Terminal redundancy and circular permutation of mycoplasma virus L3 DNA

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This communication reports the physical map of mycoplasma virus L3 (MV-L3) DNA derived from restriction patterns obtained by digestion with seven different restriction endonucleases. The length of the restriction map is 36,200 bp in contrast to the contour length of native MV-L3 DNA molecules which is 39,400 bp as determined by electron microscopy. The difference in length of 3,200 bp (corresponding to 8.1% of the native viral DNA contour length) is explained by terminal redundancy. It was possible to clone all fragments from particular restriction patterns into Escherichia coli vector pAT153, an indication of circular permutation within a population of MV-L3 DNA. However, clear evidence has been obtained from the molar ratios of fragments and from hybridization experiments. We suppose that viral DNA is packaged from a concatemeric precursor molecule starting at a specific site called pac.

Mycoplasma virus L3 (MV-L3) is a member of the Podoviridae family (Maniloff et al., 1982), which has been isolated from spontaneous virus plaques on lawns of Acholeplasma laidlawii (Gourlay & Wyld, 1973). The viral DNA is double-stranded, linear with an Mr of 26 × 10^6, which is equivalent to approximately 39.4 kbp. Host DNA synthesis is shut down within 3 h after infection (Haberer et al., 1979). Virus production starts at 90 min post-infection, reaches a maximum after 10 to 15 h and proceeds for several further hours. The infection is cytocidal rather than lytic. MV-L3 is quite an interesting virus in view of its unusual host, a wall-less, membrane-bound organism.

Earlier investigations using the polyethylene glycol-mediated transfection method resulted in failure to transfect A. laidlawii with purified MV-L3 DNA (Sladek & Maniloff, 1985), and it has been discussed that molecular size and linearity might prevent DNA uptake (Razin, 1985). However, Lorenz et al. (1988) obtained MV-L3 virus particles upon electroporation-mediated transfection with MV-L3 viral DNA. Although the efficiency of this procedure was very limited, a surprising observation was made, namely the release of two different viruses from transfected cells. By plaque hybridization techniques MV-L3 particles, derived from transfecting MV-L3 DNA, and MV-L1 particles, produced after induction of an MV-L1 provirus, were identified (Just et al., 1989).

In early experiments, MV-L3 was grown either on A. laidlawii JA1 (Liss & Maniloff, 1973) or on strain 1305/K2 (Haberer et al., 1979). Due to the release of induced MV-L1 virus during MV-L3 growth in these strains, further experiments were performed only with strain U1 (a derivative of 1305/K2) which is resistant to MV-L1. Electrophoresis of uncleaved viral DNA molecules did not show any unusual pattern or distribution in the gels. Electron microscopic length measurements showed unit length among observed molecules of native DNA.

Incubations with restriction endonucleases were done following the instructions of the enzyme manufacturers. Digestions with two enzymes were performed according to published procedures (Maniatis et al., 1982). Agarose gels used were 0.5% to 1.5% horizontal gels in Tris-acetate-EDTA electrophoresis buffer. PAGE was done in 5% to 12% acrylamide vertical gels in Tris-borate-buffered solutions. Endonuclease PstI (fig. 1, lane 6) produced 12 fragments, named A to L, which show equal molarity as determined by scanning the negative of a Polaroid photograph of the separation gel. An additional faint band, however, is located near B, thus introducing the designations B₁ (major band) and B₂ (minor band). Fragments K and L were detectable only in hybridization and/or polyacrylamide gels (6.5% acrylamide concentration). Table 1 summarizes the results for the determination of fragment sizes (mean values from 25 gels). It is noteworthy that the sum of fragment sizes is less than the value from contour length measurements.
Fragment B₂ has not been included in these calculations, and will be discussed later.

