Transfection of REP⁻ Mycoplasmas with Viral Single-Stranded DNA

TODD L. SLADEK AND JACK MANILOFF*

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

Received 4 May 1984/Accepted 13 September 1984

Double-stranded DNA from mycoplasma virus L2 can transfect *Acholeplasma laidlawii* cells in the presence of polyethylene glycol (T. L. Sladek and J. Maniloff, J. Bacteriol. 155:734-741, 1983). We report here that both single-stranded DNA and double-stranded replicative form DNA, from the single-stranded DNA mycoplasma virus L51, are also infectious in this system. For both DNAs transfection frequencies were in the range of 10^{-8} transfectants per DNA molecule and 10^{-3} transfectants per CFU. An unexpected finding was that both DNAs could transfect *A. laidlawii* strain REP⁻, a variant which is a nonpermissive host for single-stranded DNA mycoplasma viruses due to a block in viral DNA replication (Nowak et al., J. Bacteriol. 127:832-836, 1976). The number of viruses produced by transfected REP⁻ cells was comparable to the number produced by both transfected and infected wild-type cells. Therefore, transfected L51 DNAs are able to bypass the replication block in REP⁻ cells that occurs when these cells are infected by L51 virions.

Three groups of viruses infect the wall-less procaryote *Acholeplasma laidlawii* (reviewed in reference 21). Mycoplasma virus L2 is an enveloped, quasispherical virion containing circular, superhelical, double-stranded (DS) DNA of 7.8×10^6 daltons. Mycoplasma virus L3 is a T7-like virion containing linear DS DNA of about 26×10^6 daltons. Mycoplasma virus L51 is a bullet-shaped virion containing circular single-stranded (SS) DNA of 1.5×10^6 daltons.

The biochemistry of L51 DNA replication has been reviewed (20). Parental L51 DNA is converted to DS replicative form (RF) DNA during infection. Two forms, RFI (a covalently closed superhelical derivative of the viral genome) and RFII (a relaxed form of RFI), have been identified. The conversion of parental DNA to RF appears to occur during penetration, and parental RF DNA is mostly membrane associated. Membrane-associated parental RF replicates semiconservatively to produce progeny RF molecules. An *A. laidlawii* REP⁻ variant has been isolated in which RF→RF replication is blocked; in these cells parental RF is formed but no progeny RF synthesis can be detected (22). Therefore, L51 cannot propagate in REP⁻ cells. In wild-type cells, the next step in viral replication involves the release of progeny RF molecules into the cytoplasm and their asymmetric replication to produce viral SS DNA. Virus maturation presumably occurs at the cell membrane as the virion is assembled and extruded. L51 infection is noncytotoxic; infected cells continue to grow while producing viruses.

We have shown that *A. laidlawii* can be transfected by DS DNA from mycoplasma virus L2 in the presence of polyethylene glycol (PEG) (26). In this paper we extend these observations to show that both SS and DS RF DNA from L51 are also infectious in this system. Surprisingly, it was found that both of these DNAs were able to transfect the *A. laidlawii* REP⁻ strain and bypass the block that occurs when these cells are infected by virions. DNA from the DS DNA mycoplasma virus L3 is not infectious in the PEG transfection system.

MATERIALS AND METHODS

Viruses, cells, and medium. Mycoplasma viruses L51 (19), L2 (11), and L3 (12) and *A. laidlawii* strains K2 (14), JA1 (19), and REP⁻ (22) were used in these studies. Cells were assayed as CFU, viruses were assayed as PFU, and transfected cells were assayed as infectious centers on *A. laidlawii* lawns. Tryptose broth and agar plates were used for culturing cells and viruses as described previously (18).

Buffers and PEG solution. Buffers and PEG transfection solution were as previously described (26). TE buffer was 0.01 M Tris (pH 8.0)-0.001 M EDTA. TS buffer was 0.01 M Tris (pH 8.0)-0.1 M NaCl. TES buffer was TE buffer containing 0.1 M NaCl. TESN buffer was TES buffer containing 0.01 M NaCN.

Virus stocks and DNA purification. Early log-phase cultures of *A. laidlawii* were infected with L51, L2, or L3 virus at a multiplicity of infection of about 1. After overnight incubation at 37°C, cells were removed by centrifugation (10 min at 9,000 rpm and 4°C in a Beckman JA-10 rotor). Infected-cell supernatants routinely contained 10^{10} PFU/ml. These supernatants are either filtered (0.20-μm pore size) to obtain sterile virus stocks or concentrated with PEG to prepare virus DNA.

Virus was concentrated from infected-cell supernatants by PEG precipitation as previously described (26). Viral DNA was isolated from the resuspended PEG precipitate by phenol extraction and ethanol precipitation. As described below, this method yielded satisfactory preparations of L2 and L3 viral DNA. DNA recovered from L51-infected cell lysates by this method, however, contained both SS and DS L51 DNA, in addition to *A. laidlawii* chromosomal DNA. Therefore, it was necessary to use another procedure to purify L51 viral SS DNA.

