Formation of Recombinants Between Snowshoe Hare and La Crosse Bunyaviruses

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Wild-type recombinants were obtained at high frequency from coinfections of BHK cells involving temperature-sensitive, conditional-lethal mutants of snowshoe hare (SSH) and La Crosse (LAC) bunyaviruses. Analyses of two of the recombinants indicated that they have the genome compositions SSH/LAC/SSH and SSH/LAC/LAC for their respective L, M, and S virion RNA species. This evidence, together with that for the genetic stability of the recombinants, indicates that they were derived by segment reassortment of the competent genome pieces of the parental viruses. The SSH/LAC/SSH recombinant appears, from polypeptide analysis, to have the SSH type of nucleocapsid protein (N), whereas the SSH/LAC/LAC recombinant has the LAC nucleocapsid protein, suggesting that the viral S RNA codes for the N protein.

The *Bunyaviridae* are a family of enveloped RNA viruses that are unique by comparison to other enveloped, arthropod-borne viruses because their virions contain a single-stranded, segmented, RNA genome (14). This has been demonstrated by gel electrophoresis and/or sucrose gradient centrifugation of the viral RNA species for Bunyamwera, La Crosse (LAC), Lumbo, Main Drain, Melao, snowshoe hare (SSH), Tahyna, and Trivittatus viruses as well as for the serologically unrelated Uukuniemi virus (3, 5, 8, 10, 12, 13). These observations have been confirmed by oligonucleotide mapping of the individual large, medium, and small (L, M, and S, respectively) RNA species for SSH, LAC, and Uukuniemi viruses (5, 12).

Genetic studies with temperature-sensitive (*ts*), conditional-lethal mutants of SSH virus indicate that wild-type recombinant viruses can be formed at high frequency during coinfection of cells by certain *ts* mutants (7). The mutants have been divided into two nonoverlapping genetic recombination groups on the basis of the high-frequency recombination obtained between *ts* mutants of different groups and the lack of detectable recombination between *ts* mutants of the same recombination group. These results, together with the molecular evidence for a segmented genome, suggest, but do not prove, that the genetic information of the virus is resident in more than one piece of viral RNA.

From serological studies, some 90 bunyaviruses have been divided into 11 groups, with members of each group being serologically more

closely related to other members of that group than to members of other groups (2, 14). However, it has been shown that certain, but not all, members of each bunyavirus group are serologically related to one or more members of another group (2, 14). These 11 groups constitute the *Bunyamwera* supergroup of viruses (2, 14). Fifty other bunyavirus-like viruses are serologically unrelated to members of the supergroup (e.g., Uukuniemi virus), although they exhibit certain structural similarities in common with members of the supergroup (14).

With the demonstration of the formation of wild-type recombinants by coinfection of cells with temperature-sensitive mutants of SSH virus, the question of whether recombinants can be formed between bunyaviruses was raised. In this paper we document the ability of SSH and LAC viruses to form genetically stable recombinants, two of which have been characterized both by pathogenicity and molecular analyses.

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. ³²P-labeled sodium P_i, [4,5-³H]leucine (53 Ci/mmol), and U-¹⁴C-labeled L-amino acids (50 mCi/mmol) came from I.C.N., Irvine, Calif.

Viruses. The prototype wild-type LAC virus was originally obtained from J. F. Obijeski, Center for Disease Control, Atlanta, Ga. The prototype SSH virus was obtained from W. Burgdorfer, Rocky Mountain Laboratory, Hamilton, Mont. Both viruses had

been plaque-cloned in our laboratories, using monolayers of BHK-21 cells.

Isolation of temperature-sensitive, conditional-lethal virus mutants. The protocols used for obtaining temperature-sensitive, conditional-lethal mutants of SSH or LAC viruses by growth in the presence of the mutagen 5-fluorouracil have been described previously (7). Spontaneous *ts* mutants were isolated by random screening of clones obtained by plating wild-type virus clones at limit dilutions. For recombination experiments, only the mutant virus stocks were used that had nonpermissive plaquing efficiencies of less than 0.002%, (PFU assay at 39.5°C + PFU assay at 33°C) and growth efficiencies of less than 0.01% (progeny PFU after growth of infected cells at 39.5°C + progeny PFU after growth of infected cells at 33°C; progeny from both assayed at 33°C).