Using nuclease XbaI, we detected 14 bands, named A to N (Fig. 1, lane 4). In acrylamide gel electrophoresis and hybridization studies no additional bands were visible. On first examination a DNA band comparable to MV-L3* PstI B₂ (MV-L3 fragment B₂ from PstI digestion) was not detected in restriction patterns. However, in hybridization studies and in concentrated agarose gels (1.5% agarose), an additional very faint band in the gel was detected, which had been covered by MV-L3* XbaI M, leading to the designation M₂ for the minor band. Without regard to this fragment, the sum of fragment sizes from all fragments did not equal the value for the size of the native molecule as determined by electron microscopy. Restriction fragment sizes are summarized in Table 1. The HincII restriction pattern is defined by 14 bands, named A to M (Fig. 1, lane 2). In PAGE analysis no additional small bands could be detected. Fragment C is composed of two comigrating bands, which could be resolved in double digestions with two different restriction enzymes. These fragments have been named C₁ and C₂. They do not share homologies which could be demonstrated by hybridization studies.

An additional faint band comparable to the MV-L3* PstI B₂ or MV-L3* XbaI M₂ was not visible at first. Only when a double digestion with two restriction nucleases (HincII and PstI; Fig. 1, lane 1) was applied, a new faint band could be detected at the former position of MV-L3* HincII B. Thus the new band was named MV-L3* HincII B₂. The existence of this minor band was confirmed in hybridization studies indicating that it did not originate from partially cleaved material. Summing up fragment sizes, shown in Table 1, resulted in a value for the length of the physical map less than that of the native DNA molecule.

Digestion with nuclease KpnI resulted in a restriction pattern characterized by one major band and by at least three minor bands plus several additional very faint bands. The fragment size of the major band equals approximately 8800 bp, more than 25% of the length of a MV-L3 DNA molecule. The sum of all fragment sizes, including all minor bands, exceeds the genome length by at least twofold.

![Fig. 1. Restriction patterns of MV-L3-DNA with different restriction enzymes. Double digestions with these enzymes are included.](image)

**Table 1. Fragment sizes from different MV-L3 restriction patterns**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>XbaI</th>
<th>PstI</th>
<th>HincII</th>
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<tbody>
<tr>
<td>A</td>
<td>7440*</td>
<td>A1</td>
<td>A6636</td>
</tr>
<tr>
<td>B</td>
<td>6558</td>
<td>B2</td>
<td>B5459†</td>
</tr>
<tr>
<td>C</td>
<td>4883</td>
<td>B3</td>
<td>B12757</td>
</tr>
<tr>
<td>D</td>
<td>3230</td>
<td>C1</td>
<td>C5734</td>
</tr>
<tr>
<td>E</td>
<td>2621</td>
<td>C2</td>
<td>C5734</td>
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<td>D1</td>
<td>D1280</td>
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<tr>
<td>G</td>
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<td>D2</td>
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</tr>
<tr>
<td>H</td>
<td>1665</td>
<td>E1</td>
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<tr>
<td>I</td>
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<td>885</td>
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<td>F104</td>
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<td>G1</td>
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<td>G2</td>
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<tr>
<td>N</td>
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</table>

* Fragment size (bp). These are mean values from 25 gels at least. The standard deviation equals ± 0.92 to 2.6% (the latter value for small fragments).
† Fragment M₂ from the XbaI pattern, B₂ from the HincII pattern, and B₂ from the PstI pattern are minor bands in terms of their concentration in the gel.
Repeating the experiments with MV-L3* KpnI with longer incubation times and with higher enzyme concentration did not affect the restriction pattern. Using the enzyme to cleave cloned MV-L3* XbaI fragments or DNA from phage λ resulted in regular patterns, defined by equal fragment concentrations. Thus the unusual MV-L3* KpnI pattern reflects the structure of the viral DNA and is not influenced by the incubation procedure. Double digestions with either HinclI, PstI or XbaI with KpnI revealed that KpnI recognizes only two cleavage sites in MV-L3 DNA. Restriction enzyme assays with AluI, HpaI and EcoRI showed at least two bands. In combination with enzymes such as HinclI, XbaI or PstI, we could show that the first group of enzymes has only a single cleavage site in MV-L3 DNA.

