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Mapping of Mycoplasma Virus DNA Replication Origins and Termini

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A pulse-labeling protocol has been used to study DNA replication and map replication origins and termini in mycoplasma viruses L2 and L2*ins1*. The L2 genome is circular, double-stranded DNA of 11.63 kilobase pairs (kb), and the 14.89-kb L2*ins1* genome is L2 DNA containing a 3.26-kb insertion. The data show that DNA replication is bidirectional from two origins in L2 and three origins in L2*ins1*. The extra origin in L2*ins1* arises from the fact that one of the L2 origins is in one of the sequences that have been shown to be duplicated and transposed in the generation of L2*ins1* from L2.

Although mycoplasmas are the smallest free-living cells, having arisen from eubacteria by reductions in genome size (16, 21, 23), relatively little is known of the molecular biology and genetics of these cells and their viruses.

Mycoplasma virus L2 is of particular interest because of its unique morphology and infectious cycle. The heterogeneous virions are enveloped, roughly spherical particles, about 74 to 132 nm in diameter (18), and the viral genome is circular, superhelical, double-stranded DNA (17). Infection of *Acholeplasma laidlawii* cells results in a noncytocidal productive infection, with a 4- to 6-h rise period and virus maturation by budding through the cell membrane, followed by establishment of lysogeny (reviewed in references 6 and 20).

Analysis of the L2 genome has involved construction of a restriction endonuclease cleavage map (6, 7, 17) and mapping the site at which L2 DNA is integrated into the cell chromosome (6, 7), the *att* site.

To map functional sites involved in DNA replication in a mycoplasma model system, we have used the pulse-label method developed for simian virus 40 (4, 12, 13) to examine the L2 chromosome. By this method, virus-infected cells are pulse-labeled with either $^{32}P_i$ or [^{3}H]thymidine to label nascent DNA, and viral DNA molecules completed during the pulse are isolated and assayed to determine the distribution of label as a function of chromosome position. DNA molecules completed during the pulse will be labeled from the site being replicated at the start of the pulse to the replication terminus (the *ter* site). The extent of labeling along the chromosome, from the replication origin (the *ori* site) to *ter*, depends on the ratio of labeling time to the time needed to complete a round of DNA replication. For labeling times comparable to chromosome doubling times, completed viral DNA molecules have a gradient of label, with the highest specific radioactivity at *ter* and the lowest specific radioactivity at *ori*.

From an analysis of such data, using cells infected with L2 and L2*ins1* (an L2 variant containing duplications and transpositions [7]), we have mapped the DNA replication *ori* and *ter* sites and shown that L2 replicates bidirectionally from two *ori* sites. Three *ori* sites have been mapped in L2*ins1*, in agreement with one of the *ori* sequences in L2 mapping in one of the regions that was duplicated and transposed in the generation of the L2*ins1* insertion variant.

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MATERIALS AND METHODS

Cells, viruses, and media. Host cells for these studies, *A. laidlawii* K2 (9), were grown at 37°C in tryptose broth containing 1% serum fraction and 1% glucose (14). Mycoplasma viruses L2 (8) and L2*ins1*, an L2 insertion mutant (7), were assayed as PFU in soft agar overlays of *A. laidlawii* (17).

Preparation of pulse-labeled virus DNA. A 100-ml *A. laidlawii* culture was infected at a multiplicity of infection of about 5. At 2 h postinfection, the infected culture was centrifuged at 6,000 rpm for 5 min at 4 to 6°C in a JA-20 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The supernatant was removed, and the infected cell pellet was suspended in 10 ml of prewarmed medium. After a 30-min incubation at 37°C, $^{32}P_i$ or [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.) was added to a final concentration of 100 μCi/ml. At 3, 10, and 16 min, 3.3-ml culture samples were removed and centrifuged at 5,000 rpm for 5 min at 4 to 6°C in a JA-20 rotor. Each infected cell pellet was washed twice with cold buffer (10 mM Tris hydrochloride, 1 mM EDTA, 100 mM NaCl [pH 8.0]), suspended in 1 ml of an ice-cold solution of 50 mM glucose, 10 mM EDTA, and 25 mM Tris hydrochloride (pH 8.0), and kept on ice for at least 5 min. An alkaline lysis procedure (15) was used to lyse the cells and obtain viral DNA, as follows. A 1-ml volume of ice-cold cell suspension was gently mixed with 2 ml of a solution of 0.2 N NaOH and 1% sodium dodecyl sulfate, and the mixture was stored on ice. After 5 min, 1.5 ml of ice-cold 5 M potassium acetate (pH 8.0) was added with gentle mixing, and the mixture was kept on ice for another 5 min. The mixture was then centrifuged at 15,000 rpm for 10 min at 4 to 6°C in a JA-20 rotor. Under these conditions, cell DNA and debris formed a compact pellet, and viral DNA was recovered in the supernatant free from cell DNA. Viral DNA then was isolated by phenol-chloroform extraction and ethanol precipitation (17) and treated with pancreatic RNase. During this procedure, replicating viral DNA intermediates were converted to single-stranded DNA molecules by the alkaline lysis conditions and, hence, were not recovered in the final viral DNA preparation. Agarose gel electrophoretic analysis of isolated viral DNA confirmed that the DNA preparations used in these studies contained only complete circular viral DNA molecules. Therefore, DNA in the pulse-labeled samples in these studies was in the form of completed progeny viral DNA molecules.

