FIG. 2. CsCl band sedimentation analysis of Hirt supernatant samples described in Table 2. ³²P-labeled parental DNA is shown by O, and ³H-labeled newly synthesized DNA by ●. The ordinate shows the percentage of the total gradient counts in each fraction. A, Sample taken 25 min after infection; B, 40 min; C, 70 min; D, 100 min; E, 130 min; F, 160 min. Sedimentation is from left to right.

TABLE 2. Intracellular labeled DNA after MVL51 infection^a

Sample	Time after infection (min)					
	25	40	70	100	130	160
Hirt supernatant: ^b						
³² P	2,800	2,290	1,295	250	200	0
³ H	150	720	1,400	1,510	2,200	3,350
Hirt pellet: ^c						
³² P	0	500	2,490	2,600	2,555	2,610
³ H	800	1,540	2,500	3,190	4,400	4,800
Total gradient: ^d						
³² P	2,578	2,312	1,300	240	215	0
³ H	142	750	1,387	1,492	2,230	3,300

^a ³²P-labeled MVL51 was added (at an MOI of 0.1) with [methyl-³H]thymidine to unlabeled JA1 cells, and samples were taken at various times and assayed as described above.

^b Soluble low molecular weight DNA, after high salt precipitation, used for gradient analysis.

^c High molecular weight DNA precipitated by high salt treatment.

^d Sum of counts per minute from all gradient fractions shown in Fig. 2; should be equal to Hirt supernatant counts per minute.

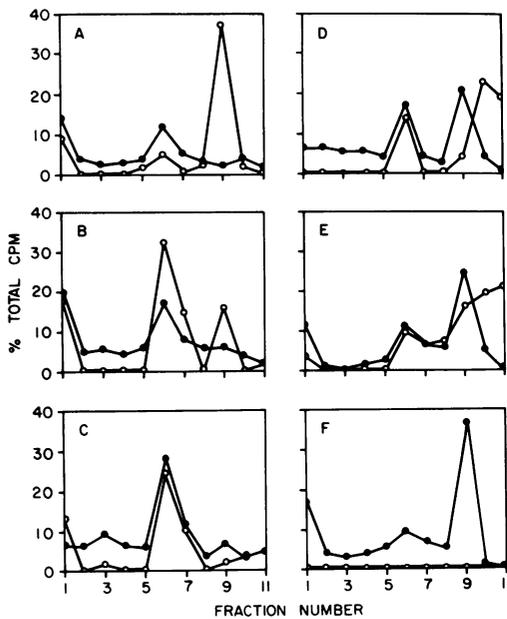


FIG. 3. *CsCl* band sedimentation analysis as described in Fig. 2, except that each Hirt supernatant was phenol extracted prior to the gradient analysis. The distribution and amount of counts per minute was almost identical to the values for the experiment shown in Table 2. ^{32}P -labeled parental DNA is shown by O, and ^3H -labeled progeny DNA by ●. The sample times are as shown in Fig. 2.

sis. The sedimentation pattern at each time is similar to that in Fig. 2, except for times late in the infection. The change is that where previously newly synthesized DNA was seen spread from fraction 7 to the bottom of the gradient (Fig. 2E and F), after phenol treatment this material banded like P-DNA (Fig. 3E and F). The fate of the parental virus DNA remained the same as before.

Further sedimentation analysis of the intracellular products involved in the replication of MVL51 DNA was done by using a pulse-chase experiment. This consisted of infecting unlabeled cells with unlabeled virus. Twenty-five minutes after the start of the infection, [^{32}P]-phosphate was added to label newly synthesized DNA. Fifteen minutes later, excess unlabeled phosphate (as phosphate buffer) was added to dilute out the radioisotopic label and stop the incorporation of the labeled precursor. At the start of the chase, 40 min after infection (Fig. 4A), the labeled material bands as R-DNA. As the infection proceeded (Fig. 4B through F), the labeled DNA gradually shifted from the R-DNA peak to the P-DNA fraction. Note that these data are of phenol-treated DNA, as described in

Fig. 3. This experiment indicates that R-DNA is the precursor of P-DNA.

Alkaline CsCl isopycnic sedimentation analysis of all P- and R-DNA fractions have been done and are identical to the one set of data shown in Fig. 5. In all cases, R-DNA appears to sediment as double-stranded DNA and P-DNA as single-stranded DNA. Combining this strandedness information with the data from Fig. 2, 3, and 4, the MVL51 replication experiments can be interpreted as follows: single-stranded DNA of the infecting virus enters the cell. After a short time, the parental virus DNA is converted into a double-stranded DNA replicative form which is then copied to produce more replicative form DNA. The parental label eventually leaves the viral DNA replication complex and is found in high molecular weight DNA material; one possible trivial explanation is that the parental DNA could be degraded and used as precursors in cellular DNA synthesis. Single-stranded DNA, synthesized on the remaining double-stranded replicative forms,

