Proc. Natl. Acad. Sci. USA
Vol. 73, No. 5, pp. 1489–1493, May 1976
Biochemistry

Replication of mycoplasmavirus MVL51: Attachment of MVL51 parental DNA to host cell membrane*

(mycoplasmas/virus replication/membrane-associated viral DNA)

JYOTIRMOY DAS AND JACK MANILOFF†

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

Communicated by Lewis Thomas, February 12, 1976

ABSTRACT The replication of the single-stranded circular DNA of MVL51 mycoplasmavirus has been studied with respect to the roles of free and membrane-associated viral DNA intermediates. Replication involves the formation of parental replicative intermediate (RF) molecules on, at most, two to three membrane sites per cell, symmetric RF replication at the membrane, and apparent asymmetric RF replication in the cytoplasm leading to single-stranded progeny chromosomes.

Mycoplasmaviruses have recently been isolated and morphologically and serologically classified into three groups (1, 2). MVL51, a group L1 virus, has been studied most extensively. These are bullet-shaped particles (about 14 × 70 nm) containing single-stranded circular DNA of molecular weight 2×10^6 (3). The intracellular replication of MVL51 has been shown (4) to involve the conversion of the parental DNA to double-stranded replicative intermediates which can exist in two forms: RFI (covalently closed circles) and RFII (relaxed or nicked circles). Single-stranded progeny chromosomes (SSI) are produced from RFII and become associated with viral proteins to form a DNA-protein complex designated SSII (5, ‡). SSII is a short-lived intermediate and can be identified only during viral infection of cells in poor nutrient medium‡, such as Eagle's basal medium, where the viral latent period is 60 min. In richer tryptose medium used in the studies reported here, there is only a 10 min latent period and the SSII component cannot be resolved by sucrose gradient analysis (4). Virus assembly and extrusion through the cell membrane occur without lysing or killing the cells (6).

The results presented here demonstrate that, depending upon the multiplicity of infection, a certain percentage of the intracellular parental viral DNA sediments with the host cell membrane and that this DNA exists mostly in RFI and RFII forms. These data also suggest that RF replication takes place at membrane sites and that the maximum number of sites available per cell is two to three. There is no transfer of parental label to progeny viruses at the multiplicities of infection used in this study.

MATERIALS AND METHODS

Organisms, Media, and Buffer. *Acholeplasma laidlawii* JA1 was used for virus propagation and as indicator host and MVL51, a group L1 mycoplasmavirus, was used in this study (6, 7). JA1 cells were assayed as colony forming units (CFU) on tryptose agar plates, and viruses were assayed as plaque forming units (PFU) on JA1 lawns.

Tryptose broth and agar plates were used for cultivating cells and viruses (3, 6, 7). The Tris–EDTA–NaCl buffer (pH 8.0) was 0.01 M Tris·HCl, 0.001 M EDTA, 0.1 M NaCl, and Tris–EDTA–NaCl–NaCN buffer was Tris–EDTA–NaCl buffer containing 10 mM NaCN. The high salt buffer used was Tris–EDTA–NaCl buffer containing 1 M NaCl.

Preparation of Labeled Virus. Radioactively labeled MVL51 virions and viral DNA were prepared as described (4).

Infection and Lysis. A logarithmic phase culture of JA1 was infected with ^{32}P-labeled MVL51 (specific activity 1.25×10^6 PFU/cpm), and after 5 min unadsorbed viruses were removed by centrifugation. The washed infected cell pellet was resuspended in warmed tryptose broth, and at specified intervals the infected culture was pulse labeled for varying periods with [^{3}H]thymidine. Labeling was terminated by adding an equal volume of ice-cold Tris–EDTA–NaCl–NaCN buffer and centrifuging at 12,000 rpm for 10 min at 5°. The cell pellet was washed twice with Tris–EDTA–NaCl buffer and finally resuspended in the same buffer. The cell suspension was gently lysed by freezing in liquid nitrogen and thawing. Three cycles of freeze-thawing were found to lyse the cell suspension completely, as judged by the loss of turbidity.

