REPLICATION OF MYCOPLASMAVIRUS MVL51: I. REPLICATIVE INTERMEDIATES

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SUMMARY

The intracellular replication of the single stranded DNA of the non-lytic bullet-shaped Group L1 mycoplasmavirus, MVL51, has been shown to involve three virus specific DNAs: RFI, RFII and SS. The relative sedimentation rates and ethidium bromide CsCl gradient analysis show that RFI is covalently closed circular double stranded DNA and RFII is a nicked form of RFI. SS is circular single stranded progeny viral DNA. RFI and RFII serve as precursors for the synthesis of progeny SS.

INTRODUCTION

Three morphologically and serologically distinct groups of viruses which can infect mycoplasmas, the smallest free living organisms, have recently been isolated and characterized (1,2). Of these groups, Group L1 viruses are naked bullet-shaped particles (about 15 nm by 80 nm) having helical capsid symmetry and containing single stranded covalently closed circular DNA of molecular weight 2×10^6 daltons (3). Viral infection has been shown to be non-lytic: infected cells continue to grow and extrude progeny viruses (4). This paper reports the isolation, characterization and role of the DNA intermediates involved in the replication of MVL51 (a Group L1 mycoplasmavirus).

MATERIALS AND METHODS

Cells and viruses, medium and buffer:

Acholeplasma laidlawii JA1 was used for virus propagation and as an indicator host for all experiments. MVL51, a Group L1 mycoplasmavirus, was originally isolated from a spontaneous plaque on an uninfected JA1 lawn (5). Cells and viruses were cultivated in tryptose broth or on tryptose agar plates, as described previously (3,4,5).

The Tris-EDTA-NaCl buffer (pH 8.0) used was 0.01M Tris, 0.001M EDTA,

0.1M NaCl and Tris-EDTA-NaCl-NaCN buffer was Tris-EDTA-NaCl buffer containing 10mM NaCN. The high salt buffer used was Tris-EDTA-NaCl buffer containing 1M NaCl.

Preparation of virus infected cell lysates:

To label viral DNA, 40 µCi/ml [^{3}H] deoxythymidine was added to the infected cells at different times during infection. After specified periods labelling was terminated by adding an equal volume of ice cold Tris-EDTA-NaCl-NaCN buffer, and centrifuging at 10,000 g for 10 min at 5°C in a Beckman JA20 rotor. The cell pellet was washed twice in Tris-EDTA-NaCl buffer and resuspended in 1 ml of the same buffer. Sarkosyl NL97 (Geigy Chemicals, Ardsley, New York) was added to a final concentration of 0.4 percent and mixed slowly to avoid DNA degradation. The suspension was incubated at room temperature for 10 min and then slowly pipetted onto the top of a 5-20 percent high salt sucrose gradient made in a nitrocellulose tube for a Beckman SW27 swinging bucket rotor. Centrifugation was carried out for 15-17 hrs at 5°C at 22,000 rpm in a Beckman L3-50 ultracentrifuge. Fractions of 1.2 ml were collected from the top of the gradient. It has been shown that high molecular weight cell DNA sediments rapidly to form a pellet in a high salt sucrose gradient, leaving low molecular weight viral DNA species to be resolved in the gradient (6).

Equilibrium sedimentation:

For equilibrium sedimentation, 2.77 g CsCl and 0.4 mg ethidium bromide were added to 3 ml of DNA solution. Centrifugation was for 24 hours at 40,000 rpm in a Beckman SW50.1 rotor at 20°C. Fractions were collected by puncturing the bottom of the centrifuge tube.

Preparation of marker DNA:

MVL51 was grown on JA1 lawns on tryptose agar plates containing 700 µCi per plate of ^{32}P-carrierfree orthophosphate. Viruses were washed off the plate with 0.1M phosphate buffer (pH 7.4) and the wash filtered through a Millipore filter (pore size 0.20 µm). The filtrate was concentrated using collodion bags (Schleicher & Schuell, Keene, New Hampshire) and extracted twice with phenol saturated with water. The aqueous phase was dialysed against Tris-EDTA-NaCl buffer. This DNA has been shown to be single stranded and circular (3). ^{32}P-labelled viral DNA or ^{32}P-labelled viruses were used as markers in all gradient analysis.