SS viral DNA was isolated from purified L51 viral particles. L51 virions were concentrated by PEG precipitation, suspended in TS buffer, treated with 0.2% Triton X-100, and dialyzed against TES buffer. The virus then was purified by sedimentation through discontinuous cesium chloride (CsCl) step gradients as described by Yamamoto et al. (27). Gradients were made in nitrocellulose tubes (0.625 by 2.5 in. [ca. 1.585 by 6.4 cm]) by adding 3 ml of a 1.58-g/ml CsCl solution

* Corresponding author.

(in TES buffer) and then successive 2-ml layers of 1.45-, 1.37-, 1.31-, and 1.25-g/ml CsCl solutions. Dialyzed virus (6 ml) was layered on top of the gradients, and the tubes were centrifuged for 20 h at 23,000 rpm and 20°C in a Beckman SW28 rotor. Fractions were collected and assayed for PFU. The fractions containing virus were dialyzed against TES buffer and phenol extracted. The DNA was ethanol precipitated and suspended in TE buffer.

The procedure for isolation of L51 DS RF DNA from virus-infected cells was essentially a modification of the method described by Guerry et al. (13) followed by equilibrium centrifugation in CsCl-ethidium bromide gradients (4). Two liters of L51-infected cells was centrifuged, washed in 1 liter of TES buffer, and suspended in 100 ml of TES buffer. This was mixed with 13 ml of 0.5 M EDTA (pH 8.0), 11 ml of 10% sodium dodecyl sulfate, and 25 ml of 5 M NaCl. The mixture was kept at 4°C overnight and then centrifuged for 30 min at 12,000 rpm and 4°C in a Beckman JA-14 rotor. The resulting supernatant was termed the cleared lysate. For every 1 ml of cleared lysate, 0.9 g of CsCl and 0.1 ml of a 10-mg/ml ethidium bromide solution were added, and this was centrifuged at 36,000 rpm for 72 h at 20°C in a Beckman type 42.1 rotor. After centrifugation, the covalently closed circular (CCC) RF DNA band (lower band) was collected with a syringe through the side of the centrifuge tube, extracted with water-saturated 1-butanol to remove the ethidium bromide, and dialyzed against TES buffer. The DNA was ethanol precipitated and suspended in TE buffer.

In some instances, intracellular DNA was isolated from virus-infected cells by extracting the cleared lysate with phenol. The DNA was ethanol precipitated and suspended in TE buffer. DNA prepared in this way contained viral RF molecules as well as *A. laidlawii* chromosomal DNA.

A. laidlawii chromosomal DNA was isolated by the method described by Cerone-McLernon and Furness (3).

DNA concentrations were estimated by absorbance at 260 nm, where 1 absorbance unit corresponds to 50 µg of DS DNA per ml or to 40 µg of SS DNA per ml (7). DNAs had a 260-nm/280-nm absorbance ratio of >1.8.

Preparation of labeled virus. A log-phase culture (500 ml) of JA1 was infected as above except that 5 mCi of carrier-free ³²P_i was added 15 min before infection and again 15 min after infection. Virus was purified on CsCl step gradients as described above.

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out as previously described (23) except that gels were 0.8% agarose and both gels and buffer contained 0.5 µg of ethidium bromide per ml.

Enzymes and lambda DNA. S1 nuclease, lambda DNA, and *Cfo*I and *Hind*III restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Restriction enzymes were used as recommended by the manufacturer. Purified L51 RF (1.2 µg) was incubated with S1 nuclease (40 U) in the recommended buffer for 2 h.

Transfection. The protocol used for transfection was as previously described (26). Details are given in the appropriate table footnotes and figure legends. In general, a transfection mixture contained 50 µl of DNA, 250 µl of cells, and 2 ml of PEG solution (36% final concentration).

Adsorption experiments. Virus adsorption and calculation of adsorption rate constants were done as previously described (16).

Sucrose gradient centrifugation. DNA replicative intermediates were analyzed by sedimentation through 38-ml 5 to 20% (wt/vol) linear high-salt sucrose gradients as previously described (5). Gradients were centrifuged for 16 h at 5°C and

24,000 rpm in an SW28 rotor. Fractions (1.2 ml) were collected from the top, using a density gradient fractionator (ISCO, Lincoln, Neb.).

Measurement of radioactivity. Gradient fractions were precipitated by adding an excess of cold 5% trichloroacetic acid. Precipitated samples were filtered, dried, and assayed for radioactivity as previously described (15).

RESULTS

Purification of L51 SS and DS DNA. We have shown that mycoplasma virus L2 DNA obtained by phenol extraction of PEG-precipitated viral lysates was active in transfection (26). In preliminary experiments we found that DNA isolated in the same way from L51-containing viral lysates was also active in this transfection system. However, when this DNA was analyzed by agarose gel electrophoresis, several DNA bands were seen (Fig. 1d). No such bands were seen when L2 or L3 DNA isolated by this method was analyzed. L2 DNA yielded a fast-migrating superhelical band and a slower-migrating open circular band (Fig. 1b; cf. reference 23). L3 DNA yielded a single band of linear DNA on gels (Fig. 1c). Since SS L51 viral DNA is replicated to RFI and RFII during the viral replication cycle, it was thought that these DNAs might be present in the L51 mixture (Fig. 1d) in addition to SS molecules. RF molecules are intracellular forms of viral DNA, so if these forms were present, cellular DNA might also be present. To determine which of the DNAs present in the L51 DNA preparation were active in transfection, it was necessary to identify and purify the infectious molecules from the mixture of DNAs.

