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Recombination and Complementation Between Temperature-Sensitive Mutants of a Bunyavirus, Snowshoe Hare Virus

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Complementation and recombination have been observed with temperature-sensitive mutants of Bunyavirus snowshoe hare virus.

Snowshoe hare virus is a member of the California encephalitis subgroup of Bunyaviridae (1). The virus was originally isolated from the blood of an emaciated *Lepus americanus* (snowshoe hare) caught in Bitterroot Valley, Mont. (4). Virus has also been isolated from lemmings, snowshoe hares, and mosquitoes in various parts of the northern United States, Alaska, and Canada (6, 8, 11, 13-16, 20, 22, 24-26). Antibodies against the virus have been detected in the northern areas of the continent in human (7, 9, 25) and various other animals (5, 6, 11-13, 16, 24, 25). Snowshoe hare virus is distinguishable from other members of the California encephalitis subgroup of viruses (i.e. Bocas, California encephalitis, Inkoo, Jamestown Canyon, Jerry Slough, Keystone, La Crosse, Lumbo, Melao, San Angelo, Tahyna, and Trivittatus viruses) by complement fixation and, in some cases, by hemagglutinin inhibition and neutralization of infectivity assays (21).

Recent analyses (3, 17) have established that of the California encephalitis subgroup viruses, Lumbo and La Crosse possess RNA genomes that are in three separate segments (molecular weight, $\sim 3 \times 10^6$, 2×10^6, and 0.5×10^6). We report here that a similar segmented genome is also present in snowshoe hare virions. A [^{3}H]uridine-labeled preparation of snowshoe hare virus was obtained by infecting confluent monolayers of BHK-21 cells with snowshoe hare virus at an input multiplicity of infection of 0.001 PFU per cell. The snowshoe hare virus was originally obtained from J. F. Obijeski of the Center for Disease Control, Atlanta, Ga., and was subsequently cloned twice in monolayers of BHK-21 cells. A plaque plug from the second cloning was used to derive a stock of virus (titer $\sim 3 \times 10^7$ PFU per ml). The authenticity of this virus preparation has been verified by R. E. Shope of the Yale Arbovirus Research Unit, New Haven, Conn. The procedures employed for labeling cells with [^{3}H]uridine and purifying snowshoe hare virus from supernatant fluids were similar to those described by Obijeski and collaborators (18). Purified [^{3}H]uridine-labeled virus was mixed with ^{32}P-labeled BHK-21 28S and 18S rRNA and then subjected to electrophoresis (2) in 2% gels of polyacrylamide (Fig. 1). Three bands of ^{3}H-labeled RNA were observed which, by comparison to the molecular weights of the rRNA species (10), possessed apparent molecular weights of 2.9×10^6, 1.9×10^6, and 0.45×10^6. The three RNA species were apparently not in equimolar proportions. Similar results have been obtained for ^{32}P-labeled virus (unpublished observations) and also reported for Lumbo virus (3). Although equimolar proportions have been obtained in other preparations of either ^{3}H- or ^{32}P-labeled snowshoe hare virus (unpublished data), the frequent occurrence of unequal proportions of RNA species may represent a defect in virus maturation and packaging of the virion nucleocapsids. The presence of three RNA segments in virions of four other Bunyaviruses has also been observed (Trivittatus, Bunyamwera, Main Drain, and Ilesha viruses; J. Gentsch, S. Pettaway, J. Obijeski, and D. H. L. Bishop, unpublished data). Uukuniemi virus, which is serologically distinguishable from the Bunyamwera supergroup of viruses (1), is reported to possess four RNA species of molecular weight 4.1×10^6, 1.9×10^6, 0.88×10^6, and 0.78×10^6 (19).

It has been shown (17) that virions of La Crosse virus possess three major proteins, including two glycoproteins, G1 and G2 (molecular weight, 120×10^3 and 34×10^3), and a nucleocapsid protein N (molecular weight, 23×10^3), as well as a minor, large, internal protein L (molecular weight, $\sim 180 \times 10^3$). Three major polypeptides and some minor larger polypeptides were found in preparations of snowshoe hare virus (Fig. 2). Whether one of the large minor polypeptides represents the L protein described by others (17) is not known. However,