Sucrose Gradient Centrifugation. A subcellular, fast sedimenting complex was isolated as follows: a 4 ml CsCl shelf, containing 1.2 g/ml of CsCl in 40% sucrose, was placed at the bottom of the centrifuge tube, over which 34 ml of 5–20% (wt/vol) linear sucrose gradient buffered by Tris–EDTA–1 M NaCl was formed. One milliliter of infected cell lysate was layered on top of the gradient and centrifuged for 3 hr at 24,000 rpm in a Beckman L3-50 centrifuge in an SW27 swinging bucket rotor.

Deproteinized fast and free sedimenting complexes were analyzed by sedimentation through a 38 ml 5–20% (wt/vol) linear high salt sucrose gradient as described (4). One milliliter of sample was layered on top of the gradient and centrifuged for 16 hr at 5° at 24,000 rpm in an SW27 rotor.

For all these gradient analyses, 1.2-ml fractions were collected from the top of the gradient using an ISCO density gradient fractionator.

Alkaline Equilibrium Gradient Centrifugation. For equilibrium sedimentation in alkaline CsCl, 2.3 g of CsCl was added to 1.2 ml of sample, 0.4 ml of 0.1 M EDTA, and 0.4 ml of 1 M NaOH. The remainder of the tube was filled with mineral oil. The solution was centrifuged for 36 hr at 20° at 35,000 rpm in an SW 50.1 rotor. Two-drop fractions were collected from the bottom of the tube.

Deproteinization. For further analysis of the DNA in the fast and free sedimenting fractions, samples were dialyzed against Tris–EDTA–NaCl buffer to remove sucrose and deproteinized by either of the two following methods: (*i*) The sample was mixed with 0.5 volume of 3 M $NaClO_4$ and 1 volume of $CHCl_3$:octanol (9:1) and vigorously shaken. The aqueous

Abbreviations: RFI and RFII, replicative intermediate forms of DNA; CFU, colony-forming units; PFU, plaque-forming units; SSI, single-stranded progeny chromosomes.
* This is paper II of a series. Paper I is ref. 4.
† To whom reprint requests should be addressed.
‡ J. Das and J. Maniloff, submitted for publication.

layer was dialyzed against Tris–EDTA buffer to remove perchlorate. (*ii*) To each sample sodium dodecyl sulfate was added to a 1% final concentration; the mixture was incubated for 1 hr at 37° and then extracted with Tris–EDTA saturated phenol. The DNA was concentrated by ethanol precipitation. The precipitate was resuspended in Tris–EDTA buffer.

Isolation of Infected Cell Membrane. Cell membranes from infected cells were isolated by osmotic lysis following the method of Razin *et al.* (8). Since mycoplasma have no membranous structure except surface cell membrane, there can be no contamination from other membranous material in these preparations.

Radioactive Assay. Macromolecular material in the gradient fractions was precipitated by adding an equal volume of ice-cold 10% trichloroacetic acid using bovine serum albumin as carrier. The precipitated samples were filtered, washed, dried, and assayed for radioactivity as described by Das and Maniloff (4).

RESULTS

Isolation of parental viral DNA attached to a host cell component

After infection the single-stranded DNA of mycoplasmavirus MVL51 is converted to double-stranded replicative forms, which serve as precursors for the synthesis of progeny single-stranded viral DNA (4). In order to follow parental DNA during infection, JA1 cells were infected with ^{32}P-labeled virus. Ten minutes after infection the cells were washed and gently lysed by freezing and thawing. The lysates were analyzed in high salt sucrose gradients over a CsCl shelf as described in *Materials and Methods*.

