The Design and Use of Specific Genetic Probes to Identify Closely Related Bunyaviruses and to Determine the Genotype of Their Recombinants

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(Accepted 14 April 1989)

SUMMARY

Viruses that are very closely related to each other at the genetic and gene product level can prove difficult to distinguish, although they may differ in phenotype (for example in their virulence or vector preferences). A chimeric genetic probe has been developed and tested to distinguish the S RNAs of two closely related bunyaviruses, snowshoe hare and La Crosse viruses. The technique is applicable to other RNA species of these two bunyaviruses.

It is commonly found that viruses which are genetically similar but which differ in phenotype may be distinguished only by sophisticated procedures such as oligonucleotide fingerprint analyses, neutralization of infectivity kinetics, monoclonal antibody (MAb) or virulence assays. Even in the use of serology, genotype analyses of recombinant progeny obtained from genetically similar parents can be difficult if the required tools are not available. An example is afforded by the two bunyaviruses, snowshoe hare (SSH) and La Crosse (LAC) (California serogroup, Bunyavirus genus, Bunyaviridae). SSH virus is considered to be a variant of LAC virus although it is usually carried by different species of mosquitoes and occurs at more northerly latitudes (Calisher, 1983) of the North American continent (e.g. in arctic regions). In the U.S.A. LAC virus is responsible, on occasion, for fatal human infections and paediatric illnesses involving seizures (Gundersen & Brown, 1983; Thompson et al., 1965). Occasionally SSH virus also infects man although these infections are considered less serious (Fauvel et al., 1980).

Bunyaviruses have genomes consisting of three species of negative-stranded RNA (L, M and S; Obijeski et al., 1976; Clewley et al., 1977; Gentsch et al., 1977a; Bishop & Shope, 1979). The L RNA codes for the viral L protein, a putative transcriptase component (Bishop & Shope, 1979; Endres et al., 1989). The M RNA codes for the viral glycoproteins (G1, G2) that are synthesized as a polyprotein precursor (Gentsch & Bishop, 1979; Eshita & Bishop, 1984; Grady et al., 1987; Fazakerley et al., 1988). The S RNA codes for the viral nucleocapsid protein (N) and a non-structural protein (NS5) read from an overlapping reading frame in the complementary S mRNA species (Gentsch & Bishop, 1978; Cash et al., 1979; Bishop et al., 1982; Fuller & Bishop, 1982; Akashi & Bishop, 1983; Fuller et al., 1983; Cabradilla et al., 1983). Related bunyaviruses such as SSH and LAC can recombine and produce new genotypes through the process of RNA segment reassortment (eight possible genotype combinations). For SSH and LAC viruses all eight genotypes have been identified using the appropriate temperature-sensitive (ts) mutants, progeny virus cloning and oligonucleotide fingerprinting procedures (Gentsch et al., 1977b, 1979, 1980; Rozhon et al., 1981). However, particular SSH–LAC virus reassortants occur only at low frequency (Rozhon et al., 1981). Similar data have been recorded for other bunyaviruses (Iroegbu & Pringle, 1981a, b; Pringle & Iroegbu, 1982; Pringle et al., 1984; Elliott et al., 1984).

Reassortant bunyaviruses are useful for the analyses of tropisms of virus infections in mosquitoes and in vertebrate hosts (Beaty et al., 1981, 1982; Shope et al., 1981; Tignor et al., 1983). However, reassortants generated through the use of mutagen-induced (or even spontaneous) ts mutants may contain silent mutations in the RNA species lacking the ts
mutation (Rozhon et al., 1981). Such mutants can affect the interpretation of data obtained with the reassortant viruses. Alternative procedures are required to distinguish reassortant viruses from wild-type virus in coinfections. For SSH and LAC viruses neither serological procedures nor DNA hybridization procedures are applicable as the viruses are too closely related. SSH virus is not readily distinguished from LAC virus by polyclonal antibodies using neutralization of infectivity or other serological assays. Certain MAbs raised against the glycoproteins or the nucleocapsid protein of LAC virus allow the gene products of the M and S RNA species of the parent viruses (and presumably their reassortants) to be identified (Gonzalez-Scarano et al., 1982, 1983; Grady et al., 1983). However no MAbs against the LAC (or SSH) L proteins of these viruses have been isolated. Consequently it has not been possible to distinguish the L genes of the parent reassortant viruses by serological procedures.

