CHAPTER 8

Genetics and Genome Segment Reassortment

C. R. PRINGLE

I. SEGMENTED GENOME VIRUSES

Nineteen of the ninety-five taxonomic groups of viruses (families and floating genera) listed in the Sixth Report of the International Committee for Taxonomy of Viruses (Murphy *et al.*, 1995) possess segmented genomes. All except the geminiviruses of plants are RNA viruses. These viruses are listed in Table I according to nucleic acid type, segment number, and virion composition. Segmentation of the genome is most commonly observed among the negative-stranded and double-stranded RNA genome viruses, and these viruses are single-component viruses, where the complete complement of genome segments is contained within one particle and the infectious unit is a single virion. Segmentation among the positive-stranded RNA genome viruses, on the other hand, is a phenomenon restricted to multicomponent viruses, where the individual genome segments are contained within different particles and the infectious unit is a full complement of particles. With the single exception of the *Nodaviridae*, these viruses are all viruses infecting plants.

A feature of some segmented negative-stranded RNA viruses is the ambisense encoding of genetic information, i.e., the nonoverlapping encoding of genes in the 5′ halves of both the viral RNA (negative sense) and the viral-complementary RNA (positive sense). Both segments of the bipartite

C. R. PRINGLE • Biological Sciences Department, University of Warwick, Coventry CV4 7AL, United Kingdom.

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arenaviruses exhibit ambisense encoding of information (Bishop, 1986; Bishop and Auperin, 1987), as do at least three of the four/five segments of the genomes of the filamentous tenuiviruses of plants (Ramirez and Haenni, 1994). The family *Bunyaviridae* is more heterogeneous in this respect since ambisense encoding of information is observed in the smaller RNA subunit only of viruses belonging to two of the five genera (Table I).

The three families of viruses with unsegmented negative-stranded RNA genomes are closely related in terms of genome structure and function, and accordingly they have been grouped together as the order *Mononegavirales*. Although the three families of viruses with segmented negative-stranded RNA genomes, the bipartite *Arenaviridae*, the tripartite *Bunyaviridae*, and the multipartite *Orthomyxoviridae*, are more diverse in terms of genome structure and function, they also form a coherent (super)group when phylogenetic trees for RNA-dependent RNA polymerases are constructed (Goldbach and de Haan, 1994). It has been argued that these three families of segmented genome negative-stranded RNA viruses should be combined into a second order, designated the *Multinegavirales* for consistency (Pringle, 1991a; Ward, 1993), both subsumed within the class *Negavirata* (Tordo *et al.*, 1992; Ward, 1993). The principal argument against the acceptance of this proposal is the potential for reassortment of genome subunits which defines the group. Reassortment of genome subunits can obscure evolutionary lineages and may result in the establishment of spurious phylogenetic associations. The absence of reassortment (and intermolecular recombination) in the case of the unsegmented negative-stranded genome RNA viruses (Pringle, 1987, 1990, 1991b; Chao, 1994), on the other hand, guarantees that phylogenetic relationships can be inferred with reasonable confidence, thereby allowing the creation of higher taxonomic categories.

Segmentation of the viral genome has several potential advantages. It maximizes the effects of positive Darwinian selection and promotes greater evolutionary plasticity; the epidemic and pandemic behavior of the human influenza A viruses are the best example of this in nature. It may also facilitate the elimination of deleterious mutations by purifying selection and a more rapid exit of defective interfering genomes. It has been suggested also that segmentation may be a device to increase the genetic content of RNA-containing viruses where the high mutation rate may impose a limit on the absolute size of informational RNA molecules (Reanney, 1984). However, this argument is difficult to sustain since the total genome sizes of segmented genome viruses are in the same range as those of nonsegmented viruses, and the maximum genome size of any segmented genome virus (the 23 kb of the nairoviruses or the 27.5 kbp of the double-stranded rice ragged stunt virus) does not reach the 31 kb of some of the nonsegmented coronaviruses. In the multicomponent plant viruses, segmentation of the genome is probably an adaptation to facilitate movement of virus within the infected plant, and a consequence of the absence of specific receptors on plant cells and the nonspecific mode of entry of the virus into the host plant. Segmenta-