Most (50–60%) of the parental label was associated with a fast sedimenting complex, which appeared as a white band on top of the CsCl shelf (Fig. 1a). The remaining 40–50% of the input parental label was recovered on top of the gradient. To ensure that the parental label in the fast sedimenting complex was not nonspecifically trapped by the cellular DNA-membrane complex [^{3}H]thymidine-labeled MVL51 single-stranded DNA was mixed with infected cells before lysis. The marker DNA sedimented independently of the fast sedimenting material (Fig. 1).

The nature of the host cell material associated with the fast sedimenting complex was examined by treating lysates with Brij 58 (a nonionic detergent), Sarcosyl NL30 (an ionic detergent), or Pronase. Treatment with 1% Brij 58 for 15 min at 37° released about 80% of the parental label from the fast sedimenting complex (Fig. 1b), while 3% Sarcosyl NL30 released all the parental label (Fig. 1c). At least 95% of the parental viral DNA can also be detached from the complex by treatment with either 200 μg/ml of Pronase for 1 hr at 37° or chloroform–methanol extraction (data not shown, gradients look like Fig. 1c). Because of its sensitivity to detergents, proteolytic enzymes, and lipid solvents, the fast sedimenting complex will be referred to as viral DNA-membrane complex.

Number of membrane sites per cell for parental DNA

To determine how many sites are available for MVL51 infection, the saturation of the fast sedimenting complex with infecting viral DNA was measured as described by Knippers and Sinsheimer (9). Logarithmic phase cultures of JA1 were infected with ^{32}P-labeled MVL51 at multiplicities of infection ranging from 1 to 30. After 15 min, unadsorbed viruses were removed by washing. The infected cells were lysed by three cycles of freezing and thawing and analyzed in 5–20% high salt sucrose

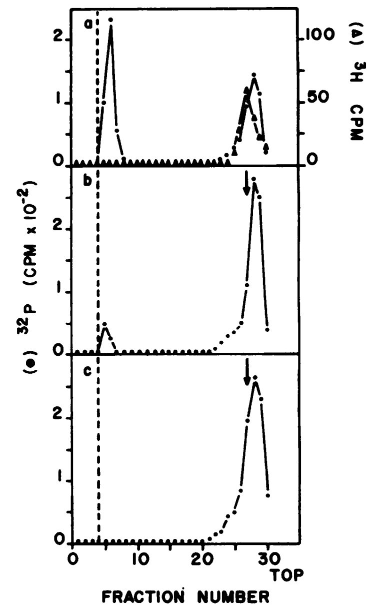

FIG. 1. Attachment of MVL51 parental DNA to the host cell membrane. JA1 cells were infected with ^{32}P-labeled MVL51 (●). Ten minutes after infection cells were harvested and washed, mixed with [^{3}H]thymidine-labeled MVL51 marker DNA, and lysed. The lysate was divided as follows: (a) untreated control; (b) treated with 1% Brij 58; (c) treated with 3% Sarcosyl NL30. Each portion was sedimented through a 5–20% high salt sucrose gradient on a CsCl shelf. The arrows in (b) and (c) indicate the position of the ^{3}H-labeled marked DNA (Δ). The dotted line indicates the position of the CsCl-sucrose boundary.

gradients with a CsCl shelf, as in Fig. 1a, to determine the amount of parental viral DNA in fast and free sedimenting material. The parental label in the fast sedimenting complex reached a saturation value at about a multiplicity of infection of 20, while the ^{32}P in the free sedimenting material increased with increasing multiplicities of infection (Fig. 2). The number of bound parental DNA molecules per cell was calculated by multiplying the saturation value of about 1400 cpm of membrane associated parental viral DNA per 7×10^{8} cells by the specific activity of the parental viral DNA, which was 1.25×10^{6} PFU/cpm. This gives a value of 2.5, from which it is concluded that there are two to three MVL51 DNA molecules bound per exponentially growing cell.