Recombination assays. Recombination experiments involved infecting confluent monolayers of 2×10^5 BHK-21 cells in a 24-place microwell dish with two temperature-sensitive mutants ($A \times B$, 5 PFU for each mutant per cell). Parallel infections involving individual mutants were also performed (e.g., A or B). After a 30-min adsorption period at room temperature, the inocula were removed and the infected cells were washed once with 1 ml of Eagle medium containing 5% (vol/vol) newborn-calf serum and then overlaid with 1 ml of the same medium. The dish was sealed in a plastic bag, charged with a 5% (vol/vol) carbon dioxide-air mixture, and incubated at 33°C for 2 h. The supernatant fluids, containing desorbed virus, were then replaced with fresh medium. After a further 22 h of incubation at 33°C (e.g., $A \times B_{33}$), the cell supernatant fluids were titered by plaque assays at 39.8 and 33°C, using confluent monolayers of BHK-21 cells [e.g., ($A \times B_{33}$)_{39.8}]. From the data obtained, the recombination percentages were calculated according to the formula:

$$\frac{\{ (A \times B_{33})_{39.8} - [(A_{33})_{39.8} + (B_{33})_{39.8}] \}}{(A \times B_{33})_{33}} \times 100 \times 2$$

All manipulations involving crosses between SSH and LAC *ts* mutants were performed in a BioGard hood in a P3 containment facility at the University of Alabama at Birmingham.

Infection of cells and virus purification. The procedures used to infect BHK-21 cell monolayers with bunyaviruses in the presence of [³H]leucine (5 μCi/ml), ¹⁴C-amino acid mixture (1 μCi/ml), or ³²P_i (250 μCi/ml) and those used to obtain and purify labeled virus from the clarified supernatant fluids have been described previously (5, 8, 9).

Purification of labeled viral RNA species, RNase T1 digestion of RNA, and separation of oligonucleotides by two-dimensional gel electrophoresis. The extraction and purification of ³²P-labeled bunyavirus L, M, and S RNA species have been described previously (5). RNA samples, dissolved in 15 μl of 0.02 M Tris-hydrochloride-0.002 M EDTA (pH 7.4) were digested with 10 U of RNase T1 at 37°C for 30 min, and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis (5, 6). After electrophoresis, each gel was autoradiographed to obtain the oligonucleotide fingerprint (5).

Polyacrylamide gel electrophoresis of virion polypeptides. Virus preparations labeled with ¹⁴C-amino acids or [³H]leucine were mixed together and dissociated by treatment with 1% (wt/vol) sodium dodecyl sulfate in the presence of 1% (vol/vol) 2-mercaptoethanol and 6 M urea at 60°C for 30 min, and the polypeptides were resolved on 8% polyacrylamide gels as described previously (11).

CF and HI tests. Complement fixation (CF) tests utilized 2 U of complement and grid titrations of antibody and sucrose-acetone-extracted and sonically oscillated mouse brain antigens incubated for 18 h at 4°C as described elsewhere (4). The same antigens were used in hemagglutination inhibition (HI) tests with acetone-extracted sera by the method of Clarke and Casals (4) as modified by Beaty et al. (1) to use 0.4% NaCl with 4 to 8 antigen units.

RESULTS

High-frequency recombination with temperature-sensitive, conditional-lethal mutants of SSH virus. The recombination percentages obtained with some 48 temperature-sensitive, conditional-lethal mutants of SSH virus are shown in Tables 1 and 2. Ten of the mutants were obtained as spontaneous *ts* mutants (*ts* no. 32, 33, 51 to 54, 56, and 59 to 61) and were recovered at a frequency of 1.7% from the clones of the wild-type virus stock. The rest were obtained from mutagenesis involving 5-fluorouracil. Apart from two possible double mutants (*ts* 14 and 27), the mutants can be categorized into two recombination groups, with group I including 26 mutants (*ts* no. 1, 9, 12, 13, 15, 16, 20, 25, 28, 35, 36, 39, 41, 42, 47 to 50, 52 to 54, 56, and 58 to 60) and group II including 20 mutants (*ts* no. 2, 7, 18, 21, 22 to 24, 26, 29, 30, 32 to 34, 37, 38, 40, 43, 51, 57, and 61). No recombination was observed between mutants within each group. The recombination percentages, which in most cases represent the average of two determinations for each cross, varied 1 to 38%. Although three unique RNA species have been identified for SSH virus (5, 8), we obtained no evidence for a third recombination group of *ts* mutants. One possible reason for the lack of mutants representing a third recombination group might be the unequal target size of the three RNA species of the virus. Another reason could be that mutations in that RNA more frequently lead to lethal alterations in the gene products than for the other RNA segments.