To identify which of the DNA bands in the mixture was SS viral DNA, L51 virus was purified on CsCl step gradients and DNA was isolated from the virus as described in Materials and Methods. Figure 2c shows that when this DNA was run on agarose gels, it comigrated with the fastest-migrating DNA band present in the PEG-precipitated

FIG. 1. Agarose gel electrophoresis of viral DNAs from phenol-extracted PEG precipitates. (a) λ + *Hind*III; (b) L2; (c) L3; (d) L51; (e) λ + *Hind*III. Mycoplasma virus DNAs were isolated from strain K2 cells. Molecular sizes of the λ *Hind*III fragments are shown in kilobase pairs.

FIG. 2. Agarose gel electrophoresis of L51 DNAs. (a) λ + *Hind*III; (b) phenol-extracted L51 DNA from PEG precipitate; (c) purified L51 SS viral DNA; (d) DNA from cleared lysate from L51-infected cells; (e) purified L51 RF DNA; (f) *A. laidlawii* chromosomal DNA; (g) purified L51 RF + S1 nuclease; (h) purified L51 RF + *Cfo*I; (i) λ + *Hind*III. Mycoplasma virus and cell DNAs were isolated from strain K2 cells. Molecular sizes of the λ *Hind*III fragments are shown in kilobase pairs on the left. The positions of L51 DS RF open circular (OC), linear (LIN), and superhelical CCC DNAs are shown on the right.

viral lysate (Fig. 2b). In addition, this DNA band was destroyed by low concentrations of S1 nuclease, an SS-specific nuclease (data not shown), which is consistent with the band being SS DNA.

When whole intracellular DNA was isolated from virus-infected cells (see Materials and Methods) and analyzed by agarose gel electrophoresis, the banding pattern in Fig. 2d was seen. Intracellular DNA isolated from noninfected cells yielded a single DNA band (Fig. 2f) which comigrated with the slowest-migrating band present in the DNA from infected cells. We conclude that this high-molecular-weight DNA band is *A. laidlawii* chromosomal DNA and that the two other major bands present in the intracellular DNA preparation from infected cells must be intracellular forms of L51 viral DNA. The SS viral DNA band was not present in these preparations.

When intracellular DNA from infected cells was centrifuged to equilibrium on CsCl-ethidium bromide gradients and the CCC DNA band was collected, chromosomal DNA was eliminated (Fig. 2e). When DNA from the CCC band was analyzed on agarose gels, three prominent bands were consistently seen. We reasoned that these were probably CCC superhelical DNA (RFI), nicked circles (RFII), and linear DS molecules, the latter two being generated from RFI molecules during preparation. To determine which DNA band on gels corresponded to each of these forms, the enzyme S1 nuclease was used. S1 nuclease has been shown to convert CCC superhelical DNA to linear molecules in a two-step reaction, first nicking CCC molecules and then cleaving the intact DNA strand opposite the nick (2, 10). Therefore, under appropriate enzymatic conditions, one should see conversion of CCC DNA to both nicked circles and linear molecules. When CCC L51 intracellular DNA from equilibrium gradients was treated with S1 nuclease as described in Materials and Methods, the pattern in Fig. 2g was seen. It can be seen that the amount of DNA in the fastest-migrating band of the untreated sample has been greatly diminished in the S1-treated sample. We conclude

that this fast-migrating band represents CCC superhelical L51 molecules.

Two other major bands were seen in the S1 nuclease-treated DNA. One band migrated near the lambda molecular-weight marker at 4.3 kilobase pairs. The amount of DNA in this band was greatly increased in the S1-treated sample compared with the untreated sample. This is the expected position for the linear DS form of the 4.5-kilobase pair L51 genome (24). Treatment of CsCl-ethidium bromide gradient-purified intracellular viral DNA with restriction endonuclease *Cfo*I converted all of the DNA forms to this band (Fig. 2h). This is consistent with the observation that the restriction endonuclease cleavage map of L51 RF DNA has a single *Cfo*I site (T. Sladek, unpublished data). The second band in the S1 nuclease-treated sample comigrated with the 6.6-kilobase pair lambda molecular-weight marker. We believe that these are open circular RFII molecules since *Cfo*I eliminated this band.

A minor band that is seen only in S1 nuclease-treated preparations migrated at a position that would be expected for a linear molecule of approximately 8.8 kilobase pairs in length. This is the molecular weight of an RF molecule which is twice the normal size. We believe that these are dimer RF molecules and these must have existed inside the cell as superhelical molecules since they purified with that DNA on CsCl-ethidium bromide equilibrium gradients. We can eliminate the possibility that these are nonviral plasmids since gradients of cleared lysates from noninfected cells (both strain JA1 and K2) yielded no superhelical DNA band.