Fate of parental DNA

In order to identify the form of the intracellular parental viral DNA during infection, we analyzed virus infected cell lysates by velocity sedimentation in 5–20% high salt sucrose gradients (Table 1). The total amount of parental label remained constant during the 60 min time studies and, since this corresponds to the period of maximal viral production (Fig. 3), there must be essentially no transfer of parental DNA to progeny viral DNA. The fraction of parental DNA in the RFI intermediate (Table 1) decreases during the infection, with corresponding increases in RFII and SS. Throughout the infection, most (70–80%) of the parental viral DNA remains in double-stranded replicative intermediates (RFI and RFII). The peak labeled SS appears not to be native single-stranded viral chromosomes, since it sediments more slowly than marker SSI DNA.

An experiment to follow the parental [^{32}P]DNA during infection (Fig. 3) showed that whereas the number of PFU in the cell-free supernatant increased normally, there was no detectable increase in cell-free acid-insoluble ^{32}P, in agreement with the data in Table 1. During this experiment, at each sample

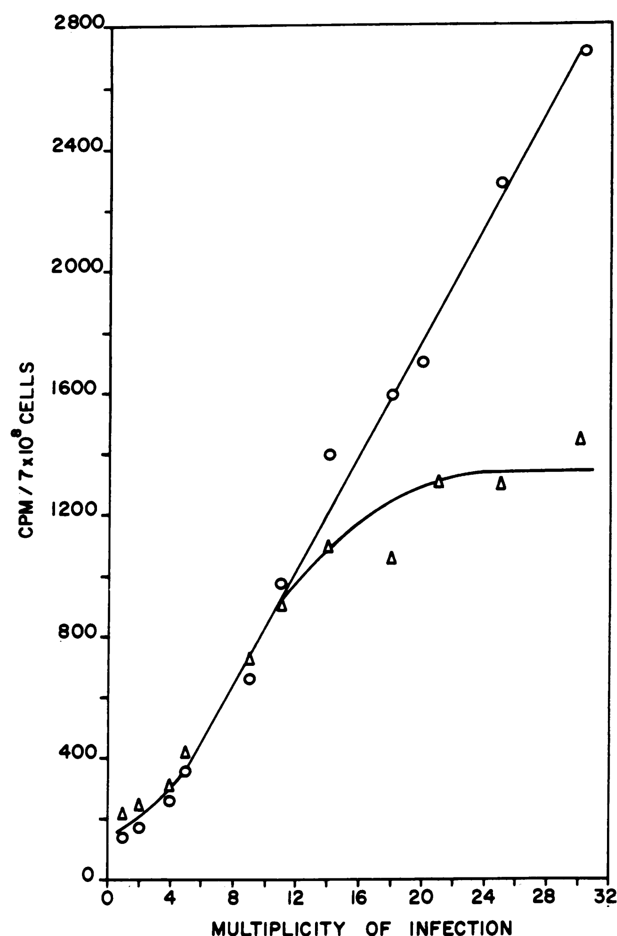

FIG. 2. Saturation of the fast sedimenting complex with intracellular viral DNA. At each multiplicity of infection, the amount of ^{32}P-labeled parental DNA was measured in the fast (Δ) and free (O) sedimenting material, as in Fig. 1.

time membranes from infected cells were isolated by osmotic shock by the method of Razin *et al.* (8), in order to follow the fate of the membrane-associated parental DNA and to confirm the identification of the fast sedimenting material as the cell membrane fraction. About 50% of the total intracellular parental label could be recovered in membranes isolated from the infected cells (Fig. 3). The amount of membrane-associated parental label did not change significantly during early infection; later in infection about 20% of membrane-associated viral DNA was released and was recovered in the cytoplasm.

Site of progeny viral DNA synthesis

The analysis of infected cell fast and free sedimenting components (as shown in Fig. 1) was repeated, except that at different times during infection [^{3}H]thymidine was added 5 min before harvesting. This enabled the determination of distribution of parental ^{32}P-labeled and nascent ^{3}H-labeled DNA (Fig. 4).

