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# Recombination Between Snowshoe Hare and La Crosse Bunyaviruses

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We have previously reported heterologous genetic recombination resulting from crosses involving temperature-sensitive (*ts*) mutants of La Crosse (LAC) group II and snowshoe hare (SSH) group I *ts* mutants (J. Gentsch, L. R. Wynne, J. P. Clewley, R. E. Shope, and D. H. L. Bishop, J. Virol. **24**: 893-902, 1977). From those crosses two reassortant viruses having the large/medium/small viral RNA segment genotypes of SSH/LAC/SSH and SSH/LAC/LAC were obtained. In this study it has been found that the reciprocal cross (SSH group II × LAC group I *ts* mutants) has not yielded the expected LAC/SSH/SSH or LAC/SSH/LAC reassortant viruses. The backcross of a SSH/LAC/SSH group II *ts* mutant with a LAC group I *ts* mutant has produced a new reassortant virus, LAC/LAC/SSH, whereas the backcross of SSH/LAC/LAC group I *ts* mutants with SSH group II *ts* mutants gave another reassortant, SSH/SSH/LAC. Backcross analyses of LAC/LAC/SSH group I *ts* mutants with group II *ts* mutants of SSH have not yielded the expected LAC/SSH/SSH reassortant virus, nor have backcrosses of SSH/SSH/LAC group II *ts* mutants with group I *ts* mutants of LAC virus yielded the expected LAC/SSH/LAC reassortant. Possible reasons why certain reassortant viruses are not produced are discussed. A procedure to screen SSH-LAC reassortant viruses which differ in their virion N polypeptides is described.

Molecular studies have established that the Bunyaviridae family of arthropod-borne viruses characteristically have a genome consisting of three unique species of RNA (4, 7, 11, 20, 22-24) designated large (L), medium (M), and small (S). Using closely related viruses, we have initiated a study of the heterologous genetic recombination potential of bunyaviruses with the object of subsequently studying distantly related viruses to define the genetic capabilities of members of Bunyaviridae.

Heterologous virus genetic recombination experiments with certain temperature-sensitive (*ts*), conditional lethal mutants of snowshoe hare (SSH) and La Crosse (LAC) viruses have shown that wild-type SSH-LAC reassortant viruses can be formed with the L/M/S genotypes of SSH/LAC/SSH and SSH/LAC/LAC (9, 12). Serological studies have suggested that SSH and LAC viruses can be considered as varieties of the LAC subtype of California encephalitis (CE) serotype virus (2, 6, 13, 16, 18, 27, 28). Serological studies also indicate that other subtypes of CE are Tahyna (varieties: Tahyna and Lumbo, Inkoo, San Angelo, and CE viruses (1, 2, 5, 6, 13, 17, 18, 25-28). Together with the serotypes Trivittatus and Melao—the latter having the subtypes Melao, Serra do Navio, Keystone, and Jamestown Canyon (varieties: Jamestown Canyon and Jerry Slough viruses)—the three serotypes are considered as a complex of viruses (CE complex) within the CE serogroup (2, 3, 8, 14, 15, 18, 25, 26, 27).

The demonstration that particular *ts* mutants of SSH and LAC viruses can produce reassortant viruses raises the question of whether all forms of SSH-LAC reassortants can be generated. From a coinfection involving two viruses with three RNA segments, one might expect to produce $2^3$ genotypes (i.e., 8) which, minus the two progenitors, would include 6 new genotype combinations. The heterologous genetic capabilities of SSH and LAC viruses have been investigated in this study. Although four SSH-LAC reassortant viruses have been generated from SSH and LAC virus coinfections, including backcross analyses with SSH-LAC reassortant viruses, two expected reassortant genotypes (LAC/SSH/SSH and LAC/SSH/LAC) have not been recovered. Possible reasons for our inability to obtain or detect these reassortant viruses are discussed.

## MATERIALS AND METHODS

**Materials.** Acrylamide and bis-acrylamide were obtained from Serva Laboratories through Accurate Chemical Corp., Hicksville, N.Y. Sankyo RNase T1

was obtained through Calbiochem, La Jolla, Calif. Radioisotopes came from I.C.N., Irvine, Calif. 5-Fluorouracil was purchased from Sigma Chemical Co., St. Louis, Mo.

**Viruses.** The origins of prototype SSH and LAC and the derivation of their spontaneous or 5-fluorouracil-induced *ts* mutants have been described elsewhere (2, 6, 10, 11, 12, 16, 28). Recombinant and putative recombinant viruses were plaque cloned in BHK-21 cells, working stocks were obtained, and their plaquing efficiencies at 33 and 39.8°C were determined as described elsewhere (9, 12).

**Isolation of *ts* conditional lethal virus mutants.** The protocols used for obtaining working stocks of *ts* conditional lethal mutants of SSH, LAC, SSH/LAC/LAC, SSH/LAC/SSH, LAC/LAC/SSH, and SSH/SSH/LAC viruses by growth in the presence of the mutagen 5-fluorouracil followed the procedures described previously (9, 12). For most recombination experiments, only those mutant virus stocks were used that had nonpermissive temperature (39.8°C) plaquing efficiencies of less than 0.01% (PFU assay at 39.8°C ÷ PFU assay at 33°C), and growth efficiencies of less than 0.01% (progeny PFU after growth of infected cells at 39.8°C ÷ progeny PFU after growth of infected cells at 33°C; progeny from both were assayed at 33°C).

**Recombination assays.** Recombination experiments involving single and dual mutant virus infections in BHK-21 cells at 33°C and assaying the 24-h progeny virus at 33 and 39.8°C were performed as described previously (9, 12). The recombination percentages were calculated as described by Gentsch and associates (9, 12).