Search for heterozygotes among the recombinant progeny of crosses involving SSH *ts* mutants. The recombinant progeny, characterized as wild types from their efficiency of plating (EOP) at permissive and nonpermissive temperatures, could either represent wild-type viruses with wild-type genomes derived by parental segment reassortment, or heterozygotes

TABLE 1. *Recombination analyses with SSH ts mutants*^a

SSH <i>ts</i> mutant	% Recombinants with SSH <i>ts</i> mutant:														
	1	2	3	7	9	12	13	14	15	16	18	20	21		
1	.	4	0	7	0	0	0	0	0	0	14	0	21		
2	.	.	17	0	18	4	1	0	16	9	0	7	0		
3	.	.	.	24	0	0	0	0	0	0	14	0	2		
7	.	.	.	.	5	4	3	0	5	5	18	4	0		
9	.	.	.	.	.	0	0	0	0	0	5	0	7		
12	.	.	.	.	.	.	0	0	0	0	25	0	4		
13	.	.	.	.	.	.	.	0	0	0	9	0	6		
14	.	.	.	.	.	.	.	.	0	0	0	0	0		
15	.	.	.	.	.	.	.	.	.	0	13	0	6		
16	.	.	.	.	.	.	.	.	.	.	6	0	2		
18	.	.	.	.	.	.	.	.	.	.	.	2	0		
20	.	.	.	.	.	.	.	.	.	.	.	.	2		

^a Assignments: Group I—*ts* 1, *ts* 3, *ts* 9, *ts* 12, *ts* 13, *ts* 15, *ts* 16, and *ts* 20 (eight mutants); group II—*ts* 2, *ts* 7, *ts* 18, and *ts* 21 (four mutants). Unassigned: *ts* 14 (possible double mutant). Recombinant percentages (average of three determinations) were determined as described in the text.

containing in their virions duplicate RNA segments (*ts* and wild type) derived from both viruses, or aggregates of both parental viruses. Since for the latter two possibilities one might expect high-frequency shedding of *ts* viruses on subsequent clonal analyses, this question was initially examined by determining the shedding rate of *ts* viruses from the 39.8°C-cloned, wild-type progeny of the *ts* 2 × *ts* 3 cross. The progeny from that cross were plated at 39.8°C and 35 were clones recovered. The EOP (equals the ratio of PFU at 39.8°C/33°C) of virus in each clone was then determined by plating at 39.8 and 33°C. All 35 clones had EOPs of ~1. Six clones were subsequently replated at 33°C, 50 plaques per original clone were recovered, and the EOPs of the virus in these plaques were determined. Of 300 clones analyzed, 298 had EOPs of 0.4 to 1.5 (i.e., comparable to that of wild-type virus). The remaining two clones had EOPs of less than 0.01. The plating efficiencies of these latter two viruses remained constant in subsequent titrations, suggesting that they were de novo, spontaneously produced *ts* mutants. These results also suggested that either the progeny viruses from the original *ts* 2 × *ts* 3 cross are stable heterozygotes, or that they represent wild-type recombinants derived by reassortment of the competent wild-type RNA species of the original parental mutants. Since we observed that there was a spontaneous shedding rate of 1.7% for *ts* mutants from our wild-type SSH stock virus, the two *ts* viruses probably reflect spontaneous production of *ts* mutants from these clones.

Similar results were obtained with 13 putative recombinant clones recovered from a 33°C plaque assay of the original *ts* 2 × *ts* 3 cross.

TABLE 2. *Recombination analyses with SSH ts mutants*^a

SSH <i>ts</i> mutant	% Recombinants with SSH <i>ts</i> mutant:																																		
	22	23	24	25	26	27	28	29	30	32	33	34	35	36	37	38	39	40	41	42	43	47	48	49	50	51	52	53	54	56	57	58	59	60	61
1	26	9	22	0	17	0	0	4	6	2	4	5	0	0	16	17	0	14	0	0	1	0	0	0	0	0	25	0	0	0	2	0	0	0	2
2	0	0	0	7	0	0	17	0	0	0	0	0	17	14	0	0	9	0	5	45	0	11	7	7	3	0	13	31	33	2	0	4	15	11	0
3	14	4	15	0	16	0	0	19	20	9	10	10	0	0	17	30	0	38	0	0	2	0	0	0	0	0	23	0	0	0	4	0	0	0	12
18	0	0	0	13	0	0	12	0	0	0	0	0	7	8	0	0	2	0	4	12	0	7	24	4	8	0	7	32	16	4	9	18	10	0	0