The amount of parental label in the fast sedimenting complex (i.e., membrane-associated) did not change significantly up to 30 min of infection (Fig. 4a, and b). However, by 60 min after infection (Fig. 4c) 10–15% of the parental label was released from the fast sedimenting complex. No further decrease of label from this component was observed during prolonged infection. The total amount of intracellular parental label did not decrease with time during infection, indicating no transfer of parental label to progeny viruses (in agreement with Table 1 and Fig.

Table 1. Distribution of intracellular parental label as a function of time*

Time after infection (min)	Amount of parental label in†		
	RFI	RFII	SS‡
10			
cpm	840	480	330
%	51	29	20
30			
cpm	500	675	430
%	30	42	28
60			
cpm	405	680	460
%	27	43	30

* Logarithmic phase culture of JA1 was infected with ^{32}P-labeled viruses at a multiplicity of infection of 5. At different times, 10-ml aliquots were removed and an equal volume of ice-cold Tris-EDTA-NaCl-NaCN buffer was added. The cells were washed, lysed by sodium dodecyl sulfate, and analyzed in 5–20% high salt sucrose gradients.

† The total label was: 1650 cpm at 10 min, 1605 cpm at 30 min, and 1545 cpm at 60 min.

‡ Material sedimenting more slowly than SSI but faster than RFI.

3). At 10 min of infection (Fig. 4a), 80% of the newly synthesized DNA was associated with the fast sedimenting complex. With increasing periods of infection, the amount of nascent DNA in the fast sedimenting complex decreased and, by 100 min after infection, 80% of the nascent DNA sedimented as free component.

To analyze which DNA components are present in the fast and free sedimenting material for each of the gradients in Fig. 4, we deproteinized and sedimented the fractions in high salt sucrose gradients as described in *Materials and Methods*.

Figure 5a–c show the virus specific DNA components from the fast sedimenting material. At 10 min after infection (Fig. 5a) most of the membrane-bound parental label was in RF

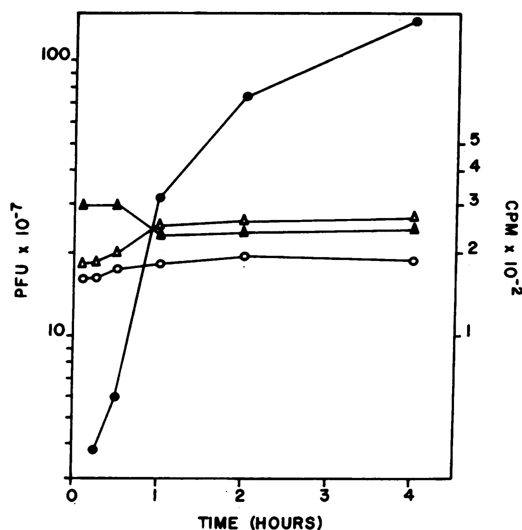

FIG. 3. Fate of parental virus DNA. A logarithmic phase culture of JA1 cells was infected with ^{32}P-labeled MVL51 of a multiplicity of infection of 5. At different times after infection aliquots were removed. The cells were pelleted, and cell-free supernatant was assayed for PFU (●) and radioactivity (O). The infected cell pellets were lysed by osmotic shock, and membrane and soluble fractions were isolated. Trichloroacetic acid-precipitable radioactivity in membrane (▲) and soluble (Δ) fractions was assayed.