**Virus purification.** The procedures used to obtain $^{32}$P-labeled virus have been described (7, 11, 19-21).

**Purification of labeled viral RNA species, RNase T1 digestion of RNA, and separation of oligonucleotides by two-dimensional gel electrophoresis.** The procedures used for extraction and purification of $^{32}$P-labeled bunyavirus individual L, M, and S RNA species have been described (8a). RNase T1 digestion of viral RNA and the resolution of the products by two-dimensional polyacrylamide gel electrophoresis, as well as the procedures used to obtain an autoradiographic fingerprint, have also been described (7).

## RESULTS

**Recombination between SSH and LAC *ts* mutants.** Coinfections of BHK-21 cells with SSH group I *ts* mutants and LAC group II *ts* mutants have led to the recovery of two L/M/S RNA genotype reassortant viruses, namely SSH/LAC/LAC and SSH/LAC/SSH (12). These results indicate that LAC group II *ts* mutants have a defective L RNA segment, whereas SSH group I *ts* mutants have a defective M RNA, and that the S RNA of each virus segregates independently of the L or M RNA species (12). Although we have not obtained any group III *ts* mutants (presumptive S RNA mutants), the reciprocal cross (i.e., SSH group II and LAC group I *ts* mutants), has been investigated with regard to the possibility of generating LAC/SSH/LAC and LAC/SSH/SSH reassortant viruses (Table 1).

No wild-type plaques were obtained from the harvests of dual virus infections involving SSH group II and LAC group I *ts* mutants (Table 1). From some of the crosses (e.g., LAC I-23 × SSH II-2) a few plaques were observed on the 39.8°C plates receiving the lowest dilution of progeny virus. They were recovered, and virus stocks were grown. For two of these cloned progeny, fingerprint analyses were undertaken. Both viruses were found to have a LAC/LAC/LAC genotype. When correlated with the 39.8°C/33°C efficiency of plating ratios of the two virus stocks (0.1 and 0.5), the results indicate that these progeny viruses probably represented revertant LAC viruses. From a LAC I-9 and SSH II-18 *ts* mutant coinfection, fingerprint analysis of a cloned progeny virus also indicated that it was a LAC revertant.

**Search for SSH group III *ts* mutants; isolation and characterization of a SSH group I/II *ts* mutant.** To select certain SSH-LAC recombinant viruses (e.g., SSH/SSH/LAC) we have sought to isolate SSH group III *ts* mutants. Two procedures have been used to obtain SSH group III *ts* mutants. The first procedure involved recombination assays of new SSH *ts* mutant virus stocks with known SSH group I and group II *ts* mutants and an analysis of the results for viruses which recombined with both group I and II *ts* mutants (9, 12). The second procedure involved recombination assays of new SSH *ts* mutant virus stocks with a group I/II double mutant. The characterization of a group I/II double mutant (SSH *ts* 31) is described below.

Initial tests of SSH *ts* 31 with selected SSH group I and SSH group II *ts* mutants (*ts* I-1, *ts* I-3, *ts* II-2, and *ts* II-18) indicated that *ts* 31 did

TABLE 1. *Recombination analyses with SSH and LAC ts mutants*

| LAC *ts* mutant | % Recombination with SSH *ts* mutants[a] | | | |
|---|---|---|---|---|
| | SSH I-1 | SSH I-3 | SSH II-2 | SSH II-18 |
| LAC I-9 | 0 | 0 | 0 | 0 |
| LAC I-13 | 0 | 0 | 0 | 0 |
| LAC II-3 | 4 | 5 | 0 | 0 |
| LAC II-4 | 7 | 6 | 0 | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0, indicating that the frequency of $ts^+$ virus in the mixed infection did not exceed the levels of $ts^+$ (e.g., revertants) detected in the single infection titers.

not recombine with any of these mutants. To determine whether *ts* 31 was a multiple mutant representing group I and II defects or even a triple mutant with group I, II, and III defects, a backcross of *ts* 31 with wild-type SSH virus was undertaken at 33°C (5 PFU per cell for each virus), and the progeny were screened for *ts* mutants. Of 32 progeny clones examined, 13 were found to be *ts* (40%), and the rest had wild-type virus efficiency of plating values. Recombination analyses with representative SSH group I mutants (*ts* I-1, *ts* I-3) and group II mutants (*ts* II-2, *ts* II-18) established that 2 of the 13 *ts* mutants did not recombine with either the SSH group I or group II mutants (i.e., like *ts* 31). Nine of the remaining mutants recombined with the group I mutants, and the other two recombined only with the group II mutants; none of these 11 mutants recombined with both the SSH group I and group II mutants. The high frequency of group I and group II progeny mutants recovered from the SSH *ts* 31 and wild-type virus cross indicated that SSH *ts* 31 is a double (group I/II) *ts* mutant.

Screening new SSH *ts* mutant virus stocks with either the group I/II double *ts* mutant or representative group I and group II *ts* mutants, has not yet yielded any viruses which can be considered as group III mutants. To date 65 SSH *ts* mutants have been analyzed and categorized into either group I (31 mutants), group II (24 mutants), group I/II (1 mutant), or possible multiple mutants (9 mutants).

**Analyses of wild-type SSH and LAC coinfections for recombinant SSH-LAC viruses.** In view of the absence of detectable recombination between SSH group II and LAC group I *ts* mutants and considering the possibility that the lack of recombination may be the result of trivial causes (e.g., defective viruses inhibiting productive infections), the progeny from a coinfection involving wild-type SSH and LAC viruses were cloned, virus stocks were produced, and their L, M, and S oligonucleotide fingerprints were obtained. From eight plaque-cloned virus stocks analyzed by this procedure, fingerprint analyses indicated that three had the genotype LAC/LAC/LAC (Fig. 1B) and three were SSH/SSH/SSH (Fig. 1A), whereas one virus had a SSH/LAC/LAC genotype (Fig. 1C) and another had a SSH/LAC/SSH genotype (Fig. 1D). Although this procedure resulted in the recovery and characterization of two recombinant viruses, the amount of work entailed in obtaining the results precluded using it to screen large numbers of progeny for new SSH-LAC recombinants.