To facilitate the construction of the physical map, we started to clone restriction fragments of individual restriction digests. Fragments were excised from the gel and electroeluted with a Biotrap device (Schleicher & Schüll). The purification of the probe by DEAE-Sephacel (LKB-Pharmacia) was as described by Maniatis et al. (1982). The MV-L3* PstI fragments were cloned via the shotgun technique into vector pAT153 (Twigg & Sherratt, 1980). The eluted MV-L3* XbaI fragments were cloned into an XbaI site, which had been introduced into the same vector by replacement of the PstI site by an XbaI linker (pATX153). The host for recombinant DNA was Escherichia coli strain C600 (Appleyard, 1954) grown in LB-broth (Miller, 1972). The plasmids were purified by NACS37 column chromatography (Gibco-BRL) or by caesium chloride–ethidium bromide density gradients. The alkaline lysis procedure prior to column chromatography was adapted from the accompanying manual for the NACS37 matrix (comparable to the published procedure of Birnboim & Doly, 1979). Column chromatography was according to the manufacturer’s manual except for the linear salt gradient ranging from 0.5 M-NaCl to 1.0 M-NaCl in Tris–EDTA 10/1 pH 7.2.

All restriction fragments from MV-L3* XbaI have been cloned into the modified vector pATX153 (Fig. 2). Only fragment M₂, which shares homology with fragment I, has not been cloned, because of its extremely low concentration. All clones have been proved to carry the particular fragment (in colony hybridization as well as in Southern blot analysis).

At first, attempts to clone the MV-L3* XbaI fragment I failed completely. Only some clones were obtained which carried the I fragment connected to another fragment of the XbaI restriction pattern. One of these clones, harbouring I and J, was used for further subcloning. After incomplete digestions of the recombinant plasmid and subsequent self-ligation, 5000 clones were tested. Only one single clone carried fragment I as shown in Fig. 2, lane I. However sequence studies showed that one terminal region of this clonable fragment I was no longer homologous to that of the ‘master’ fragment I, from which it was derived during subcloning. In Southern blots, however, this new fragment hybridized to fragment I. The sizes of the two fragments in question were indistinguishable on a gel. Due to the uncertain state of the clone discussed above, only the XbaI fragment I which orginally had been integrated into the vector together with fragment J was used for further studies. The I band was always cut out of the vector and separated from the other fragments by gel electrophoresis and subsequent electroelution. Filter hybridization studies were used to improve the data from the restriction analyses. Southern blots were performed on nitrocellulose filters using standard capillary soaking procedures. Probes for hybridization were labelled with
[\textsuperscript{32}P]dCTP or Biotin-11-dUTP (Gibco-BRL). The non-radiative detection system was as described in the manual for the BluGene detection kit (Gibco-BRL).

Probe fragment MV-L3* \textit{PstI} A hybridized to \textit{PstI} B2, to several smaller bands, and of course to \textit{PstI} A (data not shown). Hybridization to the MV-L3* \textit{XbaI} pattern revealed the existence of the band called M2. Fragments MV-L3* \textit{PstI} B1 and B2 are so similar in size that they could not be separated from each other. When jointly eluted, the mixture showed as expected hybridization signals with \textit{PstI} B1 and B2, but also with A. The names of fragments in Fig. 3 are indicated where needed, particularly in the case of the MV-L3* \textit{XbaI} pattern. In each lane of the blot shown in Fig. 3 it can be seen that strong and faint hybridization signals are produced. The strong ones derive from hybridization with B1, the other ones from B2. In addition, the faint bands hybridize only to those bands which show homology with MV-L3* \textit{PstI} A. Therefore B2 and A share homologous sequences.

Hybridization with MV-L3* \textit{XbaI} I led to an unexpected result: fragment I hybridized to itself and to M. Regarding the physical map of MV-L3 DNA in Fig. 4, it is obvious that this specific hybridization signal originates from the homology to M2. No similar results have been obtained with all other cloned probes. Another important result of hybridization studies was the finding that the faint bands MV-L3* \textit{HincII} B2 and MV-L3* \textit{PstI} B2 share homologous sequences with MV-L3* \textit{HincII} A and MV-L3* \textit{PstI} A, respectively. This indicates that faint bands of restriction patterns from \textit{HincII}, \textit{PstI} and \textit{XbaI} digestions are obviously derived from well-defined larger bands of the same restriction pattern, their precursor bands.