TABLE I. The Segmented Genome Viruses

Nucleic acid type	Sense	No. of segments	Particle type	Family or genus[a]	Principal host	Evidence of reassortment
ssDNA		1	—	4 families	—	n.a.[b]
		1 or 2	Multicomponent	*Geminiviridae*	Plant	yes
dsDNA		1	—	18 families or genera	—	n.a.
		>1	—	none	—	n.a.
ssRNA	+	1	—	26 families or genera	—	n.a.
	+	2	Multicomponent	G. *Bymovirus*	Plant	?[c]
	+	2	Multicomponent	*Comoviridae*	Plant	yes
	+	2	Multicomponent	G. *Furovirus*	Plant	yes
	+	2	Multicomponent	G. *Dianthovirus*	Plant	yes
	+	2	Multicomponent	G. *Idaeovirus*	Plant	?
	+	2	Multicomponent	*Nodaviridae*	Insect	?
	+	2	Multicomponent	G. *Enamovirus*	Plant	yes
	+	2	Multicomponent	G. *Tobravirus*	Plant	yes
	+	3	Multicomponent	*Bromoviridae*	Plant	yes
	+	3	Multicomponent	G. *Hordeivirus*	Plant	yes
	−	1	—	3 families	—	n.a.
	ambisense	2	Single-component	*Arenaviridae*	Animal	yes
	−/ambisense	3	Single-component	*Bunyaviridae*	Animal/plant	yes
	−/ambisense	4 or 5	Multicomponent	G. *Tenuivirus*	Plant	?
	−	7 or 8	Single-component	*Orthomyxoviridae*	Animal	yes
dsRNA	+/−	1	—	2 families	—	n.a.
	+/−	2	Single-component	*Birnaviridae*	Animal	yes
	+/−	2	Multicomponent	*Partitiviridae*	Plant	?
	+/−	3	Single-component	*Cystoviridae*	Bacteria	yes
	+/−	10–12	Single-component	*Reoviridae*	Animal/plant	yes
DNA/RNA	+/− or +	1	Single-component	4 families or genera	Animal/plant	n.a.

[a]The genus *Bymovirus* is classified in the family *Potyviridae*. The genera *Furovirus, Dianthovirus, Enamovirus, Tobravirus, Hordeivirus,* and *Tenuivirus* are floating genera.
[b]n.a., not applicable.
[c]?, not reported.

tion may more often be a stratagem to provide control of biosynthesis at the transcriptional level; the temporal control of gene product synthesis in the bunyaviruses is an example of this (Pennington *et al.*, 1977). The evolution of segmentation in RNA viruses has been equated with the evolution of sex by Chao (1994), allowing the participation of two, three, or more parents in the production of offspring.

A consequence of segmentation of the genome is the possibility of increasing genetic heterogeneity by reassortment of subunits during replication and morphogenesis. Plant virologists use the term *pseudorecombination* rather than *reassortment* to describe the exchange of genome subunits that can accompany the maturation of multicomponent viruses (Table I). Pseudorecombination has been observed between different viruses belonging to the family *Bromoviridae* and the floating genera *Tobravirus* and *Dianthovirus*, and between different strains of the same virus in all three genera (*Nepovirus*, *Comovirus*, and *Fabavirus*) of the family *Comoviridae*, and in the genera *Furovirus*, *Hordeivirus*, *Enamovirus*, and *Geminivirus*. It is likely that all segmented genome viruses have the ability to interchange genome subunits between related strains.