**Backcrosses of SSH/LAC/SSH *ts* mutants with SSH and LAC *ts* mutants and the recovery of a LAC/LAC/SSH reassortant virus.** As an alternative approach to obtaining new SSH-LAC recombinant viruses, backcrosses of SSH/LAC/SSH and SSH/LAC/LAC *ts* mutants with LAC and SSH *ts* mutants have been employed.

*ts* mutants of wild-type SSH/LAC/SSH were derived by growth of the wild-type SSH/LAC/SSH virus in the presence of 50 μg of 5-fluorouracil per ml of growth medium. The cloned mutant virus stocks were categorized into groups by reciprocal recombination tests (Table 2). Out of seven *ts* mutants isolated, one was assigned to one group, and six were assigned to a second group.

Backcrosses of the mutants SSH/LAC/SSH *ts* 9 and *ts* 13, with SSH and LAC *ts* mutants (SSH I-3, SSH II-21, LAC I-16, and LAC II-2) were undertaken with the results shown in Table 3. Because the SSH/LAC/SSH *ts* 9 mutant only recombined with LAC and SSH group II mutants, it was categorized as an SSH/LAC/SSH group I mutant (Table 2). Likewise, because the SSH/LAC/SSH *ts* 13 mutant recombined only with LAC and SSH group I mutants, it was categorized as an SSH/LAC/SSH group II mutant (Table 2).

On the assumption that group I mutants of LAC, SSH, and SSH/LAC/SSH viruses have defective M RNA species, whereas group II mutants of those viruses have defective L RNA species, the following predictions were made. (i) The wild-type progeny of the SSH/SSH*/SSH × SSH*/LAC/SSH cross (i.e., SSH I × SSH/LAC/SSH II) should contain only SSH/LAC/SSH progeny. (ii) The wild-type progeny of the SSH*/SSH/SSH × SSH/LAC*/SSH cross (i.e., SSH II × SSH/LAC/SSH I) should contain only SSH progeny. (iii) The wild-type progeny of the LAC*/LAC/LAC × SSH/LAC*/SSH cross (i.e., LAC II × SSH/LAC/SSH I) should contain both SSH/LAC/SSH and SSH/LAC/LAC recombinants (i.e., like those obtained originally from the LAC II × SSH I crosses). (iv) The wild-type progeny of the LAC/LAC*/LAC × SSH*/LAC/SSH cross (i.e., LAC I × SSH/LAC/SSH II) should contain both LAC virus and the recombinant LAC/LAC/SSH.

These assumptions and predictions could be tested by plaque cloning representative wild-type progeny from the different crosses and subsequent fingerprint analyses. Some of these predictions were tested as follows. For a plaque-cloned virus stock obtained from the SSH I-3 × SSH/LAC/SSH II-13 cross, fingerprint analyses indicated that it had, as predicted, an SSH/LAC/SSH genotype (Fig. 2B). For a plaque-