The sequences of DNA clones representing the S RNA species of LAC and SSH viruses have been published (Bishop et al., 1982; Akashi & Bishop, 1983; Cabradilla et al., 1983). Sequences of the M RNA species of the two viruses have been described (Eshita & Bishop, 1984; Grady et al., 1987). Partial sequence analyses of the L RNAs have also been reported (Clerx-van Haaster & Bishop, 1980; Clerx-van Haaster et al., 1982). Based on these data the genomes of the two viruses are very similar, as illustrated by Diagon analyses (Staden, 1986) of the S RNA species of the two viruses shown in Fig. 1. Of a total of 982 to 984 nucleotides for the S RNA species, there are 114 nucleotide differences between prototype SSH and LAC viruses. Of these, six are insertions or deletions and 108 are substitutions. Because of this similarity it is not possible to distinguish the S RNAs of parent or progeny viruses by conventional hybridization procedures using cloned DNA representing the entire genome and dot blot analyses (unpublished data) or Northern blot hybridization procedures (Fig. 2a) analogous to those used for other bunyaviruses (Pringle et al., 1984; Endres et al., 1989). A similar problem pertains for the SSH and LAC M RNA species (Eshita & Bishop, 1984; Grady et al., 1987) and probably for the L RNA species, based on the limited L RNA sequence data available (Clerx-van Haaster & Bishop, 1980; Clerx-van Haaster et al., 1982). In certain cases, it has been possible to deduce the genotypes of reassortant SSH/LAC viruses (described as the L/M/S genotypes, e.g. SSH/LAC/SSH or S/L/S)
Fig. 2. Use of riboprobes representing equivalent 634 nucleotide long LAC and SSH viral sequences (LAC S residues 139 to 773, and the equivalent SSH S residues 137 to 771) to identify viral S RNA in extracts of virus-infected cells (a). Vero cells (1 × 10^7) were infected at multiplicities of 20 p.f.u./cell with each of the parent (LAC or SSH) or reassortant viruses (L/M/S RNA genotypes; e.g. S/S/L has the L and M RNA species of SSH virus and the S RNA of LAC virus). RNA was phenol-extracted from cytoplasmic preparations at 24 h post-infection. RNA samples (a) were run on 1% agarose containing 10 mM-methylmercury (Bailey & Davidson, 1976) and the RNA was transferred to Hybond-N membranes, fixed by exposure to u.v. light, prehybridized for 2 h with 5 × SSC, 2 × Denhardt's solution, 50% formamide, 0·1% SDS and 250 μg/ml of yeast RNA (Maniatis et al., 1982) then hybridized in the same solution containing approx. 4 × 10^6 c.p.m. of [α-32P]UTP-labelled LAC (top) or SSH (lower) riboprobes for 12 to 16 h at 42 °C. Membranes were washed twice in 0·1 × SSC-1% SDS at 65 °C for 1 h, then autoradiographed. In (b) the authenticity (at least with respect to the S-encoded N proteins) of each virus was assessed by SDS-PAGE analyses (Laemmli, 1970; Fuller & Bishop, 1982). A similar discrimination does not pertain to the M- and L-encoded gene products of these two viruses (Gentsch & Bishop, 1979; unpublished data).

It has been observed that the sequence differences between SSH and LAC S (or M) RNA species are not randomly dispersed in the viral genome (Akashi & Bishop, 1983). Two regions of the S sequences of these viruses exhibit more variation than elsewhere. This is illustrated in Fig. 1 (a, b). In view of this observation and because individual divergence at either site was not extensive, chimeric oligonucleotide sequences representing these regions were synthesized chemically (Fig. 3). In order to generate riboprobes for Northern blot hybridization (or dot blot) procedures the oligonucleotides were cloned into the plasmid pSPT18 (Pharmacia). To
Short communication

Fig. 3. Sequences used to prepare SSH and LAC chimeric oligonucleotide probes. The origins of the LAC and SSH oligonucleotide probes that were backcopied and cloned (see text) are indicated in relation to the coding strategy of the S RNA species of these viruses. The sequences of the cloned species between the BamHI sites were confirmed by sequence analyses (Maxam & Gilbert, 1980).