Transfection with SS and DS L51 DNA. L51 SS DNA isolated from purified viral particles and L51 RF DNA purified on CsCl-ethidium bromide gradients were used in transfection experiments. The dose-response curves for both of these DNAs are shown in Fig. 3. The basic features of both curves are similar to those previously reported for L2 DNA (26). The dose-response curves were linear with a slope of about 1 (0.95 for SS, 0.99 for RF), which indicates that single DNA molecules give rise to transfectants. The curves also show that saturation was achieved at high DNA concentrations. Optimum transfection frequencies for both SS and RF DNA were in the range of 10^{-8} transfectants per DNA molecule (2.3×10^{-8} for SS, 7.3×10^{-8} for RF). Figure 3 shows that the optimum transfection frequencies per cell were higher for L51 SS DNA (2.5×10^{-3} transfectants per CFU) than for L51 RF DNA (8.0×10^{-4} transfectants per CFU).

Experiments with L3 DNA. We have also used phenol-extracted DNA from mycoplasma virus L3 in transfection dose-response experiments and have not observed plaques, even though the L3 DNA appeared to be intact (Fig. 1c). In fact, L3 DNA can compete with transfecting L2 DNA (data not shown), causing a decrease in the number of L2 plaques in transfection experiments. We found that other nontransfecting DNAs (for example, *Mycoplasma gallisepticum* chromosomal DNA) also compete with transfecting L2 DNA (data not shown).

Transfection of REP⁻ cells. The REP⁻ strain is a variant of *A. laidlawii* strain JA1 which propagates DS DNA mycoplasma viruses, but not SS DNA mycoplasma viruses (22). It has been shown that, in REP⁻ cells, parental SS DNA is converted to DS RF molecules; however, these RF molecules are not replicated further (22).

Although REP⁻ cells are not permissive for L51 replication when viruses infect these cells, we found that REP⁻ cells are permissive for L51 replication when either SS or DS L51 DNA is transfected into the cells with PEG. Table 1

shows that the transfection frequency (infectious centers per CFU) of strain REP⁻ is similar to that of strain JA1 for both SS and DS L51 DNA. Table 1 also shows that, when a given amount of L51 virus is added to cells in a normal infection, REP⁻ cells are 50-fold less efficient in forming infectious centers with L51 than are JA1 cells. However, the few infectious centers measured for REP⁻ cells are probably artifactual, as discussed below. The control experiment (virus without cells) shows that the washing conditions were such that unadsorbed viruses were removed in these experiments.

These data show that REP⁻ cells can act as hosts for L51 in transfection experiments. To determine the number of viruses produced by the transfected cells, one-step virus growth experiments were done. Figure 4a shows that production of virus by REP⁻ and JA1-transfected cells was identical; each transfected cell produced about 1,000 viruses in the 3-h period after transfection. Figure 4b shows one-step growth experiments for infection. Three hours after viruses were added, the number of PFU in an infected JA1 culture had increased about 100-fold. In the same period of time, the number of PFU in an infected REP⁻ culture had not increased over the background of unadsorbed virus, in agreement with previous observations (22).

There are two possible explanations for these transfection data. One is that L51 DNAs are able to bypass the REP⁻ replication block because DNA entry and replication after transfection are different than after infection. The second explanation is that the processing of cells during the transfection procedure (centrifugation or PEG treatment or both) alters the intracellular environment such that the REP⁻ character is lost. To distinguish between these two possibilities, virus-infected REP⁻ and JA1 cells were processed in the same manner as in transfection experiments and then assayed for production of infectious centers. Table 2 shows that L51-infected REP⁻ cells, processed for transfection,

FIG. 3. Transfection dose-response curves of L51 SS DNA and DS RF DNA. DNA was isolated from virus grown on strain K2. Transfection was performed by adding various amounts of L51 SS (●) or DS RF (○) DNA to strain K2 cells. The data are from two separate experiments and are plotted as the number of transfectants per cell (infectious centers per CFU) against the number of DNA molecules per cell. For SS DNA, there were 10^8 CFU per transfection mixture and the maximum number of transfectants was 2.6×10^5 . For RF DNA, there were 1.5×10^8 CFU per transfection mixture and the maximum number of transfectants was 1.2×10^5 . The slope of the linear part of the SS curve is 0.95, and that of the DS curve is 0.99.

TABLE 1. L51 transfection and infection of REP⁻ and JA1 cells^a

Infection with L51:	Host cells	No. of infectious centers	Infectious centers/CFU (relative no.) ^b
SS DNA	REP ⁻	1.00×10^4	1.00×10^{-4} (1.06)
	JA1	4.50×10^3	9.40×10^{-5} (1.00)
RF DNA	REP ⁻	1.70×10^4	1.70×10^{-4} (1.63)
	JA1	5.00×10^3	1.04×10^{-4} (1.00)
Virus	REP ⁻	1.17×10^3	8.93×10^{-7} (0.02)
	JA1	3.30×10^4	4.18×10^{-5} (1.00)
	None	2.00×10	

^a Transfection was carried out by the addition of 5 μ g of L51 SS or RF DNA (virus grown on strain JA1) to either 10^8 CFU of strain REP⁻ or 4.79×10^7 CFU of strain JA1. Infectious centers were assayed by plating on JA1 lawns. Infection was done by adding 5.00×10^4 PFU of L51 virus (grown and assayed on strain JA1) to either 1.31×10^9 CFU of strain REP⁻ or 7.90×10^8 CFU of strain JA1 (total volume of 1 ml). Virus was allowed to adsorb to cells for 5 min at 37°C. The cells were then centrifuged for 4 min in an Eppendorf Microfuge 5412 (Brinkmann Instruments Inc., Westbury, N.Y.), unadsorbed virus was decanted, and the cells were suspended in 1 ml of fresh medium. The cells were pelleted once more and again resuspended as described above. Cells were then plated for infectious centers on JA1 lawns. The control experiment was done in the same way except that virus was added to 1 ml of medium containing no cells.