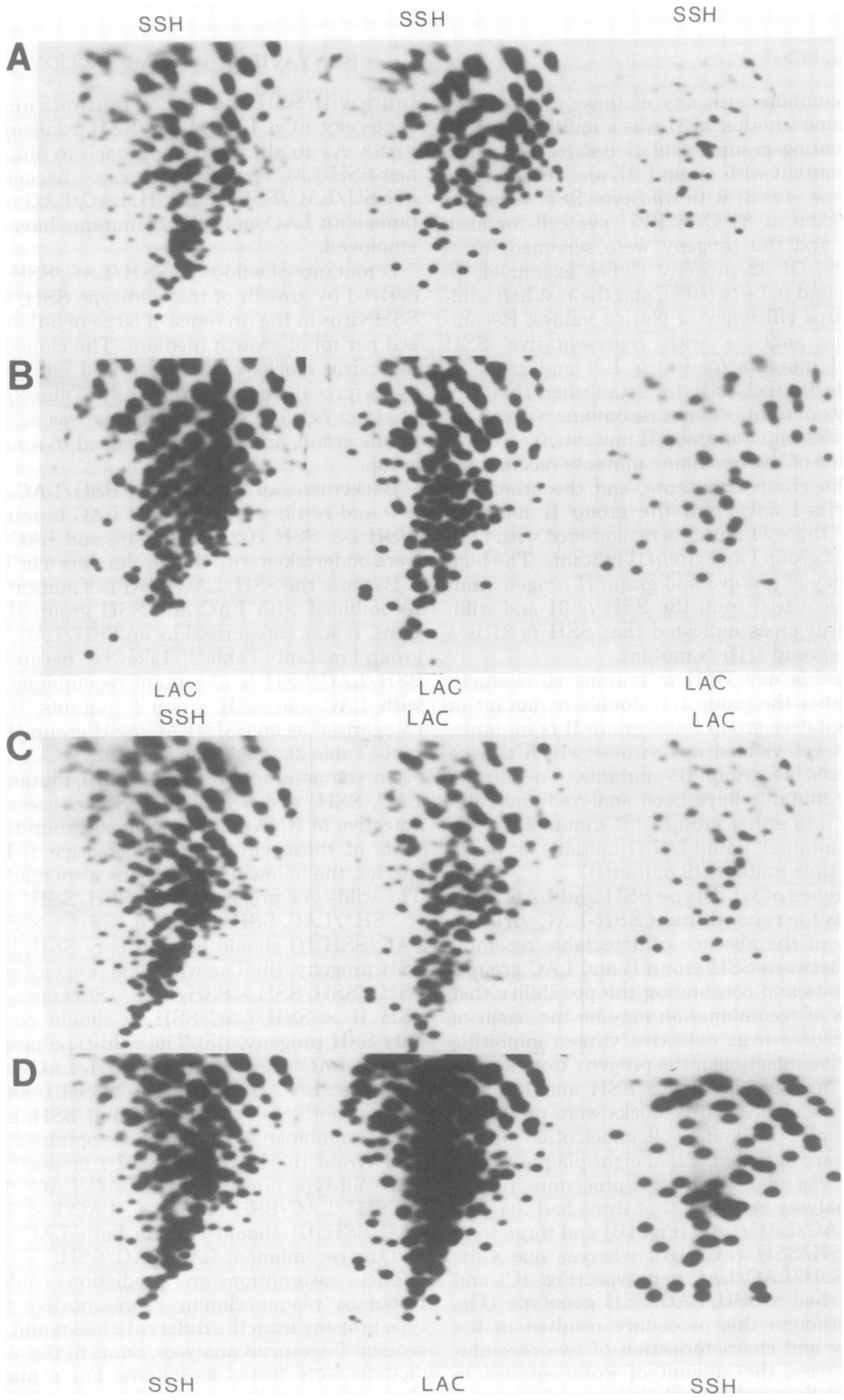


FIG. 1. *Oligonucleotide fingerprint analyses of cloned progeny from wild-type SSH and LAC virus coinfections. The L, M, and S RNA species obtained from cloned progeny virus derived from dual wild-type SSH and LAC coinfections were digested with ribonuclease T1, and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as described previously. The L (left), M (center), and S (right) RNA fingerprints are shown for four cloned progeny virus stocks (A, B, C and D). The L/M/S genotype of (A) is SSH/SSH/SSH, for (B) it is LAC/LAC/LAC, for (C) it is SSH/LAC/LAC and for (D) it is SSH/LAC/SSH.*

cloned virus stock obtained from a LAC I-16 × SSH/LAC/SSH II-13 cross, fingerprint analyses indicated that the virus had a genotype of LAC/LAC/SSH (Fig. 2A). No cloned virus stocks from other crosses were analyzed since the object of these experiments was to determine whether such a recombinant could be generated.

**Backcrosses of SSH/LAC/LAC *ts* mutants with SSH and LAC *ts* mutants and the recovery of a SSH/SSH/LAC reassortant virus.** *ts* mutants of wild-type SSH/LAC/LAC were obtained and categorized into groups by reciprocal recombination tests (Table 4). Five of the *ts* mutants that were obtained were assigned to one group, with another four mutants assigned to a second group.

Backcrosses of the mutants SSH/LAC/LAC *ts*-1 and *ts*-6 with the *ts* mutants SSH I-1, SSH I-9, SSH II-7, SSH II-21, LAC I-19, LAC I-20, LAC II-4, and LAC II-5 were undertaken, with the results shown in Table 5. Because the SSH/LAC/LAC *ts*-1 mutant recombined only with

TABLE 2. *Recombination analyses with SSH/LAC/SSH ts mutants*[a]

| *ts* mutant | % Recombination | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 8 | 9 | 13 |
| 1 | | 0 | 0 | 0 | 9 | 0 |
| 2 | | | 0 | 0 | 4 | 0 |
| 3 | | | | 0 | 17 | 0 |
| 8 | | | | | 8 | 0 |
| 9 | | | | | | 20 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0. Assignments: group I, *ts* 9; group II, *ts* 1, *ts* 2, *ts* 3, *ts* 8, *ts* 13.

TABLE 3. *Backcross recombination analyses of SSH/LAC/SSH ts mutants with group I and group II mutants SSH and LAC*[a]

| LAC or SSH *ts* mutant[b] | % Recombination with SSH/LAC/SSH *ts* mutants | |
|---|---|---|
| | ts I-9 | ts II-13 |
| SSH I-3 | 0 | 10[c] |
| SSH II-21 | 2 | 0 |
| LAC I-16 | 0 | 3[c] |
| LAC II-2 | 41[c] | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0.

[b] Control crosses of SSH I and II, or LAC I and II, and SSH/LAC/SSH I × SSH/LAC/SSH II all gave values greater than 1%.

[c] Clones were obtained from these crosses for fingerprint analyses (Fig. 2).