^a Assignments: Group I—*ts* 1, *ts* 3, *ts* 25, *ts* 28, *ts* 35, *ts* 36, *ts* 39, *ts* 41, *ts* 42, *ts* 47, *ts* 48, *ts* 49, *ts* 50, *ts* 52, *ts* 53, *ts* 54, *ts* 56, *ts* 58, *ts* 59, and *ts* 60; group II—*ts* 2, *ts* 18, *ts* 22, *ts* 23, *ts* 24, *ts* 26, *ts* 29, *ts* 30, *ts* 32, *ts* 33, *ts* 34, *ts* 37, *ts* 38, *ts* 40, *ts* 43, *ts* 51, *ts* 57, and *ts* 61. Recombination percentages were determined as described in the text.

Eleven of the 13 recombinants had plating efficiencies of between 0.5 and 1.0. When three of these clones were plated at 33°C and 50 plaques were recovered for each, subsequent EOP analyses of these 150 virus clones indicated that 145 had wild-type EOPs (~1), whereas 5 had low EOPs (<0.01) and probably represented spontaneously produced *ts* mutants. Two of the 13 original clones from the *ts* 2 × *ts* 3 cross were found to have a low plating efficiency (~0.1). On subsequent clonal analyses, these two clones were shown to contain predominantly temperature-sensitive progeny (96 of 100) with EOPs of less than 0.01, suggesting that their original clones may have contained temperature-sensitive viruses. The remaining four viruses had wild-type EOPs. Since the frequencies of reversion to wild-type of *ts* 2 and *ts* 3 are less than 10⁻⁴ (7), the recovery of 4% wild-type virus may indicate that the two original clones contained both *ts* 2 and *ts* 3 as well as recombinants. No further analyses were undertaken to investigate this possibility.

High-frequency recombination with temperature-sensitive, conditional-lethal mutants of LAC virus. The recombination percentages obtained with temperature-sensitive, conditional-lethal mutants of LAC virus are given in Table 3. Five of these mutants were obtained, at a frequency of 1%, as spontaneously occurring, temperature-sensitive mutants (*ts* no. 19 to 23). Although only 20 mutants were analyzed, high-frequency recombination was obtained for crosses involving certain mutants. From these tests, the mutants were categorized into two genetic recombination groups: group I, containing six mutants (*ts* no. 9, 13, 16, 19, 20, and 23); and group II, containing 14 mutants (*ts* no. 1 to 5, 10 to 12, 14, 15, 17, 18, 21, and 22). The designations group I and group II for the two LAC recombination groups were chosen arbitrarily.

Recombination between temperature-sensitive, conditional-lethal mutants of SSH and LAC viruses. The results of recom-

bination experiments between certain *ts* mutants of LAC and SSH viruses are shown in Table 4. High-frequency recombination was found for certain crosses (e.g., SSH I-3 × LAC II-4). Clonal analyses of eight separate plaques obtained from the 39.8°C assays gave EOP ratios of 0.1 to 1.3. Each putative recombinant was grown into a stock of virus for pathogenicity and eventual molecular analyses. The EOPs of all presumptive recombinant stocks were found to be ~1.0, which was comparable to the EOPs of the parental LAC and SSH wild-type viruses.

Pathogenicity tests of wild-type LAC and SSH viruses and certain LAC-SSH recombinants. The pathogenic potentials of the wild-type viruses (inoculated either singly or together) and six of the LAC-SSH recombinants were determined by intraperitoneal inoculation of groups of five 3-week-old mice, using two dilutions of the viruses. Groups of two or three adult hamsters and of two adult guinea pigs were also used for similar tests. The results, in terms of mortality rates and survival times, are shown in Table 5. None of the guinea pigs, all of which were inoculated intraperitoneally with 0.1 ml of a 10⁻¹ dilution of virus, died during 21 days of observation, although each received between 2.6 × 10⁶ and 1.8 × 10⁵ PFU of virus and most, by CF and HI tests, seroconverted (see Table 6).

The inoculated mice received between 2.6 × 10⁷ and 1.8 × 10⁴ PFU of virus (Table 5). All died between 2 and 6 days post-inoculation. However none of the groups of mice that re-

TABLE 4. *Recombination between-SSH and LAC ts mutants^a*

LAC <i>ts</i> mutant	% Recombinants with SSH <i>ts</i> mutant:			
	SSH I-1	SSH I-3	SSH II-2	SSH II-18
LAC II-3	3.9	5.4	0	0
LAC II-4	6.5	6.2	0	0
LAC II-5	35.7	16.9	0	0

^a Recombination percentages were determined as described in the text.