Radioactive assay:

Gradient fractions were trichloroacetic acid (TCA) precipitated, filtered, washed with 5 percent TCA and distilled water. The dried filter papers were counted in a Beckman LS-230 liquid scintillation counter using toluene-Omnifluor (New England Nuclear, Boston, Mass.) as scintillation fluid.

RESULTS AND DISCUSSION

Isolation of replicative intermediates:

JA1 cells infected with MVL51 at a multiplicity of infection of 20 were labelled with [^{3}H]-deoxythymidine for 30 minutes at different times after infection. At the end of the labelling period cells were

gently lysed and analyzed in 5-20 percent sucrose gradients containing 1M NaCl as described in Materials and Methods. The results of the velocity sedimentation of such crude lysates are shown in Fig. 1. Three virus specific DNA components (labelled RFI, RFII, and SS) were observed (Fig. 1b). The fastest sedimenting component SS cosediments with the ^{32}P-labelled MVL51 DNA used as marker. This is consistent with the fact that at high ionic strength the most rapidly sedimenting component should be single stranded DNA (7). Components sedimenting at rates 0.71 and 0.52 times that of the SS DNA are designated DNA replicative forms RFI and RFII by analogy with the terminology used for single stranded DNA bacteriophages. The distribution of labelled thymidine when an uninfected culture was treated and analyzed identically is shown in Fig. 1a. This

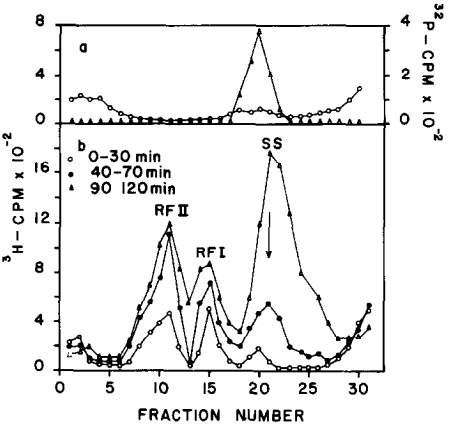

Fig. 1: Velocity sedimentation of infected cells lysed at different times during infection. JA1 cells were grown to 5 x 10^8 cells/ml and MVL51 was added to a multiplicity of infection of 20. After 5 min at 37°, the cells were spun down and resuspended in pre-warmed tryptose broth. At 0, 40 and 90 min after resuspension 20 ml aliquots were removed and 40 µCi/ml of [^{3}H]-deoxythymidine added. Labelling was continued for 30 min and terminated by adding equal volume of ice cold Tris-EDTA-NaCl-NaCN buffer. The infected cells were then washed, mixed with ^{32}P-labelled marker DNA, and lysed and sedimented for 15 hrs as described in Materials and Methods. The direction of sedimentation is from left to right. (a) uninfected JAL cells labelled for 30 min with [^{3}H]-deoxythymidine (O); (Δ) marker MVL51 DNA. (b) infected cells labelled for 30 min at 0, 40, and 90 min after absorption.

shows that there is no contamination of cellular DNA in the regions of virus specific DNA in the gradients.

Between 0 to 30 min after infection most of the incorporated label was found in RFI and RFII forms of DNA. The amount of SS DNA increased dramatically with the progress of infection. The amount of RF synthesized between 40-70 min after infection was not significantly different from the amount made between 90-120 min. The shoulder on the SS peak appearing late during infection might be due to mature virus particles. Comparison of the relative sedimentation rates of RFI, RFII, and SS DNAs observed in the present study with those reported for filamentous bacteriophages (6), whose chromosome structure is similar to that of MVL51, indicates that RFI and RFII are double stranded and that RFI should be covalently closed circles while RFII should be a nicked form of RFI. These identifications are confirmed by experiments described in the following section.

Analysis of replicative intermediates:

Fig. 2 shows the results of equilibrium sedimentation in CsCl gradients containing ethidium bromide of RFI and RFII forms of DNA obtained from gradients as shown in Fig. 1b. ^{14}C-labelled JA1 cell DNA was used as marker in these gradients. Fig. 2a and c show that RFI DNA is more dense than RFII DNA in these gradients. After mild deoxyribonuclease treatment (10^{-3} µg/ml for 10 min at 37°C), RFI DNA cosediments with RFII DNA (Fig. 2b). Both RFII and DNase treated RFI cosediment with JA1 cell DNA. This sedimentation behavior is in agreement with the identification of the two viral double stranded intermediates as RFI and RFII.