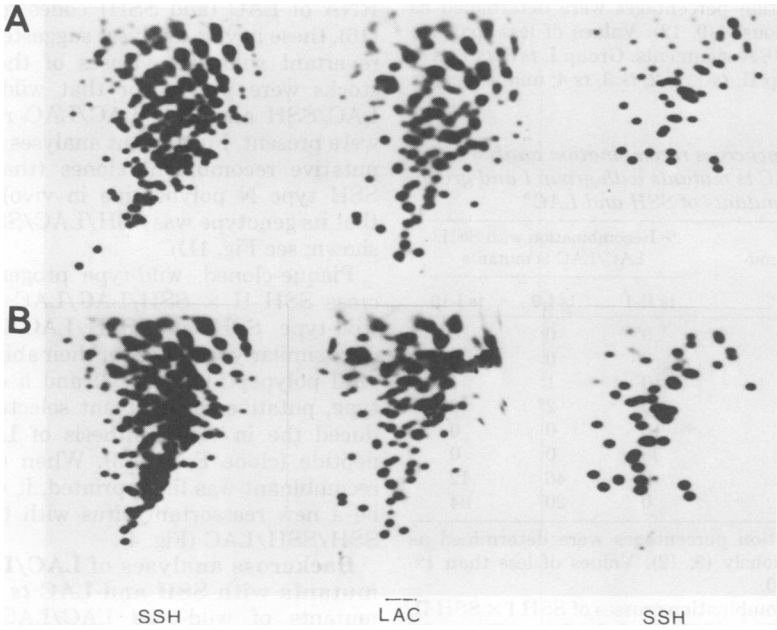


FIG. 2. *Oligonucleotide fingerprint analyses of cloned wild-type progeny from dual virus infections involving SSH/LAC/SSH ts mutants and LAC or SSH ts mutants. The L, M, and S RNA species obtained from cloned wild-type progeny derived from dual ts mutant virus infections involving (A) LAC I-16 and SSH/LAC/SSH II-13 and (B) SSH I-3 and SSH/LAC/SSH II-13 were digested with ribonuclease T1 and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as described previously. For (A) the L/M/S genotype is LAC/LAC/SSH, and for (B) it is SSH/LAC/SSH.*

SSH or LAC group I mutants, it was categorized as a group II mutant (Table 4). Likewise, because the SSH/LAC/LAC *ts*-6 mutant recombined only with SSH or LAC group II mutants, it was categorized as a group I mutant.

Assuming that the group I mutants of LAC, SSH, and SSH/LAC/LAC viruses have defective M RNA species and group II mutants of those viruses have defective L RNA species, the following predictions were made. (i) The wild-type progeny of the SSH/SSH*/SSH × SSH*/LAC/LAC cross (i.e., SSH I × SSH/LAC/LAC II) should contain the recombinants SSH/LAC/SSH and SSH/LAC/LAC (i.e., like those obtained from the SSH I × LAC II cross). (ii) The wild-type progeny of the SSH*/SSH/SSH × SSH/LAC*/LAC cross (i.e., SSH II × SSH/LAC/LAC I) should give SSH virus and a new recombinant SSH/SSH/LAC. (iii) The wild-type progeny of the LAC/LAC*/LAC × SSH*/LAC/LAC cross (i.e., LAC I × SSH/LAC/LAC II) should give LAC virus. (iv) The wild-type progeny of the LAC*/LAC/LAC × SSH/LAC*/LAC cross (i.e., LAC II × SSH/LAC/LAC I) should give the recombinant SSH/LAC/LAC.

TABLE 4. *Recombination analyses with SSH/LAC/LAC ts mutants*[a]

| *ts* mutant | % Recombination | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *ts* 1 | | 0 | 0 | 0 | 0 | 7 | 19 | 18 | 23 | 16 |
| *ts* 2 | | | 0 | 0 | 0 | 1 | 14 | 20 | 33 | 43 |
| *ts* 3 | | | | 0 | 0 | 1 | 16 | 10 | 20 | 27 |
| *ts* 4 | | | | | 0 | 5 | 15 | 18 | 8 | 16 |
| *ts* 5 | | | | | | 0 | 0 | 0 | 0 | 0 |
| *ts* 6 | | | | | | | 0 | 0 | 0 | 0 |
| *ts* 7 | | | | | | | | 0 | 0 | 0 |
| *ts* 8 | | | | | | | | | 0 | 0 |
| *ts* 9 | | | | | | | | | | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0. Assignments: Group I, *ts* 6, *ts* 7, *ts* 8, *ts* 9, *ts* 10; group II, *ts* 1, *ts* 2, *ts* 3, *ts* 4; unassigned, *ts* 5.

TABLE 5. *Backcross recombination analyses of SSH/LAC/LAC ts mutants with group I and group II mutants of SSH and LAC*[a]

| LAC or SSH *ts* mutant[b] | % Recombination with SSH/LAC/LAC *ts* mutants | | |
|---|---|---|---|
| | ts II-1 | ts I-9 | ts I-10 |
| SSH I-1 | 9[c] | 0 | 0 |
| SSH I-9 | 9[c] | 0 | 0 |
| SSH II-7 | 0 | 1[c] | 1[c] |
| SSH II-21 | 0 | 2[c] | 2[c] |
| LAC I-19 | 10 | 0 | 0 |
| LAC I-20 | 38 | 0 | 0 |
| LAC II-4 | 0 | 46 | 12 |
| LAC II-5 | 0 | 20 | 34 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0.

[b] Control recombination crosses of SSH I × SSH II, LAC I × LAC II, and SSH/LAC/LAC I × SSH/LAC/LAC II all gave values greater than 1%.

[c] Progeny virus was plaque cloned from the 39.8°C plaque assays, virus stocks were obtained, and their genomes were analyzed by oligonucleotide fingerprint analyses (see text).

To test some of these predictions, several plaque-cloned, wild-type virus stocks obtained from the SSH I × SSH/LAC/LAC II crosses, as well as wild-type SSH and SSH/LAC/LAC and their *ts* mutants, were used to infect BHK cells (multiplicity of infection, 10 to 50) and the infected cells were labeled for 1 h at 8 h postinfection with media containing 50 μCi of [$^3$H]leucine per ml. The infected cell polypeptides were recovered after sodium dodecyl sulfate lysis of the cells and resolved by slab polyacrylamide gel electrophoresis. Some of the putative recombinant virus samples were found to have a LAC type N polypeptide, whereas others had a SSH type N polypeptide (Fig. 3). Since the original SSH I × SSH/LAC/LAC II cross was expected to yield both SSH/LAC/SSH and SSH/LAC/LAC wild-type recombinants, and since the S RNA of LAC (and SSH) codes for N protein (16), these in vivo analyses suggested either that revertant wild-type viruses of the original *ts* stocks were present or that wild-type SSH/LAC/SSH and SSH/LAC/LAC recombinants were present. Fingerprint analyses of one of the putative recombinant clones (that induced a SSH type N polypeptide in vivo) established that its genotype was SSH/LAC/SSH (data not shown; see Fig. 1D).