TABLE 3. *Recombination analyses with LAC ts mutants^a*

LAC <i>ts</i> mutant	% Recombinants with LAC <i>ts</i> mutant:																			
	3	4	9	13	1	2	5	10	11	12	14	15	16	17	18	19	20	21	22	23
3	·	0	13	11	0	0	0	0	0	0	0	0	10	0	0	8	13	0	0	14
4	·	·	13	5	0	0	0	0	0	0	0	0	12	0	0	7	17	0	0	12
9	·	·	·	0	21	39	10	30	8	33	27	19	0	25	32	0	0	22	7	0
13	·	·	·	·	17	24	4	39	3	7	7	4	0	18	6	0	0	11	2	0

^a Assignments: Group I—*ts* 9, *ts* 13, *ts* 16, *ts* 19, *ts* 20, and *ts* 23; group II—*ts* 3, *ts* 4, *ts* 1, *ts* 2, *ts* 5, *ts* 10, *ts* 11, *ts* 12, *ts* 14, *ts* 15, *ts* 17, *ts* 18, *ts* 21, and *ts* 22. Recombination percentages were determined as described in the text.

TABLE 5. *Intraperitoneal pathogenicity in 3-week-old mice of LAC, SSH, and putative LAC-SSH recombinants^a*

Virus	Inoculum titer (PFU/ml)	Average Survival Time (Days).	
		Inoculum: 0.1 ml of 10 ⁻⁵ dilution	Inoculum 0.1 ml of 10 ⁻² dilution
LAC (wild type)	1.2 × 10 ⁸	2.6 ± 0.49	3.0 ± 0.0
SSH (wild type)	3.4 × 10 ⁷	2.2 ± 0.40	3.0 ± 0.0
LAC × SSH (wild types)	1.5 × 10 ⁸	3.0 ± 0.0	3.0 ± 0.0
Recombinant cross			
LAC II-3 × SSH I-1	1.7 × 10 ⁸	3.0 ± 0.0	4.0 ± 0.0
LAC II-3 × SSH I-3	2.6 × 10 ⁸	2.6 ± 0.49	2.8 ± 0.57
LAC II-4 × SSH I-1	2.3 × 10 ⁸	4.0 ± 0.0 ^b	5.8 ± 0.57 ^b
LAC II-4 × SSH I-3	2.2 × 10 ⁸	4.0 ± 0.0 ^b	4.2 ± 0.04 ^b
LAC II-5 × SSH I-1	1.8 × 10 ⁷	5.2 ± 0.40 ^b	5.8 ± 0.57 ^b
LAC II-5 × SSH I-3	1.6 × 10 ⁸	3.0 ± 0.0	3.0 ± 0.0

^a Groups of five 3-week-old mice were inoculated intraperitoneally with two dilutions of the wild-type virus stocks or an equal-volume mixture of the wild-type viruses and observed until death occurred. Virus stocks obtained from the cloned recombinants from the indicated mutant virus crosses were similarly used to inoculate mice. All inoculated mice died, and the average survival times are given.

^b The average survival time, which differed from that of either parent wild type, is significant by Student's *t*-test. *P* < 0.001.

ceived the putative recombinant viruses died significantly earlier than those receiving the one or two wild-type viruses. Some of the mice that were inoculated with the putative recombinants did, however, survive significantly longer than those that received the wild-type viruses (e.g., the recombinant derived from LAC II-4 × SSH I-1). Although no correlation has yet been attempted for the ratio PFU titer/mean lethal dose for recombinants with those of the wild-type viruses, these results suggest that certain recombinants may allow mice an increased survival time compared to those receiving the wild-type progenitors.

The hamsters that were inoculated subcutaneously received between 2.6×10^6 and 1.8×10^5 PFU of virus. One of the two hamsters inoculated with wild-type progenitor LAC virus died on day 13; the two inoculated with wild-type progenitor SSH virus died on days 8 and 16. Two of 14 hamsters that received the recombinant viruses died (LAC II-5 × SSH I-3 died on day 11; LAC II-5 × SSH I-3 died on day 13); the rest survived.