accomplish this, the LAC oligonucleotide probe was synthesized so that it consisted of an in-tandem sequence representing LAC virus-complementary S nucleotide (nt) residues 56 to 83 and 784 to 815. The corresponding SSH probe was represented by SSH virus-complementary nt residues 55 to 80 and 781 to 814, also in tandem (Bishop et al., 1982; Akashi & Bishop, 1983). The ends of each synthetic oligonucleotide included additional nucleotide sequences to allow the cloning into a BamHI site of the plasmid vector as well as a homologous region of the viral sequences to allow each oligonucleotide to be backcopied with a synthetic primer (TTGGATCCTAATTTG). After 5' phosphorylation of all the oligonucleotides, the chimeric species were backcopied into dsDNA (Maniatis et al., 1982). The products were found to be resistant to digestion by BamHI although they were shown by gel analyses to be double-stranded and of the expected length (data not shown). The reason the product could not be digested is not known but was probably due to the chemically synthesized component; when mixed with plasmid DNA containing BamHI sites the oligonucleotides remained undigested and the plasmid DNA was digested to completion (data not shown). Using standard procedures (Maniatis et al., 1982), the ds oligonucleotide samples were blunt-end cloned into the dephosphorylated SmaI site of plasmid pSPT18, recombinants were isolated and the inserts sequenced, thereby confirming that the inserted sequences were as expected. After BamHI digestion and insertion into the BamHI site of pSPT18 the plasmids were again sequenced to verify the orientation and sequences of the oligonucleotide probes. As controls, the 634 bp LAC and SSH SfnA DNA fragments were recovered from their respective DNA clones (Bishop et al., 1982; Akashi & Bishop, 1983; i.e. SSH residues 137 to 771 and LAC residues 139 to 773), ligated into the SmaI sites of pSPT18 and recombinants were analysed by restriction enzyme analyses to determine the orientation of the inserted sequences.

Virus-complementary RNA probes were synthesized from the cloned DNAs in the presence of [α-32P]UTP (800 Ci/mmol) using T7 RNA polymerase (Pharmacia) after linearization of plasmid DNA with HindIII. Alternatively, DNA samples were cleaved with EcoRI and transcribed with SP6 polymerase (Bethesda Research Laboratories). The RNA products, representing the virus-complementary RNA sequences (to hybridize to viral RNA) were purified by phenol extraction and gel filtration prior to use in Northern blot analyses. Cytoplasmic RNA preparations of SSH, LAC and six SSH–LAC reassortant viruses were prepared, resolved by electrophoresis in 1% agarose gels containing 10 mM-methylmercury (Bailey & Davidson, 1976), transferred to Hybond-N membranes (Amersham), fixed to the membrane and probed with radioactive RNA transcripts (Maniatis et al., 1982). The results for the chimeric probes are shown in Fig. 4 and the data for the longer DNA probes are shown in Fig. 2(a).

The results of our analyses unambiguously demonstrated that the SSH virus S RNA could be distinguished from that of LAC virus using the oligonucleotide probes but not when the longer
Northern blot analyses of cytoplasmic RNA recovered from SSH and LAC parent and reassortant viruses (see Fig. 2a) using radiolabelled riboprobes representing the LAC and SSH chimeric oligonucleotide sequences. The conditions used for RNA preparation and Northern blot analyses are as described for Fig. 2(a).

cDNA probes were used. Although single, non-chimeric probes could have been used (representing for instance the 18 residue sequence variation in the right-hand region of the oligonucleotides shown in Fig. 3), the chimeric species were prepared to increase the discriminatory power of the riboprobes and to allow less stringent hybridization conditions to be used (due to the increased length of the probes and the increased sequence diversity). Discriminatory sequences for the SSH and LAC M and L RNA species have been identified from the published (and unpublished) data to allow probes to be prepared for the two other RNA species of each virus. The development and use of these probes to identify particular SSH-LAC reassortants derived from SSH and LAC wild-type virus infections is currently under investigation.

The work was supported by contract DAMD17-87-C-7069.

REFERENCES


On: Sun, 11 Nov 2018 08:13:15


(Received 13 March 1989)