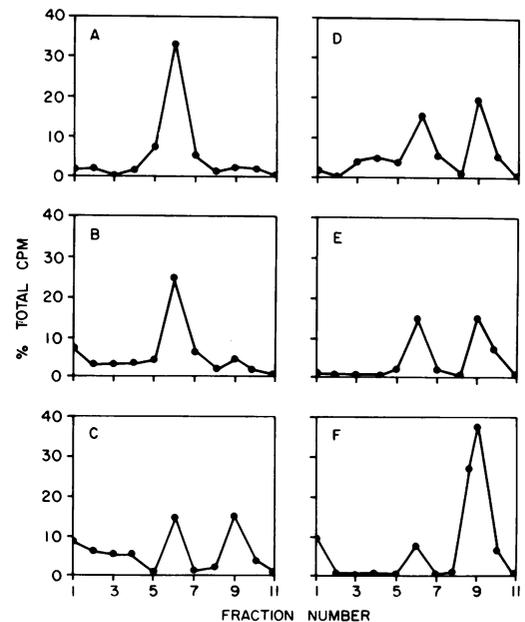


FIG. 4. *CsCl* band sedimentation analysis of pulse-chase experiment of cells infected with unlabeled MVL51 at an MOI to 0.1. The pulse labeling was begun 25 min after infection and the chase was begun 15 min later. Samples were taken and analyzed as described in Fig. 3 (i.e., Hirt extraction, phenol treatment, and band sedimentation). The samples were taken at: A, 40 min after infection (i.e., at the start of the chase); B, 50 min after infection; C, 60 min; D, 90 min; E, 105 min; F, 120 min. Sedimentation is from left to right.

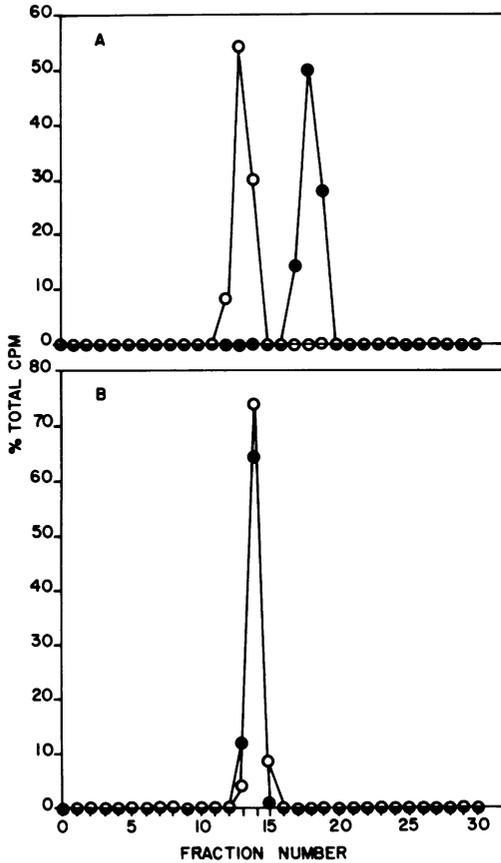


FIG. 5. A, Alkaline $CsCl$ isopycnic sedimentation of fraction 6 (R-DNA) from the $CsCl$ gradient of Fig. 2C. The sample analyzed here was obtained by pooling fraction 6 from six separate gradients. B, Alkaline $CsCl$ isopycnic gradient of fraction 9 (P-DNA) from the $CsCl$ gradient of Fig. 2A and 2F. The sample used was obtained by pooling fraction 9 from six separate gradients. In both graphs ○ represents the ^{32}P -labeled parental DNA and ● represents the 3H -labeled progeny DNA. The peak fraction in the bottom graph is at a density of 1.763 g/cm^3 ; the top graph has a peak at a similar density and one at 1.748 g/cm^3 . Sedimentation is from left to right.

forms a pool of viral chromosome size single-stranded DNA molecules, which are the progeny virus chromosomes.

Parenthetically, these data also show that, of the two possible complementary single DNA strands, only one is synthesized and is found in the viruses. If this were not so, the single-stranded viral chromosomes (P-DNA) in Fig. 5 would have both the peaks found in the double-stranded replicative forms (R-DNA).

None of these results were observed when the experiments were carried out by using uninfected cells (data not shown).

The replication model described above, which explains these data on MVL51 replication, is similar to the replication systems of other single-stranded bacteriophage (8, 10) and animal viruses (1). The fact that these diverse single-stranded DNA viruses (all with DNA molecular weights about 2×10^6) follow the same basic replication scheme must reflect the selective advantage of this replication mechanism; a slight modification to the general model has been found for two parvoviruses (the adeno-associated satellite virus and densovirus) which contain separately encapsidated complementary single DNA strands (reviewed by Tinsley and Longworth, ref. 11). It should be noted that these single-stranded DNA viruses utilize different modes of attachment to their hosts: bacteriophage like $\phi X174$ attach to *E. coli* cell wall receptors, filamentous bacteriophage to male *E. coli* F-pili, parvoviruses to animal cell membranes, and mycoplasma viruses to mycoplasma cell membranes. Another difference among these viruses involves virus release: bacteriophage like $\phi X174$ release their progeny by lysis of the infected cells, whereas the filamentous bacteriophage and the group L1 mycoplasma viruses are extruded from infected cells which continue to grow and divide.

The mechanism of mycoplasma virus MVL51 maturation remains to be elucidated. We have shown here that there is an intracellular accumulation of virus chromosome size DNA in infected cells. This must be considered with previous observations (6) that mature virus particles do not accumulate within infected cells, but that maturation occurs at the time of virus extrusion from the cell.

ACKNOWLEDGMENTS

We wish to thank Art McEvoy (New England Nuclear Corp.) for help with the formulation of the Aqualol mixture, David Gerling for his technical assistance, and J. R. Christensen and F. E. Young for many helpful discussions.

This investigation was supported in part by Public Health Service grant AI-10605 from the National Institute of Allergy and Infectious Diseases. A.L. was a Public Health Service trainee (grant GM-00592) and J.M. is the recipient of a Public Health Service Research Career Development Award (grant AI-17480).

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