Plaque-cloned, wild-type progeny from the cross SSH II × SSH/LAC/LAC I as well as wild-type SSH and SSH/LAC/LAC viruses were similarly screened for their ability to induce viral polypeptides (Fig. 3) and a cloned, wild-type, putative recombinant selected which induced the in vivo synthesis of LAC N polypeptide (clone F, Fig. 3). When this putative recombinant was fingerprinted, it was found to be a new reassortant virus with the genotype SSH/SSH/LAC (Fig. 4).

**Backcross analyses of LAC/LAC/SSH *ts* mutants with SSH and LAC *ts* mutants.** *ts* mutants of wild-type LAC/LAC/SSH virus have been derived and categorized into groups by reciprocal recombination tests, with the results shown in Table 6. Backcrosses of selected LAC/LAC/SSH *ts* mutants with SSH and LAC *ts* mutants were then undertaken (Table 7) and

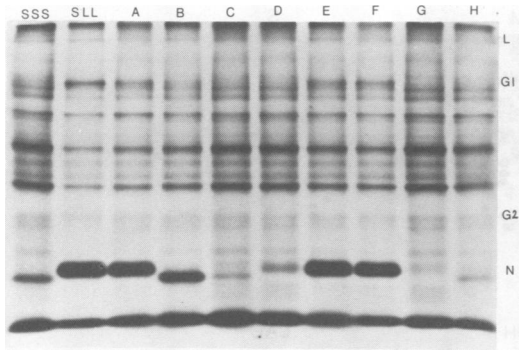


FIG. 3. *Intracellular polypeptides induced by wild-type SSH virus, wild-type SSH/LAC/LAC virus, and eight cloned putative recombinant viruses obtained from dual mutant virus infections involving SSH II and SSH/LAC/LAC I ts mutants (Table 5). Stocks of virus were used to infect confluent BHK-21 cells (multiplicity of infection, 10 to 50), and after 8 h of incubation at 33°C the intracellular polypeptides were labeled by changing the infected cell supernatant fluids for Eagle medium containing ⅒th the normal concentration of leucine and 10 μCi of [$^3$H]leucine per ml. After 1 h at 33°C, the cells were washed and resuspended in 0.2 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and heated at 100°C for 2 min, and approximately $10^5$ cpm of acid-insoluble radioactivity was placed in the wells of a 10% discontinuous polyacrylamide gel. The polypeptides were resolved by electrophoresis, and an autoradiogram of their distribution was obtained. From left to right the gel wells contained samples from infections involving: (lane 1) SSH infected cells, (lane 2) wild-type SSH/LAC/LAC, and (lanes 3 to 10) eight individually cloned wild-type putative recombinants derived from (A and B) SSH/LAC/LAC I-9 and SSH II-7 coinfections, (C and D) SSH/LAC/LAC I-10 and SSH II-7 coinfections, (E and F) SSH/LAC/LAC I-9 and SSH II-21 coinfections, (G and H) SSH/LAC/LAC I-10 and SSH II-21 coinfections. The positions where the viral L, N, G1, and G2 polypeptides were expected to be recovered are indicated. It was concluded that the putative recombinants A, D, E, and F had a LAC type N protein whereas the putative recombinants B, C, and H had a SSH type N protein. No N protein was detected in the G sample.*

used to assign the LAC/LAC/SSH mutants into group I and II categories on the basis of their genetic compatibilities with the original LAC and SSH virus mutants.

On the assumption that the group I mutants of LAC, SSH, and LAC/LAC/SSH viruses have defective M RNA species, whereas their respective group II mutants have defective L RNA species, the following predictions were made. (i) The wild-type progeny of the SSH/SSH*/SSH × LAC*/LAC/SSH cross (i.e., SSH I × LAC/LAC/SSH II) should contain the recombinant SSH/LAC/SSH. (ii) The wild-type progeny of the SSH*/SSH/SSH × LAC/LAC*/SSH cross (i.e., SSH II × LAC/LAC/SSH I) should contain a new recombinant LAC/SSH/SSH. (iii) The wild-type progeny of the LAC/LAC*/LAC × LAC*/LAC/SSH cross (i.e., LAC I × LAC/LAC/SSH II) should contain LAC/LAC/SSH and LAC virus, as should the wild-type progeny of the reciprocal cross LAC*/LAC/LAC × LAC/LAC*/SSH (i.e., LAC II × LAC/LAC/SSH I).

It should be noted that crosses involving LAC/LAC/SSH group I or II *ts* mutants with those of SSH/LAC/LAC would give rise only to LAC virus or recombinant viruses that have already been derived (i.e., SSH/LAC/LAC, SSH/LAC/SSH, and LAC/LAC/SSH). Likewise, crosses involving LAC/LAC/SSH group I or II *ts* mutants with those of SSH/LAC/SSH would give rise to SSH/LAC/SSH and LAC/LAC/SSH recombinant types.

The results of the crosses involving LAC/LAC/SSH group I and II *ts* mutants with those of SSH and LAC viruses shown in Table 7 indicate that whereas both group I and II mutants of LAC/LAC/SSH recombined with LAC II and I *ts* mutants, respectively, only the group II *ts* mutants of LAC/LAC/SSH recombined with any group I *ts* mutants of SSH virus. The reciprocal cross (LAC/LAC/SSH I × SSH II) did not yield the expected LAC/SSH/SSH recombinant virus genotype.

**Backcross analyses of SSH/SSH/LAC *ts* mutants with SSH and LAC *ts* mutants.** *ts* mutants of wild-type SSH/SSH/LAC virus were derived, categorized into groups by reciprocal recombination tests (Table 8), and backcrossed with SSH and LAC *ts* mutants to obtain the compatible group assignments (Table 9).