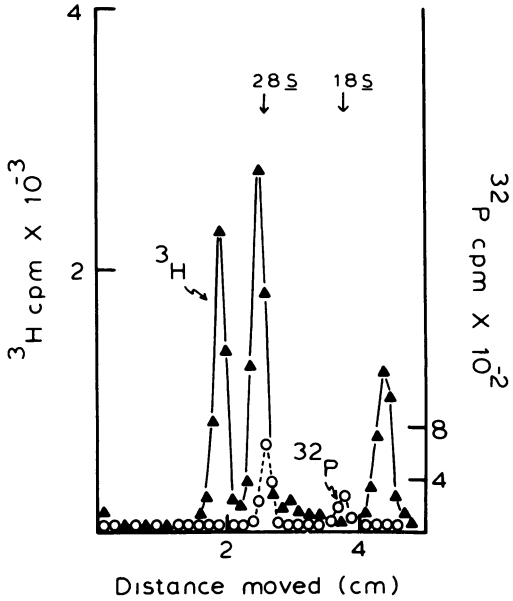

FIG. 1. *Co-electrophoresis of ^{3}H-labeled snowshoe hare viral RNA with ^{32}P-labeled BHK rRNA. A portion of purified [^{3}H]uridine-labeled snowshoe hare virus was mixed with purified 28S and 18S RNA extracted from BHK-21 cells and resolved by electrophoresis in 2% polyacrylamide gels (2).*

since two of the major polypeptides (G1 and G2) could be labeled by inclusion of [^{3}H]glucosamine in the growth medium (data not shown) and specifically removed by Pronase treatment (data not shown), it can be concluded that they are surface glycoproteins. Co-electrophoresis of ^{14}C-labeled snowshoe hare viral polypeptides with ^{3}H-amino acid-labeled La Crosse viral polypeptides indicates that there are only minor differences in the sizes of the three major polypeptides of the two viruses (unpublished data).

The interrelationships of the three RNA segments of the Bunyaviruses are not known. Do the smaller RNA pieces represent defective RNA species or are they degradation products of the largest RNA? Alternatively, do they contain separate genetic information? If the latter, then one might expect, as shown for influenza virus that possesses a segmented genome, that high-frequency recombination would occur during co-infection of cells by two temperature-sensitive, conditionally lethal mutants if those mutations were in gene products of different RNA segments. For influenza virus it has been postulated that such high-frequency recombination is due to the reassortment of the progeny segments derived from the genomes of the two parental viruses (see review by Sugiura [23]).

To ascertain if the snowshoe hare Bunyavirus has unique genetic information resident in more than one RNA species, a genetic analysis was undertaken. Temperature-sensitive, conditionally lethal mutants of snowshoe hare virus were obtained by the following protocol. Several confluent monolayers of BHK-21 cells were infected with wild-type virus at a multiplicity of infection of approximately 0.001 PFU per cell and incubated in Eagle medium containing 5% (vol/vol) fetal calf serum and 50 μg of 5-fluorouracil per ml. At 72 h postinfection the virus harvests (representing ~1% of the normal wild-type yield) were plated on BHK cells at 33°C, and from each harvest approximately 75 well-separated plaque plugs were pulled; each one was used to infect duplicate microwells of confluent BHK cells. One set of microwells was incubated at 39.5°C, and the other set was incubated at 33°C. The progeny yields, as determined by a 33°C plaque assay, were compared 48 h later, and those viruses for which the harvest from the 33°C infection was at least

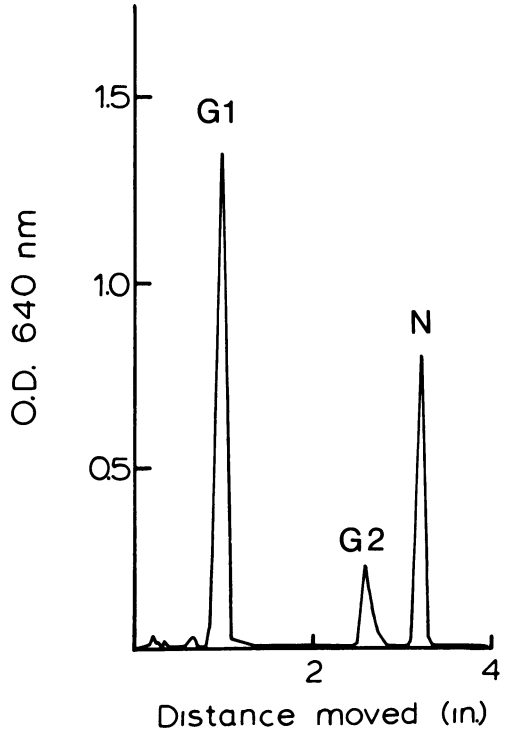

FIG. 2. *Electrophoresis of snowshoe hare viral proteins. A sample of purified snowshoe hare virus was dissociated in 1% (wt/vol) sodium dodecyl sulfate and 1% mercaptoethanol and then resolved by electrophoresis in 8% polyacrylamide gels, stained by Coomassie brilliant blue, and scanned at 640 nm (16, 17).*

1,000-fold greater than that of the 39.5°C infection were selected for further study (usually one or two per original harvest). From the 33°C plaque assay of the 33°C harvests of those viruses a plaque plug was again obtained and used to grow a virus stock at 33°C using a confluent monolayer of BHK-21 cells. Each virus stock was tested for (i) its ability to give plaques at 33°C, (ii) its ability to give plaques 39.5°C, and (iii) its yield of viruses upon infection at 33°C as opposed to 39.5°C (i.e., its leakiness or reversion rate). Of some twenty initial candidates, six were sufficiently nonleaky (see Table 1) to be used in the complementation-recombination assays described below.