II. THE *BUNYAVIRIDAE* GENOME

The viruses belonging to the family *Bunyaviridae* are well suited to genetic study because of their antigenic and ecological diversity. This provides a wealth of phenotypic variation as a substrate for genetic analysis. The tripartite nature of the genome favors the analysis of the phenomenon of reassortment, since the number of reassortant genotypes is small enough to allow complete analysis of the progeny from individual crosses. Table II shows the number of possible genotypes generated as a consequence of reassortment for the different groups of single-component segmented genome viruses. The number of reassortant genotypes from crosses of the bipartite arenaviruses is too small to address critically questions such as the randomness of the reassortment process; in addition, the ambisense encod-

TABLE II. The Number of Reassortant Genomes in Relation to Segment Number

Virus family or group	Number of genome segments	Number of reassortant genotypes in progeny (less parental types)
Arenaviridae	2	$2^2 - 2 =$ **2**
Bunyaviridae	3	$2^3 - 2 =$ **6**
Tenuiviruses	4 or 5	$2^4/2^5 - 2 =$ **14** or **30**
Orthomyxoviridae	7 or 8	$2^7/2^8 - 2 =$ **126** or **254**
Reoviridae	10, 11, or 12	$2^{10}/2^{11}/2^{12} - 2 =$ **1022**, **2046**, or **4094**

ing of information in both segments of the genome may place constraints on mutation type and frequency. The number of progeny genotypes from crosses of tenuiviruses (Table II) might be manageable, but their multicomponent nature and the vagaries of assay of plant viruses *in vitro* render these viruses unsuitable. The number of progeny genotypes from crosses of the orthomyxoviruses or the reoviruses is too large for comprehensive analysis and genetic analysis must rely on statistical estimation. The importance of complete progeny analysis will become clear in the following sections. The genetic consequences of ambisense encoding can be investigated best in the *Bunyaviridae* because this family comprises conventional negative-stranded viruses, and several where one of the three segments exhibits ambisense encoding of information.

It is a characteristic of negative-stranded RNA viruses, whether segmented or not, that almost all of the nucleotide sequence is protein encoding. The nucleotide sequence of the genome of several viruses in the family *Bunyaviridae* (Bunyamwera virus of the genus *Bunyavirus*, Hantaan, Puumala, and Seoul viruses of the genus *Hantavirus*, and Rift Valley fever virus and Uukuniemi virus of the genus *Phlebovirus*) has been completely determined and in each case more than 95% of the sequence consists of open reading frames. Calculation of the coding potential of the bunyavirus genome has to take into account the encoding of the NSs nonstructural protein in an overlaping reading frame in the S RNA of viruses belonging to the genus *Bunyavirus*. The NSs protein gene of viruses of the *Phlebovirus* genus, however, does not overlap the N protein gene as the two genes are encoded in an ambisense fashion in opposite halves of the S RNA (see Chapter 5). Likewise the ambisense encoding of genetic information in the M RNA and S RNA of members of the genus *Tospovirus* does not involve any extension of coding capacity. The NSs protein gene is absent in viruses belonging to the *Hantavirus* and *Nairovirus* genera. The NSm nonstructural protein gene located in the M RNA is likewise not a universal feature of viruses of the family *Bunyaviridae* and does not extend the coding potential since it is derived by nascent cleavage of a polyprotein; it is absent in hantaviruses and variably present in the phleboviruses.

III. STRATEGY OF REPLICATION OF THE *BUNYAVIRIDAE*

Only those features that distinguish the viruses of the family *Bunyaviridae* from other negative-stranded RNA viruses and are relevant to the genetic properties of the *Bunyaviridae* are considered here. Replication occurs in the cytoplasm and is not inhibited by preexposure of cells to actinomycin D or α-amanitin. Nonetheless, infectious Bunyamwera virus is not released from enucleated susceptible cells, although viral protein synthesis is initiated. Furthermore, infectious virus was not released from infected BS-C-1 cells enucleated at times up to 6 hr after infection, suggesting that the

presence of the nucleus may be required for normal maturation (Pringle, 1977). Membranes of the Golgi apparatus are the site of maturation of bunyaviruses, and the Golgi apparatus, although present after enucleation of BS-C-1 cells, is rapidly degraded. The presence of a functional nucleus may be necessary to maintain the function of the Golgi apparatus. On the other hand, bunyavirus-specific intranuclear inclusions have been reported in the nuclei of cells infected with the phlebovirus Rift Valley fever virus (Swanepoel and Blackburn, 1977; Struthers and Swanepoel, 1982) and the bunyavirus Ilesha virus (Campbell, 1992; and Fig. 1). In the case of Rift Valley fever virus a nonstructural protein appears to be associated with these inclusion bodies (Struthers and Swanepoel, 1982).