As discussed previously, on the assumption that group I mutants of LAC, SSH, and SSH/SSH/LAC viruses have defective M RNA species whereas their group II mutants have defective L RNA species, the following predictions were made. (i) The wild-type progeny of the SSH/SSH*/SSH × SSH*/SSH/LAC cross (i.e., SSH I × SSH/SSH/LAC II) should contain SSH and SSH/SSH/LAC viruses, as should the reciprocal SSH II × SSH/SSH/LAC I cross. (ii) The wild-type progeny of LAC/LAC*/LAC × SSH*SSH/LAC (i.e., LAC I × SSH/SSH/LAC II) should contain a new recombinant LAC/SSH/LAC. (iii) The wild-type progeny of LAC*/LAC/LAC × SSH/SSH*/SSH (i.e., LAC II × SSH/SSH/LAC I) should contain the recombinants SSH/LAC/LAC and SSH/LAC/SSH that have already been derived.

Again it should be noted that crosses involving

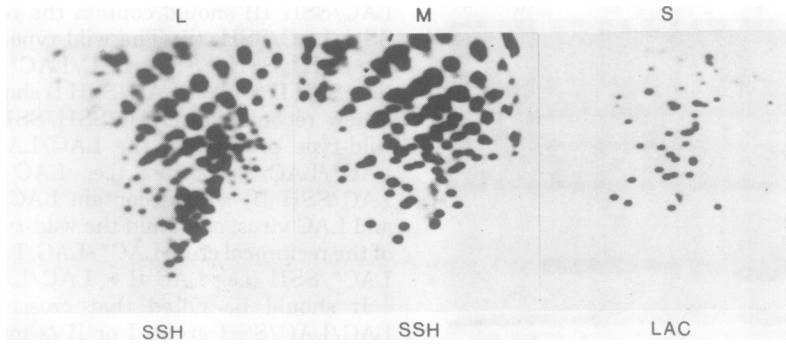


FIG. 4. *Oligonucleotide fingerprint analyses of cloned wild-type progeny from dual virus infections involving SSH/LAC/LAC and SSH ts mutants. The L, M, and S RNA species obtained from cloned wild-type progeny derived from dual ts mutant virus infections involving SSH II-7 and SSH/LAC/LAC I-10 viruses (see Fig. 3, lane F) were digested with ribonuclease T1, and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis. The L/M/S genotype is SSH/SSH/LAC.*

TABLE 6. *Recombination analyses with LAC/LAC/SSH ts mutants*[a]

| *ts* mutant | % Recombination | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 6 | 8 | 13 | 15 | 16 |
| *ts* 5 | | 0 | 0 | 0 | 0 | 3 |
| *ts* 6 | | | 0 | 0 | 0 | 7 |
| *ts* 8 | | | | 0 | 0 | 4 |
| *ts* 13 | | | | | 0 | 8 |
| *ts* 15 | | | | | | 3 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0. Assignments, group I, 16; group II, 5, 6, 8, 13, 15.

TABLE 7. *Backcross recombination analyses of LAC/LAC/SSH ts mutants with group I and group II mutants of SSH and LAC*[a]

| LAC or SSH *ts* mutant[b] | % Recombination with LAC/LAC/SSH *ts* mutants | | |
|---|---|---|---|
| | *ts* I-16 | *ts* II-5 | *ts* II-15 |
| SSH I-1 | 0 | 38 | 14 |
| SSH II-22 | 0 | 0 | 0 |
| LAC I-20 | 0 | 11 | 8 |
| LAC II-4 | 18 | 0 | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0.

[b] Control recombination crosses of SSH I × SSH II, LAC I × LAC II, and LAC/LAC/SSH I × LAC/LAC/SSH II all gave values greater than 1%.

SSH/SSH/LAC group I or II *ts* mutants with those of SSH/LAC/SSH would give rise to SSH virus and recombinant viruses that have already been obtained: SSH/SSH/LAC, SSH/LAC/LAC and SSH/LAC/SSH viruses. Likewise, crosses involving SSH/SSH/LAC group I or II *ts* mutants with those of SSH/LAC/LAC should

TABLE 8. *Recombination analyses with SSH/SSH/LAC ts mutants*[a]

| *ts* mutant | % Recombination | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 3 | 4 | 5 | 6 | 7 |
| *ts* 1 | | 0 | 0 | 2 | 0 | 0 |
| *ts* 3 | | | 0 | 10 | 0 | 0 |
| *ts* 4 | | | | 3 | 0 | 0 |
| *ts* 5 | | | | | 0 | 0 |
| *ts* 6 | | | | | | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0. Assignments: group I, 1, 3, 4; group II, 5; unassigned, 6, 7.

TABLE 9. *Backcross recombination analyses of SSH/SSH/LAC ts mutants with group I and group II mutants of SSH and LAC*[a]

| *ts* mutant[b] | % Recombination with SSH/SSH/LAC *ts* mutants | |
|---|---|---|
| | ts I-1 | ts II-5 |
| SSH I-9 | 0 | 9 |
| SSH II-22 | 5 | 0 |
| LAC I-20 | 0 | 0 |
| LAC II-4 | 11 | 0 |
| LAC II-17 | 6 | 0 |
| L/L/S I-16 | 0 | 0 |
| L/L/S II-5 | 43 | 0 |

[a] Recombination percentages were determined as described previously (9, 12). Values of less than 1% were scored as 0.

[b] Control recombination crosses of SSH I × SSH II, LAC I × LAC II, SSH/SSH/LAC I × SSH/SSH/LAC II, and LAC/LAC/SSH I × LAC/LAC/SSH II (L/L/S I × L/L/S II) all gave values greater than 1%.

give rise to the recombinants SSH/LAC/LAC and SSH/SSH/LAC.