The computation of MV-L3 DNA restriction fragment sizes obtained with several restriction endonucleases, combined with double digestion analyses and hybridization studies, led to the construction of the physical map shown in Fig. 4. Fragments L and M from the restriction patterns \textit{PstI} and \textit{HincII}, respectively, could be mapped only in comparison with the map position of MV-L3* \textit{XbaI} B. By cleaving the latter fragment either with \textit{HincII}/\textit{XbaI} or with \textit{PstI}/\textit{XbaI} from the vector, it was always found that there was one very small band missing, namely \textit{PstI} L and \textit{HincII} M, respectively.

We could show that the restriction enzyme analysis of MV-L3 DNA led to unexpected results. In contrast to other linear DNA, e.g. DNA from bacteriophage \(\lambda\), there was not a single restriction pattern showing complete homogeneity in terms of the concentrations of fragments. Using restriction endonucleases with only one or two cleavage sites in MV-L3 DNA, fragmentation patterns were obtained which could not be explained easily. On the other hand, digestions with nuclease with several recognition sites results in a restriction pattern with at least one band of reduced concentration.

The mean value for the length of the physical map is 36.2 kbp. This differs by 3.2 kbp (equivalent to 8.1\% from the length of native MV-L3 DNA molecules, that is, 39.4 kbp as determined by electron microscopy. This fact can be explained by either internal duplication or terminal redundancy. Duplication of internal or terminal regions should be recognizable in gels, because certain fragments should than be present in twice the concentration with respect to others. We have never observed such cases. However in the \textit{HincII} restriction pattern a band with double intensity (C1/C2) has been found but later identified as consisting of two comigrating bands. Terminal redundancy has been found in many viral systems such as \textit{Salmonella} phage P22 (Tye \textit{et al.}, 1974a, \textit{b}) and \textit{Bacillus subtilis} phage SPP1 (Deichelbohrer \textit{et al.}, 1974).
an ethidium bromide-stained gel did not show any irregular fragment concentrations other than those discussed above. The minor fragment B₂ from the PstI restriction pattern was recovered. Additionally, the minor fragment B₂ from the HincII restriction pattern could also be identified after double digestion with HincII/PstI. In this case the dominant fragment B₁ was cleaved by PstI whereas the uncleaved HincII B₂ fragments remained at the original position. However, the minor fragment from the XbaI pattern, M₂, could not be detected by this method.

One of the best known bacteriophages having a circularly permuted and terminally redundant genome is Salmonella phage P22. For its DNA replication cycle the term 'pac' for packaging site has been introduced (Jackson et al., 1978a, b). With respect to the P22 model which has been supported by a variety of other bacteriophage systems (B. subtilis phage SPP1 (Deichlebohrer et al., 1982), E. coli phage P1 (Sternberg & Coulby, 1987a, b) we apply this term for our viral DNA system too. As already mentioned, restriction nucleases having several cleavage sites in MV-L3 DNA (e.g. PstI, HincII and XbaI) produce regularly concentrated bands but also one defined minor band. We assume that the regularly concentrated bands are produced by cuts with a restriction endonuclease whereas the single less concentrated fragment is due to the headful packaging cleavage at pac and one restriction site. Pac site-specific cleavage is assumed to occur in a concatemeric precursor DNA molecule during the processing of replicative intermediates. Concatemeric molecules have been found in several bacteriophage systems, particularly in those with circularly permuted and terminally redundant genomes. Haberer & Maniloff (1980) have reported fast sedimenting material isolated from A. laidlawii cells infected with MV-L3. These findings will be further studied in our laboratory.

From the relative positions of the restriction sites and from the discussion of the model, a circular representation of the physical map was derived (Fig. 4). The inner circle is a length scale. Physical maps for PstI, HincII and XbaI are shown as concentric circles. The inner part of the PstI map also carries the restriction sites for EcoRI, AluI, HpaI and KpnI. Bold lined sections represent the minor fragments of each restriction pattern, showing that all of them are located in the same area. The arrow points to the start site of packaging, the pac site, which is common to all replicative intermediate molecules to be processed and determines the left-hand side of the so-called minor fragments of the first unit molecule.

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References


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