The Large Viral RNA Segment of California Serogroup Bunyaviruses
Encodes the Large Viral Protein

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SUMMARY

Reassortant bunyaviruses derived from two members of the California serogroup (La Crosse/original and Tahyna/181-57) viruses were used to demonstrate that the large Mr viral protein (L) is encoded by the L RNA segment. Radiolabelled viral proteins were analysed by discontinuous SDS–PAGE. The L protein of La Crosse virus was observed to migrate ahead of its Tahyna virus counterpart when electrophoresed through a 5% acrylamide resolving gel. Among the reassortant viruses, the L protein phenotype segregated with the viral L RNA segment. After confirming the genotype of the viruses used in this study, it was concluded that the L RNA species of California serogroup viruses codes for the L protein, the presumed viral polymerase.

Bunyaviruses are enveloped viruses containing a tripartite, single-stranded RNA genome of negative polarity. The three viral RNA species are designated as the large (L RNA), medium (M RNA), and small (S RNA) segments (Obijeski et al., 1976b; Clewley et al., 1977). Among members of the bunaviruses genus, the S RNA codes for the nucleocapsid protein (N) and a non-structural protein (NSs) which are read from overlapping reading frames (Gentsch & Bishop, 1978; Bishop et al., 1982; Fuller et al., 1983). Two viral glycoproteins (G1 and G2) as well as another non-structural protein (NSm) are encoded by the M RNA segment (Gentsch & Bishop, 1979; Fuller & Bishop, 1982).

In addition to the three major bunyavirus structural proteins (N, G1 and G2), a large (L) protein is associated with the virion (Obijeski et al., 1976a). It has been assumed, but never proved, that the L protein is encoded by the L RNA and functions as the viral polymerase (Clerx-van Haaster et al., 1982; Schmaljohn et al., 1987). In this communication we report that the L RNA segment does indeed code for the L protein based upon SDS–PAGE analysis of viral polypeptides from two California serogroup bunyaviruses, La Crosse/original (LAC) and Tahyna/181-57 (TAH), as well as from selected LAC/TAH reassortant viruses.

SDS–PAGE analyses of reassortant viral proteins provides a powerful tool for delineating viral genome segment coding assignments, provided that the parent viruses exhibit phenotypic differences for their respective protein products. By using reassortants of LAC and snowshoe hare viruses, this approach has been successfully employed to map the N and NSm polypeptides to the appropriate RNA segments (Gentsch et al., 1977; Fuller & Bishop, 1982).

Six different plaque-purified viruses having the following L/M/S RNA segment compositions were employed in this study: LAC/LAC/LAC (parent LAC), TAH/TAH/TAH (parent TAH), LAC/TAH/TAH (clone B1-1a), TAH/LAC/LAC (clone P1-2gg), LAC/TAH/LAC (clone F2-18a) and TAH/LAC/TAH (clone B1-11a). The origin of these four reassortant viruses has been described previously (Janssen et al., 1986) and is summarized in Table 1. All but one of these

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Short communication

Table 1. Characterization of parental and reassortant viruses*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Source</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>LACoriginal</td>
<td>Parent</td>
<td>LAC/LAC/LAC</td>
</tr>
<tr>
<td>TAH/181-57</td>
<td>Parent</td>
<td>TAH/TAH/TAH</td>
</tr>
<tr>
<td>B1-1a</td>
<td>LAC/LAC/LAC  × TAH/TAH/TAH</td>
<td>TAH/LAC/LAC</td>
</tr>
<tr>
<td>P1-2gg</td>
<td>TAH/LAC/LAC  × TAH/TAH/LAC</td>
<td>TAH/LAC/LAC</td>
</tr>
<tr>
<td>F2-18a</td>
<td>LAC/LAC/LAC  × TAH/TAH/TAH</td>
<td>LAC/TAH/LAC</td>
</tr>
<tr>
<td>B1-11a</td>
<td>LAC/LAC/LAC  × TAH/TAH/TAH</td>
<td>TAH/TAH/TAH</td>
</tr>
</tbody>
</table>

* Adapted from Janssen et al. (1986).

Reassortants were generated by co-infection of BHK-21 cells with parent LAC and TAH viruses. The one exception was clone P1-2gg which was obtained by co-infecting BHK-21 cells with two reassortant viruses, TAH/LAC/TAH and TAH/TAH/LAC. Reassortant viruses were directly genotyped with respect to all three segments by a modification of an RNA–RNA hybridization procedure originally reported by Hay and co-workers (1979). These results have been previously published and are summarized in Table 1 (Janssen et al., 1986).