Although crosses of SSH/SSH/LAC group I *ts* mutants with the group II *ts* mutants of LAC/

LAC/SSH should give the recombinants SSH/LAC/SSH and SSH/LAC/LAC, the reciprocal cross (SSH*/SSH/LAC × LAC/LAC*/SSH) should give two new recombinants: LAC/SSH/LAC and LAC/SSH/SSH. For this reason the results of such a cross are given in Table 9.

As seen in Table 9, neither the LAC I × SSH/SSH/LAC II cross nor the LAC/LAC/SSH I × SSH/SSH/LAC II cross gave any evidence for the formation of wild-type recombinant viruses.

## DISCUSSION

Serological studies have established that within the CE serogroup there are three virus serotypes, CE, Trivittatus, and Melao (2, 14–16, 18, 25). These three serotypes are easily distinguished by cross-neutralization of infectivity and complement fixation tests. Guaroa virus which, on the basis of complement fixation tests, is a member of the Bunyamwera serogroup of bunyaviruses (2, 29) has been shown to be related to members of the CE serogroup when neutralization of infectivity tests are used (e.g., to Trivittatus virus). We have initiated a study to determine the genetic recombination potential of bunyaviruses belonging to the CE serogroup to determine whether all members of the serogroup can recombine with each other or whether only selected viruses (or serotypes) can recombine.

The lack of observed recombination between certain SSH and LAC *ts* mutant viruses may be attributed to either basic genetic incompatibilities or our inability to experimentally detect recombinants that are formed between these viruses. Genetic incompatibilities could involve RNA polymerase enzymes which are specific for their own templates and which are unable to faithfully replicate or transcribe heterologous RNA species. Alternatively, they may reflect viral proteins which cannot interrelate with proteins of another virus to form functional structures. Previous studies have shown that the S RNA of SSH and LAC virus codes for the major nucleocapsid protein N (10). Unpublished studies indicate that the M RNA codes for the two viral glycoproteins, G1 and G2 (J. Gentsch and D. H. L. Bishop, unpublished data). Although the gene products of the L RNA have not been determined, one logical candidate is the virion large protein L. From the four SSH-LAC reassortant viruses so far derived, it can be concluded that the SSH L gene products can function with LAC M (G1 and G2) and S (N) gene products in all possible SSH and LAC M and S combinations. If one of the L gene products is an RNA polymerase, then from the recombinants we have generated it can be concluded that SSH polymerase can function with LAC N protein on the L and M RNA species of SSH virus as well as on the S RNA of LAC virus. Similarly, it can be concluded that the LAC polymerase can function with SSH N protein on LAC L and M RNA species as well as on SSH S RNA. If LAC/SSH/LAC and LAC/SSH/SSH reassortant viruses cannot be formed due to genetic incompatibilities, then if the only L gene product is an RNA polymerase, either gene product-RNA combinations involving viral RNA coated with LAC or SSH N protein cannot be transcribed or replicated by LAC RNA polymerase when SSH G1 and G2 polypeptides are formed in the infected cell, or virus particles are not produced with SSH glycoprotein in the presence of LAC L structural gene products and either SSH or LAC N gene products (e.g., a maturation problem), or they are not infectious.

The selection procedure that we have adopted to screen the progeny of dual-mutant virus infections for wild-type recombinants has involved selecting genotypically stable, cytolytic viruses. If certain recombinant viruses, for one reason or another, are noncytolytic or have a substantially decreased ability to mature and give infectious extracellular virus (e.g., 100-fold), then their presence would have been overlooked. Alternative procedures to detect noninfectious or noncytopathic recombinant viruses will have to be used to determine whether recombinant viruses are generated which, because of their genotype and functional capabilities, have eluded our screening procedures. In addition, as an alternate approach to the study of SSH-LAC genetic incompatibilities, heterologous in vitro template-transcriptase analyses and in vivo phenotypic mixing and genetic complementation experiments could be studied.

The recombinant viruses generated from certain SSH and LAC dual virus infections raise the question of the heterologous recombination potential between other CE serotype viruses (i.e., CE, LAC, SSH, Inkoo, Tahyna, San Angelo, and Lumbo). This question is of interest in view of the limited geographic distribution of these viruses. Four of the CE viruses (CE, LAC, San Angelo, SSH) have only been isolated in the North American continent (2, 6, 13, 15, 16, 27, 28). Of these four viruses, SSH virus appears by both isolation and serological studies to be widely distributed throughout Alaska and Canada, and sympatric with LAC in the northern states of the United States (16). LAC has been isolated primarily from the upper midwest and northeastern States of the United States, although several isolates have been reported from other parts of the country (27). By contrast CE virus has only been isolated from California (2, 13). Although Tahyna is known to be widely

distributed throughout Central Europe (11), Inkoo virus (2, 5) appears to be prevalent in the more northernly regions of Europe (Finland and Lapland). Neither virus has been recovered elsewhere. Lumbo virus was isolated in Mozambique (17), and its distribution in Africa is not known. Although Lumbo and Tahyna are serologically closely related to each other (2, 17), oligonucleotide fingerprint analyses indicate that the three RNA species of Lumbo and TAH viruses are easily distinguished and quite distinct from those of Trivittatus, Guaroa, LAC, and SSH viruses (7, 12; El Said et al., in press).

In view of the geographic distribution of the CE group viruses and the limited SSH-LAC recombination that we have observed, the question of whether cytolytic recombinant viruses can be formed only between the North American CE subtype isolates (or also with the European and African CE subtype isolates) is raised. Genetic studies are being initiated to investigate this question.

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