Role of replicative intermediates:

To examine the kinetics of [^{3}H]-thymidine incorporation into the different virus specific DNA components and to investigate the role of the replicative intermediates in the synthesis of progeny viral DNA, 70 min after infection cells were pulse labelled for 5 min with [^{3}H] deoxythymidine and then the label was chased for 1 hour in unlabelled

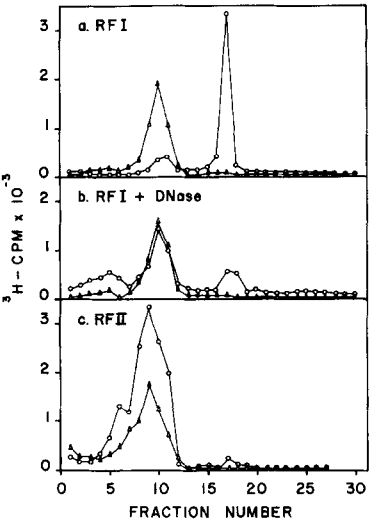

Fig. 2: Equilibrium CsCl density gradient sedimentation of [^{3}H]-labelled RFI and RFII in presence of ethidium bromide. (a) RFI DNA obtained from gradients as shown in Fig. 1b; (b) RFI DNA treated with 10^{-3} µg/ml pancreatic deoxyribonuclease for 10 min at 37°; (c) RFII DNA obtained from gradients as shown in Fig. 1b. ^{14}C-labelled JAI cell DNA was used as marker. (O)^{3}H; (Δ) ^{14}C. Sedimentation is from left to right.

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medium. To measure the distribution of the label at the end of the pulse, a sample was removed and added to an equal volume of ice cold Tris-EDTA-NaCl-NaCN buffer, washed and resuspended in the same buffer without cyanide. For the chase, an excess amount of unlabelled thymidine was added to the remaining portion of the pulse labelled sample and the sample was centrifuged in the cold for 4 min at 8000 rpm. The pellet was resuspended in prewarmed tryptose broth and incubated at 37°. Samples were then removed at 30 and 60 min after the transfer. All samples were lysed and analyzed as described in the Materials and Methods. The results of such a pulse-chase experiment are shown in Fig. 3. Fig. 3a shows that this pulse-labelled viral DNA distribution is the same as was found in the longer labelling experiments (Fig. 1b). At the start of the chase, the fastest sedimenting pulse-labelled DNA cosediments with added marker

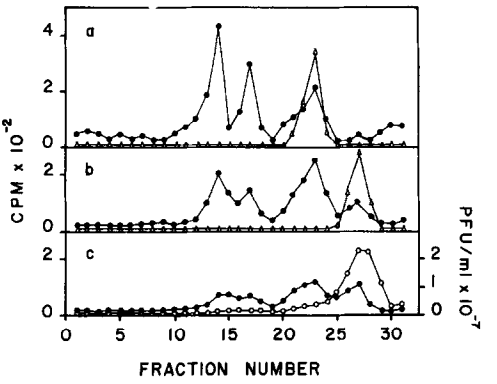

Fig. 3: Velocity sedimentation of infected cells pulse labelled with [^{3}H]-deoxythymidine (●), 50 µCi/ml, for 5 min at 70 min after infection (a) and chased for 30 min (b) and 60 min (c), as described in the text. ^{32}P-labelled MVL51 virus or viral DNA was mixed with infected cells before lysis and was used as marker. In (a) ^{32}P-labelled MVL51 viral DNA (Δ) was used as the marker and in (b) ^{32}P-labelled MVL51 virus (Δ) was the marker. In (c) no marker was added and infectivity (O) of the gradient fractions was measured.

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viral DNA. During the chase, the amount of label in double stranded forms (RFI and RFII) decreases more rapidly than the label in SS DNA and a labelled faster sedimenting material appears. This fast sedimenting material is infectious (Fig. 3c) and cosediments with added marker virus (Fig. 3b). These data also show that the lysis procedure does not destroy viral infectivity. By the end of the 60 min chase, only 15-20 percent of the label remained in viral DNA intermediates.

These results indicate that RFI and RFII are precursors in the synthesis of progeny SS DNA. This progeny DNA must then be extruded without lysing the cells.

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