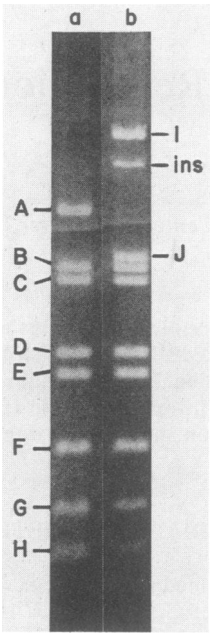

FIG. 1. Agarose gel electrophoresis of mycoplasma virus DNAs after digestion with restriction endonucleases *Pst*I, *Bst*EII, and *Hin*dIII. Lanes: a, L2 DNA with eight fragments marked A to H in order of decreasing size; b, L2*ins1* DNA with nine fragments. Because of the insertion, fragment A in L2 is replaced by two new fragments, marked I and J, in L2*ins1*. In addition, as discussed in Results, L2*ins1* DNA preparations contain linear 3.26-kb minivirus DNA (ins). The molecular sizes of the fragments are given in Results, and the fragment order is shown in Fig. 2.

Restriction endonuclease digestion. With a 30-μl reaction mixture, about 2 μg of viral DNA, isolated as described above, was digested with 5 U each of *Pst*I and *Hin*dIII at 37°C for 3 h, followed by the addition of 5 U of *Bst*EII and a further 3-h incubation at 60°C. The reaction buffer was 50 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol (15). All the enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). This digestion procedure cleaved the L2 genome into eight fragments and the L2*ins1* genome into nine fragments (Fig. 1).

Agarose gel electrophoresis. After restriction endonuclease digestion, the 30-μl reaction mixture was mixed with 5 μl of gel loading buffer (4% sodium dodecyl sulfate, 25% glycerol, 60 mM EDTA, 0.1% bromphenol blue), and the DNA fragments were analyzed by electrophoresis at constant voltage in gels of low-gelling-temperature 0.8% agarose (Marine Colloids, Rockland, Maine), with an electrophoresis buffer of 89 mM Tris borate–2 mM EDTA, pH 8.0. Electrophoresis was carried out in a cold room (4 to 6°C) for 15 h at about 1 V/cm, gels were then stained with ethidium bromide, and DNA bands were visualized by UV-light illumination.

Radioactive assays. Each agarose gel band that contained a DNA fragment was excised and placed in a glass liquid scintillation vial. A small amount (0.5 ml) of Protosol (New England Nuclear Corp., Boston, Mass.) was added, and the vial was capped tightly. Vials were incubated at 60°C for 4 h (by which time gels either had dissolved or become transparent) and then cooled at room temperature for about 15 min. A 10-ml volume of Econofluor (New England Nuclear) was added, and the contents of the vial were assayed for radioactivity in a liquid scintillation counter.