The structural proteins from these six different viruses were radiolabelled before SDS–PAGE analysis. Monolayers of BHK-21 cells were infected at an m.o.i. of 1 p.f.u./cell and viruses were labelled in the presence of 10 μCi of [3S]methionine per ml of infection medium (Gonzalez-Scarano, 1985). Viruses were precipitated from clarified supernatants with polyethylene glycol and purified by centrifugation (Obijeski et al., 1976a).

After purification, the viruses were diluted 1:1 in 2 × Laemmli sample buffer, boiled for 5 min, then quickly chilled on ice. Samples containing approximately equal c.p.m. of radioactivity were subjected to discontinuous SDS–PAGE (Laemmli, 1970). In order to detect differences in electrophoretic migration between LAC and TAH L proteins, a gel system was employed in which the stacking and resolving portions of the gel were 2-5% and 5% polyacrylamide, respectively. Radiolabelled G1 and N polypeptides were analysed by discontinuous gel electrophoresis on a 10% polyacrylamide gel. Gels were treated with En3Hance (New England Nuclear), dried and autoradiographed.

LAC and TAH L proteins could be distinguished by their different electrophoretic mobilities in the 5% polyacrylamide gel, with the LAC L protein migrating slightly faster than its TAH counterpart (Fig. 1, lanes 1 and 2). Examination of radiolabelled proteins from the four different reassortants revealed that the electrophoretic pattern of the L protein correlated with the L RNA segment (Fig. 1). For example, viruses whose only common RNA segment was the L RNA (i.e. LAC/LAC/LAC, LAC/TAH/TAH and LAC/TAH/LAC) all coded for L proteins of identical electrophoretic mobility (Fig. 1, lanes 1, 3 and 5). On the other hand, L proteins derived from viruses having a TAH L RNA segment (i.e. TAH/TAH/TAH, TAH/LAC/LAC and TAH/LAC/TAH) migrated more slowly in the same 5% polyacrylamide gel (Fig. 1, lanes 2, 4 and 6). Provided that each of the six viruses used in this study was genotypically pure, it may be concluded that the L RNA segment of California serogroup viruses codes for the viral L protein.

To confirm the genetic homogeneity of each of these viruses, two independent approaches were utilized. One method was to examine the radiolabelled G1 and N structural polypeptides on a 10% polyacrylamide gel. Since LAC and TAH viruses display differences in the mobilities of both of their G1 and N proteins when electrophoresed on a 10% polyacrylamide gel, the genotypic homogeneity of the M and S RNA segments from each of the six viruses could be inferred by examining their phenotypic purity. Such an analysis indicated that each virus was phenotypically homogeneous (Fig. 2). For example, each of the four reassortant clones possessed a G1 protein which displayed an electrophoretic mobility identical to either parent LAC G1 or to parent TAH G1, but not to both (Fig. 2). The same was also true with respect to the N polypeptides of these viruses (Fig. 2).

The second approach involved genotyping viral RNA by dot blot hybridization using cloned cDNA probes. The advantage of such a strategy was that it allowed all three RNA segments to be genotyped directly and was far less cumbersome than the RNA–RNA hybridization.
Fig. 1. Autoradiograph of California serogroup virion-associated L proteins. [35S]Methionine-labelled viral proteins were obtained from purified virions and separated by SDS-PAGE. The G1, G2 and N polypeptides were run off the 5% polyacrylamide gel and are not shown. Viruses containing a LAC L RNA segment all coded for L proteins of identical electrophoretic mobility; LAC/LAC/LAC (lane 1), LAC/TAH/TAH (lane 3) and LAC/TAH/LAC (lane 5). L proteins from viruses having a TAH L RNA segment migrated more slowly; TAH/TAH/TAH (lane 2), TAH/LAC/LAC (lane 4) and TAH/LAC/TAH (lane 6).

Fig. 2. Autoradiograph of California serogroup virion-associated polypeptides. [35S]Methionine-labelled viral proteins were obtained from purified virions and separated by SDS-PAGE on a 10% polyacrylamide gel. The phenotypic purity of the G1 and N polypeptides from each of the following viruses is evident: LAC/LAC/LAC (parent LAC), lane 1; TAH/TAH/TAH (parent TAH), lane 2; LAC/TAH/TAH, lane 3; TAH/LAC/LAC, lane 4; LAC/TAH/LAC, lane 5; and TAH/LAC/TAH, lane 6.
Fig. 3. The genotyping of California serogroup reassortants by dot blot hybridization. Three serial threefold dilutions of cytoplasmic extracts obtained from cells infected with either LAC, TAH or various LAC/TAH reassortants were spotted in duplicate on separate nitrocellulose filters. The filters were hybridized to an L segment probe (pLAC 4.16), an M segment probe (pLAC 4.27), or an S segment probe (pLAC 4C-26). Cells were infected with one of the following viruses: LAC/LAC/LAC (parent LAC), lane 1; TAH/TAH/TAH (parent TAH), lane 2; LAC/TAH/TAH, lane 3; TAH/LAC/LAC, lane 4; LAC/TAH/LAC, lane 5; and TAH/LAC/TAH, lane 6.