^b In each experiment (with SS DNA, RF DNA, or virus), the data are normalized relative to the value for JA1 cells. The normalized data are given in parentheses.

with or without PEG treatment, were 50- to 100-fold less efficient in forming infectious centers than were JA1 cells. This is consistent with the results shown in Table 1. REP⁻ and JA1 cells were also processed for transfection (but without transfecting DNA) before being infected with L51 virus. Table 2 shows that, in this case, the REP⁻ sample was only 5- to 20-fold less efficient in infectious-center formation than the JA1 sample. To determine if this increase in infectious centers was due to productive L51 infection of REP⁻ cells, the experiment was repeated (cells processed for transfection before addition of virus) and virus production was measured in a one-step growth experiment. We found (data not shown) that JA1 cells (with and without PEG treatment) produced virus whereas REP⁻ cells (with and without PEG treatment) did not produce detectable amounts of progeny virus (as in the experiment shown in Fig. 4b). Hence, the transfection protocol (with or without PEG) may cause nonspecific binding of virus to REP⁻ cells, but REP⁻ cells do not become productively infected. In JA1 cells, such a low level of nonspecifically bound virus would probably be masked by the larger number of infected cells. The transfection event itself, and not cell processing or PEG treatment, results in L51 virus production by REP⁻ cells.

That the REP⁻ strain can be transfected by L51 DNA but cannot be infected by intact virus is interesting: entry of L51 DNA by transfection allows it to avoid a block in its replication cycle in REP⁻ cells. The conclusion that parental L51 SS DNA is converted to RF in REP⁻ cells, but that the molecules are not replicated further, was based on the finding that, after infection, labeled parental viral DNA (but not detectable nascent DNA) was found in RFI and RFII molecules (22). Our finding that viral lysates contain RFI and RFII molecules (Fig. 1d) raises question about the conclusions of these earlier experiments. The viral lysates (5) used in those experiments probably contained free RF, and, since *A. laidlawii* cells can bind free DNA (8), it is possible that labeled RF DNA was present in the lysates, became bound to cells, and was then erroneously interpreted as intracellular replication intermediates produced during infection in

FIG. 4. One-step virus growth in REP⁻ (●) and JA1 (○) cells transfected and infected with L51. (a) Transfection was performed by adding 15 µg of purified L51 SS DNA (virus grown on JA1 cells) to 250 µl of REP⁻ or JA1 cells. After transfection, cells were diluted 10⁴-fold in tryptose broth. At various times after transfection, samples were centrifuged to remove cells and the supernatant was assayed for PFU on JA1 lawns to measure progeny virus production. Transfection of REP⁻ and JA1 produced 10⁴ and 1.2 × 10⁵ infectious centers, respectively. (b) For infection, cultures of REP⁻ and JA1 were infected with L51 (grown on JA1) at a multiplicity of infection of 1 to 10 and incubated at 37°C for 10 min for adsorption. The cultures were then diluted 10⁶-fold into tryptose broth, and at various times PFU were assayed as above.

REP⁻ cells. If so, it may be that the block in REP⁻ cells is really a block at the level of virus adsorption or penetration (processes that were previously only inferred to be normal in REP⁻ cells). We therefore felt it necessary to do further experiments to investigate the REP⁻ block.

To examine virus adsorption, we determined the first-order rate constants (*k*) from adsorption curves as previously described (16). Table 3 shows that the L51 adsorption constant for REP⁻ cells was about 3.5 times less than that for wild-type JA1 cells. The conclusion is that, although L51 virus adsorbs slightly slower to REP⁻ cells than to JA1 cells, virus adsorption to REP⁻ cells is still comparable to that to JA1 cells and cannot explain the block in L51 replication in REP⁻ cells. We note that our *k* value for adsorption of virus to JA1 cells is an order of magnitude less than the value calculated by others (9). However, for adsorption of mycoplasma virus L3 to *A. laidlawii* cells, it has been shown that the absolute value of adsorption rate constants varied by a factor of 5 depending on the physiological condition of the cells and the composition of different lots of medium (16).