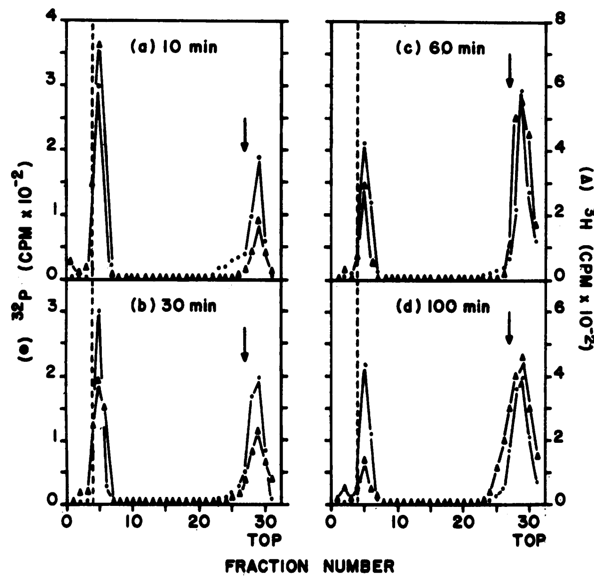

FIG. 4. Distribution of viral DNA in fast and free sedimenting components at different times during infection. A logarithmic phase culture of JA1 cells (about 6 to 7 × 10^8 CFU per ml) was infected with ^{32}P-labeled MVL51 (●) at a multiplicity of infection of 5, and unadsorbed viruses were removed. At different times samples were removed and labeled with 40 μCi/ml of [^{3}H]thymidine (▲) for 5 min. Labeling was terminated and cells were washed, lysed, and analyzed on a high salt sucrose gradient over a CsCl shelf. The dotted line indicates the position of the CsCl-sucrose boundary. The arrow indicates the position of added marker MVL51 DNA.

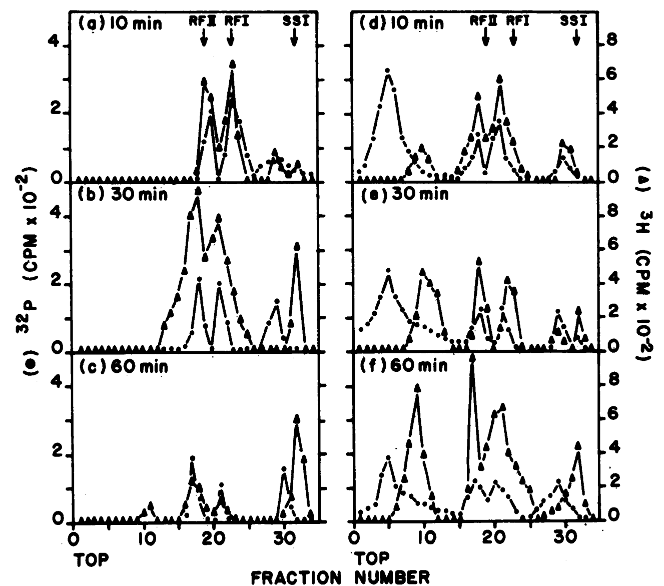

FIG. 5. Velocity sedimentation analysis in high salt sucrose gradients of deproteinized fast and free sedimenting fractions from the gradients shown in Fig. 4. (a, b, and c) Fast sedimenting complexes at 10, 30, and 60 min after infection, respectively; (d, e, and f) free sedimenting fractions at 10, 30, and 60 min, respectively. Infecting parental viral DNA was labeled with ^{32}P (●) and nascent DNA by a 5-min [^{3}H]thymidine pulse (▲). The SSI position was determined from a parallel gradient analysis of purified viral DNA, and the positions of RFI and RFII were calculated from their sedimentation rates relative to SSI, as given in Das and Maniloff (4).

components; about 20% of this parental label sedimented faster than RFI but more slowly than SSI. Most of the newly synthesized DNA at this time also sedimented as RF forms (Fig. 5a). The amount of parental label in the fast sedimenting material remained constant (Fig. 5b and c), as was seen in the experiment described in Fig. 3. Most of this parental label remained in RF forms, but about 30% sedimented in a new peak, slightly more slowly than SSI. At 30 min (Fig. 5b) nascent DNA was mainly in RFI and RFII, but a nascent SSI peak could also be seen. By 60 min after infection (Fig. 5c) most of the nascent fast sedimenting material was SSI. In view of the nonlytic nature of the viral infection, this SSI probably represents progeny virus maturation and extrusion.