Oligonucleotide fingerprint analyses of the L, M, and S RNA species of the LAC-SSH recombinants. After the pathogenicity tests in the three model animals systems indicated that the recombinant viruses were no more pathogenic than their wild-type progenitors, molecular analyses were undertaken to determine the origin and genome compositions of the recombinants. All of the virus preparations used for these experiments were grown and analyzed in the Virology P3 containment facility at the University of Alabama in Birmingham.

The oligonucleotide fingerprints of the L, M,

and S RNA species of wild-type SSH and wild-type LAC viruses are shown in Fig. 1. The fingerprints of the L, M, and S RNA species of the recombinant obtained from the LAC II-4 × SSH I-1 and LAC II-5 × SSH I-3 crosses are given in the upper and lower parts of Fig. 2, respectively. Comparison of the fingerprints of the two recombinants with those of the parental wild-type viruses indicates that both recombinants have SSH L RNA and LAC M RNA. One recombinant has SSH S RNA, whereas the other has LAC S RNA.

No evidence was obtained for either recombinant having either SSH M RNA or LAC L RNA or both SSH and LAC S RNA species, indicating that neither recombinant is heterozygous with regard to any single RNA size class. The results also suggest that the recombinants were formed by segment reassortment rather than by intrasegment recombination, since the fingerprints of all of the recombinant RNA species correspond totally to the RNA species of one or the other progenitor, and not partially to one progenitor and partially to the other. The recombinants can therefore be designated SSH/LAC/SSH (Fig. 2, top) and SSH/LAC/LAC (Fig. 2, bottom) to signify the origin of their respective L, M, and S RNA species.

Polypeptide compositions of the recombinant bunyaviruses; indication that the S RNA codes for N protein. Although the electrophoretic mobilities of viral G1, G2, and L proteins of prototype SSH and LAC viruses are essentially indistinguishable by polyacrylamide gel electrophoresis, the N protein of the latter has a slightly slower electrophoretic mobility

FIG. 1. Oligonucleotide fingerprints of the L, M, and S virion RNA species of LAC and SSH viruses. The oligonucleotide fingerprints of RNase T₁ digests of the individual ³²P-labeled L, M, and S RNA species of LAC and SSH viruses were obtained as described previously (5, 6). The X's on the radioautograms indicate the positions of the dye markers as has been previously described (5).

than that of the former (8).

The small sizes of the SSH and LAC S RNA species (5, 8; molecular weight, 0.45×10^6 to 0.50×10^6) preclude their coding for the L or G1 polypeptide (respective molecular weights, $\sim 180 \times 10^3$ and 115×10^3). Also, it appears unlikely that the S RNA species could code for both G2 (molecular weight, $\sim 38 \times 10^3$) and N protein (molecular weight, 24×10^3 to 21×10^3).

Since the SSH/LAC/SSH and SSH/LAC/LAC recombinants differ only with respect to their S RNA species, the possibility that the S RNA codes for N protein was examined by comparing the virion polypeptides of a ¹⁴C-amino acid-labeled SSH/LAC/SSH recombinant to those of a [³H]leucine-labeled SSH/LAC/LAC recombinant. The results (Fig. 3) indicate that the SSH/LAC/LAC recombi-

nant has an N protein with a slower electrophoretic mobility than that of the SSH/LAC/SSH recombinant. The G1 and G2 proteins of the two recombinants were indistinguishable. The results suggest that the S RNA codes for N protein. Peptide analyses will be needed to confirm this assignment.

Serological analyses of the animals that survived wild-type LAC, SSH, or LAC-SSH recombinant inoculations. The results of CF and HI analyses using test SSH and LAC antigens and the 21-day sera obtained from the animals that survived the virus inoculation are shown in Table 6.

The individual sera of the guinea pigs that received wild-type LAC virus, although clearly cross-reactive by both CF and HI tests, could be typed as containing LAC antibodies (2). The

individual sera of the guinea pigs that received wild-type SSH virus were less cross-reactive than those obtained for LAC and clearly contained SSH antibodies (2). A LAC CF response and an ambivalent HI response were obtained for the sera recovered from the guinea pigs that received both viruses.

No hamsters survived the wild-type SSH virus infections; however, the one hamster that survived the wild-type LAC infection gave a serum sample that, by CF and HI tests, as in the corresponding guinea pig serum tests, was compatible with it containing LAC antibodies. The hamster that survived the dual wild-type virus infections gave a serum sample that, by CF and HI tests, contained antibodies directed against SSH virus.