To look at viral penetration and replication intermediates in infected cells, L51 virus was labeled with ³²P and purified on CsCl step gradients as described in Materials and Methods. This purified virus was added to growing JA1 and REP⁻ cells at a multiplicity of infection of 100. After 5 min of adsorption, unadsorbed viruses were removed by centrifugation. Cells were suspended in prewarmed medium, incubated at 37°C for 5 min, lysed, and sedimented through high-salt sucrose gradients as described in Materials and

TABLE 2. Effect of transfection procedure on L51 infection of JA1 and REP⁻ cells^a

Cells processed for transfection:	Host cells	PEG treatment	Infectious centers/CFU (relative no.) ^b
After viral infection	REP ⁻	Yes	1.86 × 10 ⁻⁶ (0.02)
	REP ⁻	No	1.71 × 10 ⁻⁶ (0.02)
	JA1	Yes	2.36 × 10 ⁻⁴ (2.17)
	JA1	No	1.09 × 10 ⁻⁴ (1.00)
Before viral infection	REP ⁻	Yes	3.79 × 10 ⁻⁵ (0.16)
	REP ⁻	No	3.10 × 10 ⁻⁵ (0.13)
	JA1	Yes	7.97 × 10 ⁻⁴ (3.33)
	JA1	No	2.39 × 10 ⁻⁴ (1.00)

^a REP⁻ and JA1 cells were grown to an optical density of 0.1 at 610 nm, measured with a Gilford model 240 spectrophotometer (26). The cells were pelleted (10 min at 6,000 rpm in a Sorvall SS-34 rotor) and suspended in the original volume of fresh medium. Cell number was determined by plating for CFU. For cells processed after infection, 1.25 × 10⁴ PFU of L51 virus were added to 250 µl of cells and virus was allowed to adsorb to cells for 5 min at 37°C. The cells were then centrifuged and washed (as described in Table 1, footnote a) and suspended in 250 µl of fresh medium. A 2-ml portion of PEG transfection solution or 2 ml of 0.01 M Tris (pH 8.0) was added; after 2 min, the mixture was diluted with 10 ml of 0.01 M Tris and cells were pelleted (12 min at 12,000 rpm in an SS-34 rotor). The cells were suspended in 1 ml of tryptose broth and immediately assayed for infectious centers on JA1 lawns. For cells processed before infection, the procedure was identical except that cells were first treated with PEG or Tris solution and then infected with L51 virus. In both cases, infectious centers were counted and expressed as number of infectious centers per number of cells in 250 µl. For each set of experiments the data are normalized to that for JA1 cells that were not treated with PEG.

^b Data are normalized relative to the value for JA1 cells. Normalized data are given in parentheses.

Methods. Figure 5 shows that a single peak of ³²P-labeled material was found in both the JA1 and REP⁻ gradients. This peak has been identified previously as RFI DNA (22). It should be noted that purification of virus on CsCl step gradients removed all contaminating free RF molecules found in the original viral lysates. Therefore, the RFI molecules in the gradients in Fig. 5 could not be a contaminant from the virus preparation. Instead, these molecules

TABLE 3. First-order rate constants for L51 adsorption to JA1 and REP⁻ cells^a

Cell strain	Cells (CFU/ml)	MOI	<i>k</i> (cm ³ /min)
JA1	6.5 × 10 ⁸	0.07	1.43 × 10 ⁻¹⁰
	5.5 × 10 ⁸	0.08	8.40 × 10 ⁻¹¹
	2.1 × 10 ⁸	0.13	1.94 × 10 ⁻¹⁰
	3.6 × 10 ⁸	0.19	2.22 × 10 ⁻¹⁰
	3.0 × 10 ⁸	0.23	2.64 × 10 ⁻¹⁰
	5.0 × 10 ⁸	1.34	4.22 × 10 ⁻¹¹
	2.1 × 10 ⁸	3.22	1.35 × 10 ⁻¹⁰
Avg			1.55 (±0.78) × 10 ⁻¹⁰
REP ⁻	7.6 × 10 ⁸	0.06	6.05 × 10 ⁻¹¹
	6.1 × 10 ⁸	0.07	5.99 × 10 ⁻¹¹
	3.4 × 10 ⁸	0.08	2.96 × 10 ⁻¹¹
	6.8 × 10 ⁸	0.10	6.29 × 10 ⁻¹¹
	6.3 × 10 ⁸	0.11	1.53 × 10 ⁻¹¹
	7.3 × 10 ⁸	0.92	4.09 × 10 ⁻¹¹
Avg			4.48 (±1.96) × 10 ⁻¹¹

^a These experiments used L51 virus grown on JA1 cells. For each strain the average (± standard deviation) was calculated from the measured *k* values. MOI, Multiplicity of infection.

represent parental viral DNA which has been replicated to RFI molecules in both JA1 and REP⁻ cells. Therefore, SS viral DNA is replicated to RF molecules in REP⁻ cells, but no progeny viruses are produced. Our conclusion, then, is that the block in REP⁻ cells is a true block in viral replication and not a block in viral adsorption or penetration. In addition, since REP⁻ cells can be transfected by L51 DNA, the replication block can be bypassed by transfecting DNA.

DISCUSSION

The data in this paper show that the PEG transfection method previously used for mycoplasma virus L2 DS DNA (26) also can be used to transfect *A. laidlawii* with SS and DS mycoplasma virus L51 DNA. We found that methods previously used to obtain pure L2 and L3 viral DNA were not satisfactory for L51, since virion preparations obtained from cell cultures infected by L51 contained DS L51 RF DNA and cellular DNA in addition to L51 SS DNA. Although L51-infected cells continue to divide while producing progeny viruses (19), this finding indicates that at least some cells in the virus-infected culture lyse during infection and liberate intracellular DNA.