Anomalous nuclear inclusions have also been observed in paramyxovirus-infected cells at both early and late times after infection. Peeples (1991) has suggested that the role of the paramyxovirus protein located in the nucleus may be either to modify ribosome assembly in order to favor translation of viral rather than host mRNA, or to function as a transport protein for transfer of a nuclear factor promoting replication or assembly. The protein located in the nucleus of Newcastle disease virus-infected cells, however, has been identified using monoclonal antibodies as the viral matrix protein, a protein that viruses of the family *Bunyaviridae* lack. In the case of the *Bunyaviridae* the function of such a factor might be involved in cap transfer. Primary

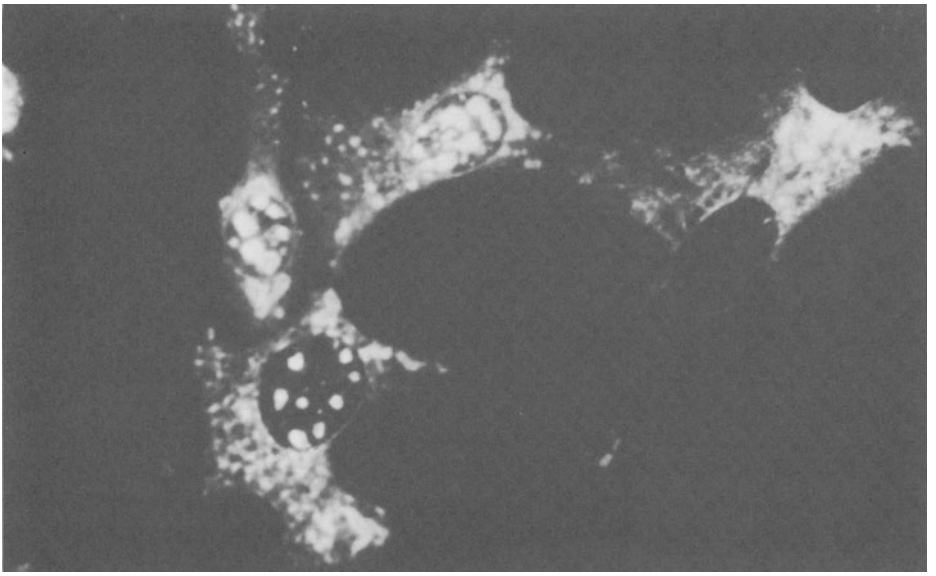

FIGURE 1. Nuclear inclusions in Ilesha virus (Bunyamwera serogroup)-infected Vero cells (photograph provided by the late P. V. Shirodaria). Ilesha virus-infected acetone-fixed Vero cells were stained at 18 hr postinfection with monoclonal antibody 6B10 and FITC-conjugated goat anti-mouse IgG. Monoclonal antibody 6B10 specifically immunoprecipitates a 30-kDa polypeptide from Ilesha virus-infected cells (Campbell, 1992).

transcription is mediated by a virion-associated RNA-dependent RNA polymerase. Synthesis of mRNA is primed by a cap-transfer mechanism analogous to that of the orthomyxoviruses, except that in the case of the *Bunyaviridae* it occurs in the cytoplasm, and the mRNAs do not appear to be polyadenylated.