The recombinant virus obtained from the LAC II-3 $\times$ SSH I-1 cross gave an LAC-type HI response and an SSH-type CF response for both the guinea pig and hamster sera. All of the

other recombinants gave LAC-type CF and HI responses for sera obtained from guinea pigs or the available hamsters.

The serological results obtained for the LAC II-5 $\times$ SSH I-3 recombinant agree well with the observation that the recombinant has an SSH/LAC/LAC genome and an LAC-type N protein, and could be interpreted as indicating that the LAC M RNA and/or S RNA provide LAC gene products as assayed by the HI and CF tests. The results obtained for the LAC II-4 $\times$ SSH I-1 recombinant, which has an SSH/LAC/SSH genome and an SSH-type N protein, do not fit the interpretation that the S RNA codes for gene products that were measured by the CF or HI tests. Although that may be true for that particular recombinant, we have found that virion protein analyses of the LAC II-3 $\times$ SSH I-1 recombinant indicate that it has an SSH-type N protein (data not shown). Although no fingerprint analyses have been under-

FIG. 2. Oligonucleotide fingerprint of the L, M, and S virion RNA species of the recombinants obtained from LAC II-4 $\times$ SSH I-1 and LAC II-5 $\times$ SSH I-3 crosses. The oligonucleotide fingerprints of the RNase T_1 digests of the individual ^{32}P -labeled L, M, and S RNA species of the recombinants derived from the (top) LAC II-4 $\times$ SSH I-1 cross and (bottom) LAC II-5 $\times$ SSH I-3 cross were obtained as described previously. Comparison of the fingerprints with those of the progenitor wild-type SSH and LAC viruses (Fig. 1) indicates that the recombinant from the LAC II-4 $\times$ SSH I-1 cross has an SSH/LAC/SSH genotype and that the recombinant from the LAC II-5 $\times$ SSH I-3 cross has an SSH/LAC/LAC genotype.

FIG. 3. Co-electrophoresis of the ^{14}C -amino acid-labeled recombinant virus obtained from the LAC II-4 $\times$ SSH I-1 cross with the ^3H -leucine-labeled recombinant virus obtained from the LAC II-5 $\times$ SSH I-3 cross. Virions of the labeled recombinants were mixed, dissociated with sodium dodecyl sulfate, and resolved by polyacrylamide gel electrophoresis (8, 9).

taken for the LAC II-3 $\times$ SSH I-1 recombinant, the serological analyses indicate an SSH-type CF response and an LAC-type HI response, suggesting that for this recombinant SSH L and/or S RNA code for the gene products that were responsible for generating the SSH-type CF responses.

Although indicative, due to the cross-reactive nature of the LAC CF and HI antibody responses (and, to a lesser extent, those obtained for SSH virus), the serological results are not conclusive. Further analyses with individual viral antigens, as well as more refined immunological tools, will be needed to establish what antigens are involved in the HI and CF tests.

It is noteworthy that some of the groups of cohabitating hamsters that received a particular recombinant virus included one or two hamsters that did not seroconvert. Although the animals were only kept for 21 days post-inoculation, the lack of seroconversion might mean that no direct transmission of the virus between cage mates occurred. Further experiments involving cohabitating uninoculated and inoculated mice and other animals will be used in future pathogenicity tests to investigate this point.

DISCUSSION

Evidence has been obtained for the formation of two new recombinant viruses during coinfection of cells with group I temperature-sensitive

mutants of SSH virus and group II temperature-sensitive mutants of LAC virus. The results indicate that these recombinants are genetically stable and have genomes derived from both parents, presumably by segment reassortment. The recombinants are neither heterozygotes nor mixtures of both parental *ts* viruses, nor do they represent recombinants obtained by intrasegment recombination.

We conclude from the results obtained that the SSH group I mutants have a defect in their M RNA, since only the M RNA of LAC virus was recovered in both recombinants. Likewise, we conclude that the group II mutants of LAC have a defect in their L RNA, since only the L RNA of SSH virus was recovered in both recombinants. The fact that one recombinant had SSH S RNA and the other had LAC S RNA suggests that the S RNA represents a genome segment that was not defective for any of the progenitor mutants tested. Recombinant virus gene product analyses suggest that the S RNA codes for N protein—an observation that needs confirmation by peptide analyses.