To determine which DNAs in the mixture were infectious in transfection assays, methods were developed to purify both L51 SS and DS RF molecules. In the course of this work we identified RF molecules which were twice the size of normal RFs. The role these dimers may play in virus replication is not clear. However, the finding that RF DNA could be isolated in large quantities from infected cells has allowed us to map this DNA with restriction endonucleases. These data will be published elsewhere.

We found that the overall parameters for transfection with L51 SS and RF DNA were similar to those previously described for transfection with L2 DNA: all dose-response curves had a slope of 1, indicating that transfection results from a single DNA molecule being taken up by a cell and saturate at high DNA concentrations. The optimum transfection frequency of about 10^{-8} transfectants per DNA molecule for both L51 SS and DS RF DNA contrasts with that of 10^{-7} transfectants per DNA molecule for L2 DNA (26). For both L51 DNAs the transfection frequency is around 10^{-3} transfectants per CFU. For L2 transfection the value is about 10^{-4} transfectants per CFU (26).

L3 DNA was not active in PEG transfection even though, by gel electrophoresis, the molecules did not appear to be sheared. The reason for this inactivity is not clear; however, the L3 DNA molecule is linear whereas both L51 and L2 DNAs are circular, and we have shown previously (26) that linear L2 DNA transfects at a much lower frequency than circular L2 molecules. L3 DNA is also much larger than L2 and L51 DNAs. Nontransfecting DNA, such as L3 DNA, was able to compete with transfecting DNA and cause a decrease in the number of plaques produced in transfection assays.

The finding that L51 DNAs can transfect REP⁻ cells was unexpected. Production of L51 by REP⁻ cells was shown to result from DNA transfection and not from processing or PEG treatment of cells in the transfection procedure. REP⁻ cells had been reported to allow conversion of L51 SS DNA to DS RF molecules, but not to allow further L51 DNA replication (22). Since free RF DNA was present in viral lysates, it seemed possible that this conclusion was incorrect and that the REP⁻ block was really a block in viral adsorption or penetration. We therefore looked at viral adsorption to REP⁻ cells and found it to be only slightly slower than

adsorption to JA1 cells. It was also found (Fig. 5) that virus could penetrate REP⁻ cells and that parental SS viral DNA was converted to RF molecules. Therefore, we confirm the previous conclusion (22) that, in REP⁻ cells, SS viral DNA is converted to DS RF molecules which are not replicated further.

The data in Table 1 show that the REP⁻ strain is 50 times less efficient at plaquing L51 virus than is the wild-type JA1 strain. That the REP⁻ strain can plaque L51 at all may indicate that this strain was leaky. However, in one-step growth experiments (Fig. 4b) no virus production by infected REP⁻ cells was seen. If the REP⁻ strain is leaky, this result indicates that only a smaller number of viruses are made by these cells. Alternatively, it may be that the REP⁻ strain is not leaky and that, in the experiment in Table 1, some viruses that had adsorbed to REP⁻ cells desorbed and infected the lawn of JA1 cells. It should be noted that, in other systems, it has been shown that adsorption of phages to cells is a stepwise process involving a reversible step (1).

Previously it was stated that the *A. laidlawii* REP⁻ phenotype was similar to that of *Escherichia coli* rep-3 mutants since these mutants, like *A. laidlawii* REP⁻ cells, are not impaired in cell growth, have increased sensitivity to UV irradiation, and are not able to propagate SS DNA

FIG. 5. Velocity-sedimentation analysis of viral DNA intermediates containing parental label. Log-phase cultures (20 ml) of (a) JA1 and (b) REP⁻ cells were infected with ³²P-labeled L51 at a multiplicity of infection of about 100. Five minutes after infection, the cells were harvested by centrifugation (10 min, $15,000 \times g$, 4°C), suspended in 20 ml of cold TS buffer, and spun again as above. The cells were resuspended in 20 ml of prewarmed tryptone broth. After 5 min of incubation at 37°C, 20 ml of cold TESN buffer was added. The cells were harvested, washed in TES buffer, suspended in 1 ml of TES, lysed by adding Sarkosyl (final concentration, 0.4%), and layered onto 5 to 20% high-salt sucrose gradients as described in the text. Under these conditions, the RFI peak is in fraction 17 (22). Sedimentation is from left to right.

viruses due to a block in the replication of parental RF to progeny RF molecules (22). It has been shown that the *E. coli rep* protein is an ATP-dependent duplex DNA unwinding protein which separates the duplex DNA strands at the replicating fork (17, 25). Our data showing that L51 DNA can transfect the *A. laidlawii* REP⁻ strain and that this strain adsorbs L51 virus slightly slower than the JA1 strain indicate that the *A. laidlawii* REP protein is probably not a helicase that unwinds DNA at a replication fork.

One possible explanation of these data is that the *A. laidlawii* REP protein may be a transmembrane protein that functions in viral adsorption and penetration and also to anchor viral DNA to those membrane sites (6) at which parental RF is replicated to progeny RF molecules. The REP⁻ strain would then have a protein altered such that its ability to position RF DNA at membrane sites for replication is impaired. This alteration would also have a small effect on viral adsorption. Therefore, in infection, viral SS DNA enters REP⁻ cells and is replicated to RF DNA, but cannot replicate further. In transfection, since entry of DNA does not require viral adsorption, the protein is bypassed. Perhaps, in transfection, viral DNA is able to attach to membrane sites that do not contain the REP protein or, alternatively, the DNA may be able to replicate free of the membrane in the cytoplasm. This model explains how two phenotypes of REP⁻ cells, slightly reduced adsorption rate and blocked L51 replication, could be caused by a single mutation. However, the model by which L51-transfecting DNA bypasses the RF replication block in REP⁻ cells must account for the observation that both SS and DS L51 RF DNA can transfect REP⁻ cells.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grant GM32442 from the National Institutes of Health.