The analysis of virus DNA components in the free sedimenting material is shown in Fig. 5d–f. At 10 min (Fig. 5d), most of the free sedimenting parental label was in a slowly sedimenting peak at about 5 S. The amount of free sedimenting parental label remained the same at 30 and 60 min after infection (Fig. 5e and f), but the amount of 5S parental material progressively decreased and there was an increase in parental label in a faster sedimenting peak. This faster material sedimented a bit more slowly than SSI, similar to that seen in fast sedimenting material (Fig. 5b and c). The remainder of the free sedimenting parental label (Fig. 5d–f) was in RF forms, and the amount remained constant through 60 min.

At 10 min (Fig. 5d), free sedimenting nascent label was in RFI, RFII, and a more slowly sedimenting 10S peak. The amount of nascent 10 S and RF synthesis increased with the infection time (Fig. 5e and f). At 60 min (Fig. 5f), increased nascent DNA was seen in SSI and as a fast sedimenting shoulder on the RFI peak.

The difference between nascent RFII in fast and free sedimenting fractions was investigated. RFII from fast and free sedimenting materials was isolated as described in Fig. 5 and then sedimented to equilibrium in alkaline CsCl gradients, which separates viral and complementary strands. As shown in Fig. 6, the pulse label was in both viral and complementary strands in RFII from the fast sedimenting complex, while that from free sedimenting material has pulse label only in the viral strand. Hence, membrane-associated nascent RFII is the product of symmetric replication, involving the synthesis of both viral and complementary strands, whereas free sedimenting nascent RFII is the product of asymmetric replication, with new DNA only in viral strand.

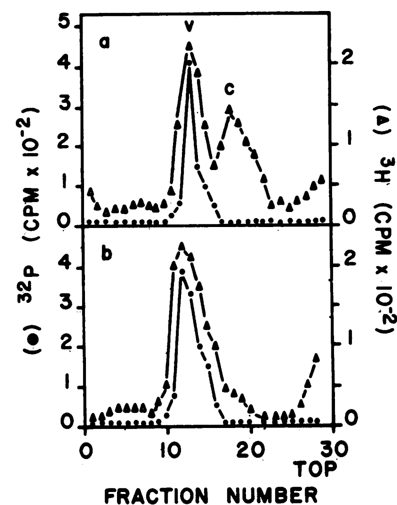

FIG. 6. Alkaline equilibrium CsCl sedimentation of RFII DNA from the fast and free sedimenting components from Fig. 5. The parental virus was labeled with ^{32}P (●), and nascent DNA was labeled with a 5-min [^{3}H]thymidine (▲) pulse. V and C indicate the positions of viral and complementary strands of the RF. (a) Membrane-bound RFII; (b) free sedimenting RFII.

DISCUSSION

The results presented here show that after infection, MVL51 DNA becomes associated with the host cell membrane. The binding involves protein, since proteolytic enzymes can release the viral DNA from the cell membrane.

Considerable evidence has accumulated to suggest that cell membranes play important roles in viral replication and morphogenesis (reviewed in ref. 10). The present study shows that for MVL51 infection the maximum number of membrane sites available is only two to three per cell and an apparent saturation of DNA attachment sites is reached at a multiplicity of infection of about 20. This saturation value is consistent with the estimate of Fraser and Fleischmann (11) that each cell has about 10 virus binding sites and suggests that only two to three of these sites lead to functional intracellular viral replication.