Complementation and recombination assays were performed as follows. Duplicate microwells of confluent BHK-21 cells (2×10^5 cells per microwell) were infected with 5 PFU per cell of either one mutant or another mutant or 5 PFU of each mutant. The viruses were allowed to absorb at room temperature for 30 min. The inocula were removed, and prewarmed media were added (33°C for one set of microwells and 39.5°C for the other set). Each set of microwells was incubated at its respective temperature for 2 h, and then the media were replaced with fresh prewarmed media to remove desorbed virus. The incubations were continued at their respective temperatures for a further 22 h (which represents the single-cycle growth period [17]), and the virus harvests were recovered. Virus yields were determined by plating the harvests at 33 and 39.5°C. The results obtained, as well as those obtained for comparable infections with wild-type virus, are expressed in Tables 1–3 in terms of the observed complementation indexes. In each case in which complementation was obtained (complementation indexes greater than 2), recombinant virus was also obtained as judged by the plaquing efficiencies of the progeny of the mixed virus infections by comparison with the plaquing efficiencies of the single-mutant virus infections and wild-type infection. This was confirmed by clonal analyses of the progeny of the single- and dual-virus infections. Clones picked from the 39.5 or 33°C harvests of the single-mutant virus infections were temperature sensitive—as judged by their plating efficiencies at 33 and 39.5°C (i.e. no revertants were found). When 48 clones of the *ts*3 × *ts*7 dual-virus infection (33°C harvest) were similarly analyzed, 13 were found to have a wild-type phenotype and the rest were temperature-sensitive. How many of the latter were double mutants was not determined. The fact that recombination indexes varied from 2 to 43% may reflect the differences in growth potential of the two mutants and therefore the efficiency of segment reassortment to give recombinant progeny.

The results indicate that both complementa-

TABLE 1. *Virus yields after single and mixed infections with snowshoe hare virus ts mutants*

Inoculum viruses	Incubation temp (°C)	24-h yield assayed at:		C index[a]	% Recombinants[b]
		33°C	39.5°C		
*ts*1	33	4.1×10^7	6×10^3		
	39.5	8×10^3	$<10^3$		
*ts*2	33	5×10^7	3×10^3		
	39.5	8×10^3	$<10^3$		
*ts*3	33	6.8×10^7	10^3		
	39.5	$<10^3$	$<10^3$		
*ts*1 × *ts*2	33	1.1×10^8	2.3×10^6		4
	39.5	4.3×10^5	3.5×10^4	25	
*ts*1 × *ts*3	33	1.5×10^8	4×10^4[c]		<0.05
	39.5	$<10^3$	$<10^3$	<0.1	
*ts*2 × *ts*3	33	7.5×10^7	5.5×10^6		15
	39.5	9.6×10^5	3.4×10^5	78	
Wild type	33	2.3×10^7	2.7×10^7		
	39.5	4.6×10^6	2.7×10^6		

[a] Complementation (C) index = $([AB_{39.5}]_{33} - [AB_{39.5}]_{39.5})/([A_{39.5}]_{33} + [B_{39.5}]_{33})$.
[b] Percentage of recombinants = $\{[AB_{33}]_{39.5} - ([A_{33}]_{39.5} + [B_{33}]_{39.5}) \times 100 \times 2\}/[AB_{33}]_{33}$.
[c] Tiny plaques.

TABLE 2. *Complementation indexes*[a]

ts Mutant	C indices with *ts* mutant:					
	1	2	3	6	7	9
1		25	<0.1	0.1	62	0.8
2			78	26	0.8	158
3				1	7	1
6					5	<0.1
7						6

[a] Complementation (C) indexes were calculated as described in Table 1, footnote *a*. Assignments: (group I) *ts*1, *ts*3, *ts*6, *ts*9; (group II) *ts*2, *ts*7.

TABLE 3. *Percentage of recombinants*[a]

ts Mutant	% Recombinants with *ts* mutant:					
	1	2	3	6	7	9
1		4	<0.05[b]	<0.2[b]	7	<0.02
2			15	28	<0.01	12
3				<0.1	43	<0.01
6					2	<0.5[b]
7						4

[a] Percentage of recombinants was calculated as described in Table 1, footnote *b*.
[b] Tiny plaques.

tion and recombination can be obtained between *ts* mutants of snowshoe hare virus. The fact that recombinants were formed at relatively high frequencies suggests that genome segment reassortment occurs in mixed virus infections. Further analyses are underway to investigate this possibility.

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