LITERATURE CITED

- Adams, M. H. 1959. Bacteriophages, p. 147-150. Interscience Publishers, New York.
- Beard, P., J. F. Morrow, and P. Berg. 1973. Cleavage of circular, superhelical simian virus 40 DNA to a linear duplex by S1 nuclease. *J. Virol.* **12**:1303-1313.
- Cerone-McLernon, A. M., and G. Furness. 1980. The preparation of transforming DNA from *Mycoplasma hominis* strain Sprott tet^r and quantitative studies of the factors affecting the genetic transformation of *Mycoplasma salivarium* strain S9 tet^s to tetracycline resistance. *Can. J. Microbiol.* **26**:1147-1152.
- Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. U.S.A.* **62**:1159-1166.
- Das, J., and J. Maniloff. 1975. Replication of mycoplasma virus MVL51. I. Replicative intermediates. *Biochem. Biophys. Res. Commun.* **66**:599-605.
- Das, J., and J. Maniloff. 1976. Replication of mycoplasma virus MVL51. Attachment of MVL51 parental DNA to host cell membrane. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1489-1493.
- Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics, p. 225. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Folsome, C. E. 1968. Deoxyribonucleate binding and transformation in *Mycoplasma laidlawii*. *J. Gen. Microbiol.* **50**:43-53.
- Fraser, D., and C. Fleischmann. 1974. Interaction of mycoplasma with viruses. I. Primary adsorption of virus is ionic in mechanism. *J. Virol.* **13**:1067-1074.
- Germond, J.-E., V. M. Vogt, and B. Hirt. 1974. Characterization of the single-strand-specific nuclease S1 activity on double-stranded supercoiled polyoma DNA. *Eur. J. Biochem.* **43**:591-600.
- Gourlay, R. N. 1971. Mycoplasma virus-laidlawii 2, a new virus isolated from *Acholeplasma laidlawii*. *J. Gen. Virol.* **12**:65-67.
- Gourlay, R. N., and S. G. Wyld. 1973. Isolation of mycoplasma virus-laidlawii 3, a new virus infecting *Acholeplasma laidlawii*. *J. Gen. Virol.* **19**:279-283.
- Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. *J. Bacteriol.* **116**:1064-1066.
- 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.
- Haberer, K., and J. Maniloff. 1980. Virus and host cell DNA syntheses during infection of *Acholeplasma laidlawii* by MVL3, a nonlytic cytocidal mycoplasma virus. *J. Virol.* **33**:671-679.
- Haberer, K., and J. Maniloff. 1982. Adsorption of the tailed mycoplasma virus L3 to cell membranes. *J. Virol.* **41**:501-507.
- Kornberg, A., J. F. Scott, and L. L. Bertsch. 1978. ATP utilization by *rep* protein in the catalytic separation of DNA strands at a replicating fork. *J. Biol. Chem.* **253**:3298-3304.
- Liss, A., and J. Maniloff. 1971. Isolation of Mycoplasma viruses and characterization of MVL1, MVL52, and MVL51. *Science* **173**:725-727.
- Liss, A., and J. Maniloff. 1973. Infection of *Acholeplasma laidlawii* by MVL51 virus. *Virology* **55**:118-126.
- Maniloff, J., J. Das, and J. A. Nowak. 1978. Single-stranded DNA mycoplasma viruses, p. 177-184. In D. T. Denhardt, D. Dressler, and D. S. Ray (ed.), *The single stranded DNA phages*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maniloff, J., K. Haberer, R. N. Gourlay, J. Das, and R. Cole. 1982. Mycoplasma viruses. *Intervirology* **18**:177-188.
- Nowak, J. A., J. Das, and J. Maniloff. 1976. Characterization of an *Acholeplasma laidlawii* variant with a REP⁻ phenotype. *J. Bacteriol.* **127**:832-836.
- Nowak, J. A., and J. Maniloff. 1979. Physical characterization of the superhelical DNA genome of an enveloped mycoplasma virus. *J. Virol.* **29**:374-380.
- Nowak, J. A., J. Maniloff, and J. Das. 1978. Electron microscopy of single-stranded mycoplasma virus DNA. *FEMS Microbiol. Lett.* **4**:59-61.
- Scott, J. F., S. Eisenberg, L. L. Bertsch, and A. Kornberg. 1977. A mechanism of duplex DNA replication revealed by enzymatic studies of phage ϕ X174: catalytic strand separation in advance of replication. *Proc. Natl. Acad. Sci. U.S.A.* **74**:193-197.
- Sladek, T. L., and J. Maniloff. 1983. Polyethylene glycol-dependent transfection of *Acholeplasma laidlawii* with mycoplasma virus L2 DNA. *J. Bacteriol.* **155**:734-741.
- Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**:734-744.