The L RNA subunit has a single major open reading frame (ORF) encoding the virion polymerase; there are several additional short ORFs which do not appear to be expressed. The M RNA subunit encodes a precursor polyprotein that yields by cotranslational proteolytic cleavage the G1 and G2 envelope proteins and in some viruses a nonstructural protein NSm. The S RNA subunit encodes the nucleoprotein (N) and in some viruses a nonstructural protein (NSs). The precise functions of the NSm and NSs proteins are not known. However, the NSm protein of the tospovirus tomato spotted wilt virus, encoded in an ambisense mode in the M RNA, appears to function as a viral movement protein mediating the movement of ribonucleocapsid structures between cells and the spread of infection within plant tissue (Kormelink *et al.*, 1994).

Morphogenesis of viruses of the family *Bunyaviridae* occurs by accumulation of the G1 and G2 glycoproteins in the Golgi apparatus where terminal glycosylation takes place. The circularized nucleocapsids are enveloped by modified host cell membrane, and virions are formed by budding into the cisternae of the Golgi vesicles. The virions are released by fusion of cytoplasmic vesicles with the plasma membrane, or by direct release from ruptured cells. The absence of a matrix protein in these viruses may be related to the internal site of morphogenesis of the virions.

All viruses of the family *Bunyaviridae*, other than those classified in the genus *Hantavirus*, replicate in invertebrate and vertebrate hosts. In common with most arboviruses, little tissue damage accompanies replication in the invertebrate host. Many viruses have very narrow host ranges in nature and their biological properties are determined by vector behavior and distribution. Venereal and transovarial transmission are common and the virus can overwinter in the dormant egg. Infection of a vertebrate host is not an obligatory part of their life cycle, and there is generally less host restriction in the vertebrate host. Human and domestic animals seldom play the role of amplifying hosts and generally represent dead-end infections. The vector and ecological specificity of these viruses limits opportunities for genetic interaction and is probably responsible for the graded spectrum of restrictions on reassortment that is characteristic of viruses of the family *Bunyaviridae*.

IV. GENETICS OF THE *BUNYAVIRIDAE*

A. The Diversity of the *Bunyaviridae*

The family *Bunyaviridae* is perhaps the most diverse of virus groups, at least in terms of named viruses. The five genera represent distinct groups of

viruses with distinctive modes of transmission (Beaty and Calisher, 1991). Viruses of the genera *Bunyavirus*, *Nairovirus*, and *Phlebovirus* are maintained by vertical transmission in arthropods and by a dynamic interaction with specific vertebrate hosts, usually small rodents. The bunyaviruses are associated with mosquitoes, the nairoviruses and uukuviruses with ticks, and the phleboviruses with sand flies. The plant tospoviruses are transmitted by thrips and can multiply in a number of plant hosts. The hantaviruses are maintained in rodents and have no known vectors. Identification of the determinants of these complex relationships is one of the challenges facing genetics.

The taxonomic relationships of viruses in the family *Bunyaviridae* are reflected in their molecular properties. In general, viruses classified in different genera show little or no sequence homology. Therefore, it is not surprising that genetic interactions have not been recorded between viruses belonging to different genera, although nongenetic interactions in the form of phenotypic mixing and pseudotype formation may be possible. The intracellular site of maturation, however, limits opportunities for pseudotype formation with heterologous enveloped viruses. Viruses classified within the same genus generally exhibit some sequence similarity, the extent of homology increasing in viruses that show serological relationship. The degree of sequence divergence among viruses within the same serogroup is sufficient, however, to authenticate as distinct biological entities the often bizarrely and arbitrarily named viruses.

B. Genetic Interactions

1. Mutants

Temperature-sensitive (ts) mutants of several representatives of the *Bunyaviridae* have been isolated from both untreated and mutagen-treated wild- type stocks to provide defined genetic material for study of gene function, elucidation of the process of reassortment, and identification of the determinants of host range and virulence. The mutants described so far have been derived from various viruses belonging to the genera *Bunyavirus*, *Phlebovirus*, and *Uukuvirus*. Gentsch *et al.* (1977) observed that spontaneous ts mutants were present in stocks of La Crosse virus and snowshoe hare virus (California serogroup bunyaviruses) at frequencies of 1.0 and 1.7%, respectively, whereas the frequency of ts mutants in a stock of Maguari virus (a Bunyamwera serogroup bunyavirus) was reported to be higher and estimated at 2.7% (Iroegbu, 1981). The majority of the ts mutants described have been isolated following replication in the presence of noninhibitory concentrations of 5-fluorouracil, 5-azacytidine, or *N*-methyl-*N'*-nitrosoguanidine (NTG). The data obtained by Ozden and Hannoun (1978) suggested that 5-fluorouracil was more effective as a mutagen than NTG.