Table 2. Characterization of La Crosse virus recombinant clones used in dot blot hybridization

<table>
<thead>
<tr>
<th>Recombinant plasmid*</th>
<th>Segment specificity</th>
<th>Vector</th>
<th>Size of insert (bases)</th>
<th>Method of cloning</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLAC 4.16</td>
<td>L RNA</td>
<td>pBR322</td>
<td>1100</td>
<td>BamHI linker</td>
</tr>
<tr>
<td>pLAC 4.27</td>
<td>M RNA</td>
<td>pBR322</td>
<td>3600</td>
<td>BamHI linker</td>
</tr>
<tr>
<td>pLAC 4C-26</td>
<td>S RNA</td>
<td>pBR322</td>
<td>981</td>
<td>GC tailing</td>
</tr>
</tbody>
</table>


procedure developed by Hay et al. (1979). Pringle and co-workers were the first to demonstrate the usefulness of dot blot hybridization for genotyping bunyavirus reassortants (Pringle et al., 1984). Using this simple yet rapid technique, they were able to determine the parental origin of the L and M RNA segments for reassortants generated by heterologous crosses among members of the Bunyamwera serogroup.

We used three LAC cDNAs to genotype LAC/TAH reassortants with respect to L, M and S RNA segments. Each cDNA was specific for a different RNA segment by Northern analysis and could clearly distinguish LAC from TAH viral RNA in dot blot hybridizations. The characteristics of these cDNA probes are summarized in Table 2. Cabradilla et al. (1983) cloned LAC 4C-26, a cDNA corresponding to the entire S RNA subunit of LAC virus. The L and M segment-specific cDNA transcripts were constructed in our laboratory (D. R. Jacoby & F.
Gonzalez-Scarano, unpublished results). All three cDNAs were cloned into pBR322. Plasmid DNAs were purified, radiolabelled with \( \alpha^{-32}\text{P} \)dCTP according to the oligolabelling procedure of Feinberg & Vogelstein (1984), and used to probe viral RNA immobilized on nitrocellulose.

Total cytoplasmic RNA obtained from infected BHK cell lysates served as the source of viral RNA. Monolayers of BHK cells were infected at a multiplicity of 10 p.f.u./cell with the same parental and reassortant virus stocks used to prepare the radiolabelled viral proteins. After incubation at 37 °C for 7 h, total cytoplasmic RNA was prepared according to the methods of White & Bancroft (1982). Samples were blotted to nitrocellulose filters using a 96-well manifold (Bio-Dot; Bio-Rad), dried, and then baked in vacuo for 2 h at 80 °C. Filters were prehybridized in a solution containing 5 × SSC (1 × SSC is 150 mM-NaCl, 15 mM-sodium citrate), 5 × Denhardt’s solution, 50 mM sodium phosphate, 250 µg/ml of salmon sperm DNA, and 50% formamide for 6 to 8 h at 42 °C. Radiolabelled recombinant plasmid was added to the hybridization solution (5 × SSC, 1 × Denhardt’s solution, 20 mM-sodium phosphate, 50% formamide) and filters were hybridized at 42 °C for 24 to 36 h. Subsequently, filters were washed once with 3 × SSC, 0-1% SDS at 42 °C. This was followed by two washes with 3 × SSC, 0-1% SDS, three washes with 1 × SSC, 0-1% SDS, and three washes with 0-1 × SSC, 0-1% SDS. These washes were performed at room temperature for hybridizations employing L or M RNA segment-specific probes and at 56 °C when the LAC S RNA segment cDNA was used. Filters were then autoradiographed using intensifying screens.

Dot blot analyses of viral RNA confirmed the genotypic purity for all six viruses used in this study (Fig. 3).

Through the use of LAC/TAH reassortant viruses in conjunction with SDS–PAGE analysis of viral proteins, differences in L protein electrophoretic migration have been clearly shown to segregate with the L RNA segment. Having established both the phenotypic and genotypic homogeneity of each of the viruses employed in this study, it was concluded that the L RNA segment of California serogroup viruses does indeed encode the L protein. Such a finding, though not surprising, provides the first experimental evidence in support of the long held assumption that the L protein of bunyaviruses is encoded by the L RNA segment.

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Short communication


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