The fact that two recombinants have been identified for the particular mutant virus coinfections investigated indicates that, as far as these LAC and SSH mutants are concerned, the reassortment process is independent. Whether this will be true for the recombinants generated by other LAC-SSH mutant combinations remains to be determined. The potential to obtain and produce recombinants with other prototype bunyaviruses remains to be determined. If reassortment of the genomes of LAC and SSH viruses is indeed independent in all the possible combinations one could produce, we would expect to generate $2^3 - 2$ (i.e., 6) new viruses from these two progenitors (e.g., LMS $\times$ lms $\rightarrow$ the parental LMS and lms and the six recombinant LMs, Lms, LmS, lmS, lMS, and lMS). We do not know whether recombinants can be obtained between SSH and the 11 other bunyaviruses in the California group. If they can, then the numbers of new viruses one could generate (i.e., $12^3 - 12$) are considerable. Likewise, we do not know whether recombinants can be obtained between SSH virus and other viruses in the Bunyamwera supergroup.

The potential for producing recombinants raises questions of whether recombinants can be detected in nature, the pathogenicity of the recombinants, their host range, and how they should be handled. We elected to perform our recombination experiments in P3 facilities, using the guidelines established for work on recombinant DNA. Pathogenicity tests were undertaken before any large quantities of the recombinant

TABLE 6. *Serological analyses of guinea pigs and the surviving hamsters that were inoculated with LAC virus and/or SSH virus, or the LAC-SSH recombinants^a*

Inoculum	Test antigen	Guinea pig		Serotype (CF-HI)	Hamster		Serotype (CF-HI)
		CF	HI		CF	HI	
LAC (wild type)	LAC	32	40	LAC-LAC	32	10	LAC-LAC
	SSH	32	20		32	<10	
SSH (wild type)	LAC	8	10	SSH-SSH	NS ^b	NS	
	SSH	128	160		NS	NS	
LAC × SSH (wild types)	LAC	64	20	LAC-?	32	20	SSH-SSH
	SSH	32	20		128	80	
Recombinant cross							
LAC II-3 × SSH I-1	LAC	32	80	SSH-LAC	16	80	SSH-LAC
	SSH	64	40		64	20	
LAC II-3 × SSH I-3	LAC	32	40	LAC-LAC	128	80	LAC-LAC
	SSH	16	20		64	80	
LAC II-4 × SSH I-1	LAC	128	40	LAC-LAC	128	80	LAC-LAC
	SSH	64	20		128	40	
LAC II-4 × SSH I-3	LAC	64	80	LAC-LAC	32	40	LAC-LAC ^c
	SSH	64	40		32	20	
LAC II-5 × SSH I-1	LAC	32	20	LAC-LAC	64	40	LAC-LAC ^d
	SSH	32	10		64	20	
LAC II-5 × SSH I-3	LAC	64	80	LAC-LAC	NR ^e	NR	
	SSH	32	20		NR	NR	

^a CF and HI antibody responses were determined in sera of surviving animals by using SSH or LAC test antigens as described previously (4). The guinea pig and hamster sera were tested on an individual basis, with the alternate sera giving the same results as those shown in these assays, except when no HI or CF response was detected, suggesting that the animal was not infected.

^b NS, No survivors.

^c One of two animals not infected, as judged by no CF or HI response.

^d Two of three animals not infected, as judged by no CF or HI response.

^e NR, No response.

viruses were produced for molecular analyses (i.e., up to 10^{10} PFU). The pathogenicity tests indicate that the recombinant viruses were either no more pathogenic than the prototype viruses in the three animal systems employed, or appeared to be less pathogenic. Further tests involving intranasal inoculation, cohabitation of inoculated animals with uninoculated controls, and use of other model animal systems are in progress.

A nomenclature system (e.g., SSH/LAC/SSH) is proposed for the recombinant viruses which signifies the origin of the recombinants' respective L, M, and S RNA segments. This system allows us to identify recombinants that may be produced from any three progenitor virus stocks.

The serological results obtained for the LAC II-3 × SSH I-1 recombinant (genotype not determined, but possessing a virion SSH-type N protein) compared with those obtained for the

LAC II-5 × SSH I-3 recombinant (SSH/LAC/LAC genotype, LAC-type N protein) are compatible with the interpretations that the HI response measures the LAC M RNA gene product, and the CF response measures an S RNA gene product (N protein). However the serological result obtained for the LAC II-4 × SSH I-1 recombinant (SSH/LAC/SSH genotype, SSH type N protein) do not agree with this interpretation. Due to the cross-reactive nature of the responses obtained with the prototype viruses, the utility of these tests in delineating the CF and HI serotypes of the LAC-SSH recombinant viruses is limited.

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