The following results represented here show that, in MVL51 infection, RF replication takes place at the cell membrane: (*i*) during the first 15 min of infection, when most nascent DNA is in RF forms, 80% of the newly synthesized DNA is associated with the fast sedimenting (membrane) complex, and (*ii*) equilibrium sedimentation in alkaline CsCl gradients showed nascent DNA in both viral and complementary strands of RFII from the fast sedimenting complex, indicating symmetrical replication of both strands of the double-stranded RF. At later times in infection, when progeny SSI synthesis predominates, free sedimenting nascent DNA is in RF and SSI. RFI has a fast sedimenting shoulder, which may be heavier replicating molecules. The fact that nascent DNA was found only in the viral strand of free sedimenting RFII material suggests that SSI synthesis may take place in the cytoplasm and may proceed by a rolling circle mechanism.

In addition to the viral replicative intermediates that have been previously observed, the gradients presented here (Fig. 5) show three other peaks whose identification is not known. First, gradients of both fast and free sedimenting material show a constant amount of the parental label sedimenting more slowly than viral marker DNA. This could be defective or partially degraded viral chromosomes. Second, in free sedimenting material, some of the parental label sediments at 5 S. This might represent degraded viral DNA, which can serve as precursors for later DNA replication, explaining the decrease in 5S parental label during infection. The small amount that might get into progeny viruses would not be detectable in these studies. Third, some nascent free sedimenting DNA appears at 10 S. Similar nascent trichloroacetic acid-precipitable DNA has been seen near the tops of gradients of uninfected and infected cells (4) and may be Okazaki fragments.

Although cell growth continues during MVL51 infection (6), there is little or no contamination of the gradients of viral DNA species presented here by cellular DNA. In the separation of fast and free sedimenting subcellular fractions (Fig. 4), cell DNA sediments with the fast sedimenting material. However, most of this cell DNA is unlabeled and not seen in this analysis. The lack of appreciable nascent DNA in fast sedimenting material late in infection, when most viral DNA replication is cytoplasmic, indicates that little nascent cell DNA is being seen. Any nascent cell DNA recovered from the gradient in Fig. 4 is sedimented to the bottom of the subsequent high salt gradient (Fig. 5) and does not contaminate the viral DNA peaks, since uninfected cells prepared in an identical way show no DNA in these gradients (data not presented here; similar data shown in ref. 4).

We thank Mr. David Gerling for his technical assistance. This investigation was supported in part by Grant AI-10605 from the U.S. Public Health Service, National Institute of Allergy and Infectious Diseases, and the University of Rochester Atomic Energy Project. J.M. is the recipient of a USPHS Research Career Development Award (Grant AI-17480). J.D. is on leave of absence from Calcutta University, Department of Physics, Calcutta, India.

1. Gourlay, R. N. (1974) *CRC Crit. Rev. Microbiol.* 3, 315–331.
2. Maniloff, J. & Liss, A. (1974) in *Virus, Evolution, and Cancer*, eds. Kurstak, E. & Maramorosch, K. (Academic Press, New York), pp. 583–604.
3. Liss, A. & Maniloff, J. (1973) *Biochem. Biophys. Res. Commun.* 51, 214–218.
4. Das, J. & Maniloff, J. (1975) *Biochem. Biophys. Res. Commun.* 66, 599–605.
5. Maniloff, J. & Das, J. (1975) in *DNA Synthesis and Its Regulation*, eds. Goulian, M., Hanawalt, P. & Fox, C. F. (W. A. Benjamin, Inc., Reading, Mass.), pp. 445–450.
6. Liss, A. & Maniloff, J. (1973) *Virology* 55, 118–126.
7. Liss, A. & Maniloff, J. (1971) *Science* 173, 725–727.
8. Razin, S., Morowitz, H. J. & Terry, T. M. (1965) *Proc. Natl. Acad. Sci. USA* 54, 219–225.
9. Knippers, R. & Sinsheimer, R. L. (1968) *J. Mol. Biol.* 34, 17–29.
10. Siegel, P. J. & Schaechter, M. (1973) *Annu. Rev. Microbiol.* 27, 261–282.
11. Fraser, D. & Fleischmann, C. (1974) *J. Virol.* 13, 1067–1074.