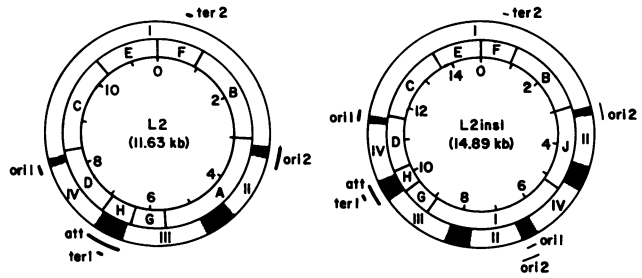

FIG. 2. Maps of DNA fragments of mycoplasma viruses L2 and L2*ins1* after digestion with restriction endonucleases *Pst*I, *Bst*EII, and *Hin*dIII. Fragment sizes are given in the text. The size of each map is marked in kilobase pairs; the 14.89-kb L2*ins1* genome is the 11.63-kb L2 genome with a 3.26-kb insertion (7). The zero point for each map is the single *Bst*EII cleavage site (7). The L2 map has been divided into four regions (labeled I, II, III, and IV) based on the duplications which generate L2*ins1* (7); L2*ins1* is related to L2 by duplications and transpositions of regions II and IV, with the order shown on the L2*ins1* map. Since the junctions between these regions have not been mapped exactly (7), they are shown as shaded areas between the regions. Sites which have been mapped are indicated by heavy arcs. The location of the *att* site was determined by Dybvig and co-workers (6, 7), and the *ori* and *ter* sites were mapped as described in this paper. The range shown for each *ori* and *ter* site marks the standard deviation of the values from the 3-, 10-, and 16-min pulse-label experiments. Tentative locations of some *ori* and *ter* sites in L2*ins1* were estimated as described in Results and are indicated by thin arcs.

RESULTS

Experimental considerations. To analyze the gradient of radioactivity in pulse-labeled viral DNA, completed viral chromosomes must be cleaved by restriction endonucleases, the resulting DNA fragments must be separated by agarose gel electrophoresis, and the amount of radioactivity in each fragment must be measured (4, 12, 13). For the experiments described here, mycoplasma virus L2 was digested with a mixture of restriction endonucleases *Pst*I, *Bst*EII, and *Hin*dIII. As expected from the L2 cleavage map (6, 7, 17), this produced eight fragments (Fig. 1, lane a), labeled A to H in order of decreasing molecular size. The fragment sizes were 2.64, 2.07, 1.96, 1.41, 1.29, 0.94, 0.73, and 0.59 kilobase pairs (kb), and the fragment order is shown on the cleavage map in Fig. 2.

For these studies, DNA fragment sizes were determined by comparison of their electrophoretic mobilities with those of molecular size standards (a *Hin*dIII digest of bacteriophage lambda DNA). The size of the L2 genome determined in this way (from the sum of the eight fragments) was 11.63 kb. We believe this is a more accurate measurement than our earlier value of 11.8 kb (17), determined from the electrophoretic mobility of linearized L2 DNA molecules. The exact size of the L2 genome will be determined from an L2 sequencing project that is in progress.

Pulse-labeling L2 virus. Progeny virus L2 DNA molecules (which had been pulse-labeled with $^{32}P_i$ for 3, 10, or 16 min during replication) were isolated and digested with a mixture of restriction endonucleases (*Pst*I, *Bst*EII, and *Hin*dIII). The resulting eight fragments were separated by agarose gel electrophoresis (Fig. 1, lane a), and each band was excised and assayed for radioactivity. The data were expressed as specific radioactivities (cpm per kilobase pair) relative to that of fragment H (Fig. 3a).

Data for L2 DNA pulse-labeled for 3, 10, and 16 min were found to fit gradients of specific radioactivity converging to

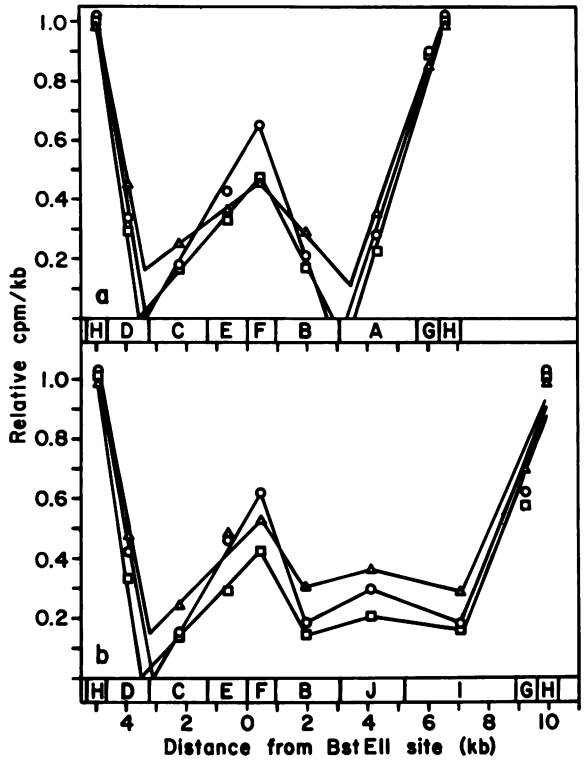

FIG. 3. Distribution of label in DNA fragments of mycoplasma viruses L2 (a) and L2*ins1* (b). Each virus was pulse-labeled, for 3 (○), 10 (□), or 16 (△) min, with $^{32}P_i$ during replication. Completed progeny viral DNA molecules were isolated and digested with restriction endonucleases, and DNA fragments were separated and assayed for radioactivity as described in Materials and Methods. The total amount of radioactivity (with background subtracted) recovered in L2 DNA fragments was 1,215 cpm for the 3-min pulse, 1,879 cpm for the 10-min pulse, and 2,917 cpm for the 16-min pulse; in L2*ins1* DNA fragments, the radioactivity was 1,817 cpm for the 3-min pulse, 2,131 cpm for the 10-min pulse, and 2,614 cpm for the 16-min pulse. The amount of radioactivity per kilobase pair for each fragment relative to the amount in fragment H is plotted on the ordinate. The midpoint of each fragment relative to its distance (in kilobase pairs) from the single *Bst*EII cleavage site is plotted on the abscissa. The fragment sizes and order are shown at the bottom of each graph.

two points on the L2 map (Fig. 3a), one near the junction of fragments D and C and the other near the junction of fragments B and A. Hence, L2 DNA replicates bidirectionally from two replication origins.

For a circular DNA, like L2 viral DNA, extrapolations of pulse-label gradients (as in Fig. 3) intersect at low specific radioactivities marking positions of replication origins and at high specific radioactivities marking positions of replication termini (4, 12, 13). From the data for L2 DNA (Fig. 3a), one of the origins (designated *ori1*) mapped at −3.4 ± 0.1 kb (or 8.2 ± 0.1 kb on the circular map in Fig. 2), and the other (*ori2*) mapped at 3.4 ± 0.3 kb (Fig. 2). These *ori* positions are the means ± standard deviations of the sites mapped in six experiments, two at each of the three time points. There is more uncertainty in the *ter* positions, and therefore, these can only be given as the midpoint of the fragment in which *ter* maps ± one-half the distance to the fragment end. Hence, one of the termini (*ter1*) is at about −4.9 ± 0.3 kb (or 6.7 ± 0.3 kb on the circular map), and the other (*ter2*) is at about 0.5 ± 0.5 kb on the L2 cleavage map (Fig. 2).

These experiments were repeated with [^{3}H]thymidine to pulse-label nascent DNA during L2 replication. Data from these experiments had to be corrected for variation in thymidine content among L2 DNA fragments. The correction factor for each fragment was determined from L2 viral DNA uniformly labeled with [^{3}H]thymidine.

Data (not shown) from the [^{3}H]thymidine pulse-label experiments were in agreement with data from the $^{32}P_i$ experiments described above; L2 replicates bidirectionally from two *ori* sites, one mapping at about −3 kb and the other mapping at about 3 kb. However, the lower specific radioactivity and slower labeling kinetics of [^{3}H]thymidine relative to those of $^{32}P_i$ preclude more quantitative conclusions from the [^{3}H]thymidine pulse-label experiments.

Pulse-labeling of L2 insertion virus. To investigate multiple replication origins in an L2-related virus, the pulse-label experiments were repeated with L2*ins1*, an insertion variant of L2 containing two genomic duplications and transpositions as shown in Fig. 2 (7). Digestion of L2*ins1* viral DNA with a mixture of restriction endonucleases (*Pst*I, *Bst*EII, and *Hin*dIII) yielded nine fragments (Fig. 1, lane b) because (owing to the insertion) fragment A in L2 is replaced by two fragments in L2*ins1* (Fig. 2); these new fragments were labeled I (with a size of 3.72 kb) and J (with a size of 2.18 kb). These data give sizes of 14.89 kb (the sum of the nine fragments) for L2*ins1* and 3.26 kb for the insert DNA, which (as discussed above for L2 DNA) are believed to be more accurate than our previous size determinations (7). In addition to the nine L2*ins1* fragments, L2*ins1* digests also contained a fragment at 3.26 kb (Fig. 1, lane b). This arose because L2*ins1* stocks contain small amounts of minivirus, which is a circular form of the 3.26-kb insert DNA with one *Pst*I site and no *Bst*EII or *Hin*dIII sites (7).

Pulse-labeling and datum analysis with L2*ins1* were carried out as described for L2. The 3-, 10-, and 16-min pulse-label data for L2*ins1* were found to fit gradients of specific radioactivity with three minima on the L2*ins1* map (Fig. 3b). Although the small number of datum points and low specific activities limit the mapping of *ori* sites in L2*ins1*, the reproducibility of the curves (showing three minima) indicate the presence of three *ori* sites in L2*ins1*. Hence, L2*ins1* has three *ori* sites; the first is near the junction of fragments D and C, the second is within fragment B or J, and the third is within fragment I.

From the three experiments in Fig. 3b, the mean ± standard deviation of the first *ori* site in L2*ins1* was at −3.4 ± 0.1 kb (or 11.5 ± 0.1 kb on the circular map), the same position mapped for *ori1* in L2. The position of the other two *ori* sites in L2*ins1* could not be mapped as exactly because of the small number of datum points. However, from the data for L2 (Fig. 2 and 3a), *ori2* would be expected to map at 3.4 ± 0.3 kb (Fig. 2) and is probably the second *ori* site seen in L2*ins1* (Fig. 3b). It cannot be determined from these data whether the third *ori* site in L2*ins1* results from a duplication of *ori1* or *ori2*, since the locations of the expected duplications overlap (Fig. 2) and are near the position of the third L2*ins1* *ori* site (Fig. 3b). Similarly, the data allow one *ter* site to be mapped at about −4.9 ± 0.3 kb in L2*ins1*, the same position as the *ter1* site in L2 (Fig. 2). The other two *ter* sites in L2*ins1* map in fragments F and J (Fig. 3b), one near the expected *ter2* site at 0.5 ± 0.5 kb and the other near a possible duplication of *ter1* at about 4.8 to 5.0 kb.

At all three time points, the measured specific radioactivity of the 3.26-kb minivirus DNA was much less than the mean specific radioactivity of L2*ins1* DNA but was close to the mean of the values for fragments I and J. This might be

a coincidence or may reflect the mechanism of minivirus replication, since fragments I and J contain the insert 3.26-kb DNA which is homologous to the 3.26-kb minivirus DNA.

The L2*ins1* experiments were repeated with [^{3}H]thymidine (data not shown) as the pulse-label. As discussed above for L2, the [^{3}H]thymidine data for L2*ins1* were in agreement with the $^{32}P_i$ data.

DISCUSSION

This is the first report that identifies and maps functional sites of mycoplasma DNA replication. For mycoplasma virus L2 grown in *A. laidlawii*, we have shown that DNA replication is bidirectional (with both replication forks moving away from the origin at similar rates) and mapped two replication origins and termini. The *ori1* and *ori2* sites map at 8.2 ± 0.1 kb and 3.4 ± 0.3 kb, respectively, on the L2 map, and *ter1* and *ter2* map at 6.7 ± 0.3 kb and 0.5 ± 0.5 kb, respectively (Fig. 2). From these data, we cannot determine whether these *ter* sites are site-specific sequences, located approximately halfway between *ori* sites, or whether they are simply where two replicating forks meet. It should be noted that *ter1* is either near or overlaps *att*, the site at which L2 DNA recombines with the cell chromosome DNA during lysogeny.

Multiple replication origins have been described in bacteriophages T4 (literature reviewed in reference 11) and T7 (22) and plasmid R6K (2, 3, 10). In most cases, replication proceeds bidirectionally from a single primary origin on each DNA molecule; however, a few percent of T4 (5) and R6K (3) molecules were found to replicate simultaneously from two origins. The symmetry of the pulse-label data around the two L2 origins (Fig. 3a) indicates that both *ori* sites are being used roughly equally during L2 DNA replication. However, additional data are needed to decide whether half the L2 DNA molecules replicate from one of the *ori* sites and the other half replicate from the other *ori* site, or whether most L2 DNA molecules replicate simultaneously from both *ori* sites.

Since mycoplasmas arose by degenerative evolution from gram-positive eubacteria (16, 21, 23), mycoplasma evolution involved a significant loss of genetic material. Hence, mycoplasmas allow us to investigate the details of DNA replication in cells containing limited amounts of genetic information. We expect mycoplasma virus L2 to be a useful model for examination of mycoplasma DNA replication because the L2 genome is too small to code for many of its own replication proteins and must use the DNA replication proteins of the cells. The limited data available do indeed indicate that L2 DNA replication uses cell enzymes: (i) *A. laidlawii* has a novobiocin-sensitive DNA gyrase activity which is required for L2 DNA replication (19); and (ii) 6-(*p*-hydroxyphenylazo)-uracil, an inhibitor of DNA polymerase III in gram-positive eubacteria (reviewed in reference 1), inhibits DNA replication in *A. laidlawii* cells and L2 replication in these cells (R. J. McDonald and J. Maniloff, unpublished data). Hence, replication of the small circular L2 chromosome should model cell DNA replication in genome-limited mycoplasmas.

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