2. Recombination of Genetic Markers by Subunit Reassortment

A total of 210 ts mutants isolated from different viruses have been described in some detail. Ten of these eleven viruses belong to two serogroups in the genus *Bunyavirus* and the remaining one to the genus *Uukuvirus* (Table III). These mutants have been employed principally to demonstrate that recombination of genetic markers in crosses of bunyaviruses is mediated by reassortment of genome subunits (Gentsch *et al.*, 1977, 1979; Iroegbu and Pringle, 1981a). The majority of these mutants have been assigned to reassortant groups, such that non-ts virus is only present in the progeny from mixed infections with parental ts mutants classified in different reassortant groups. An unexpected finding, however, was the predominant recovery of mutants assignable to only two reassortant groups rather than the three predicted by the tripartite nature of the viral genome.

These reassortant groups were designated groups I and II and their equivalence in the different viruses included in the same serogroup was established by analysis of the progeny from mixed infections with heterologous parental viruses. Reassortment was restricted to viruses within the same serogroup. Attempts to obtain reassortment in crosses of viruses belonging to the California and Bunyamwera serogroups have not succeeded. However, the combinations of viruses examined have been rather limited. The homologies of the reassortant groups listed in Table III do not extend beyond the boundaries of the serogroups.

Maguari virus of the Bunyamwera serogroup is anomalous in that 1 of the 46 ts mutants isolated from mutagenized wild-type virus, designated tsMAG23(III), generated non-ts reassortants in mixed infections with all mutants belonging to reassortant groups I and II tested and also with two putative double I + II mutants, suggesting that the third reassortant group predicted by the tripartite structure of the genome had been identified (Pringle and Iroegbu, 1982). Mutant tsMAG23(III) exhibited a pronounced host restriction and a small plaque phenotype which may account for the failure to isolate similar mutants in other viruses. The plaque-forming ability of tsMAG23(III) on BS-C-1 cell monolayers was 100-fold lower, and yields from mixed infections 1000-fold less than in BHK-21 cells. On the other hand, mutants of Maguari virus and other bunyaviruses classified in groups I and II did not exhibit such marked host-dependent differences. However, conclusive evidence that the three reassortment groups correspond to the three genome segments has been difficult to obtain because of conflicting evidence in the characterization of representative mutants of the three groups at the phenotypic level (see below).

3. Complementation

Intergenic complementation is difficult to discriminate from reassortment in bunyaviruses, because reassortment is an early event in the multi-

TABLE III. Classification of Temperature-Sensitive Mutants into Reassortant Groups

Genus	Serogroup	Virus	No. of ts mutants	Reassortment groups I	II	III	Unclassified	References
Bunyavirus	Bunyamwera	Batai	5	1	4	0	0	Iroegbu (1981); Iroegbu and Pringle (1981a,b); Pringle and Iroegbu (1982); Elliott *et al.* (1984)
		Bunyamwera	8	5	3	0	0	
		Maguari	46	12	31	1	2	
		Germiston	8	2	6	0	0	Ozden and Hannoun (1980)
		Guaroa	4	2	2	0	0	Pringle (unpublished data)
			9	3	5	1	0	Bishop (1979)
	California	La Crosse	20	6	14	0	0	Bishop (1979); Gentsch and Bishop (1976); Gentsch *et al.* (1977, 1979, 1980)
		Snowshoe hare	48	26	20	0	2	
		Tahyna	20	1	17	0	2	
		Trivittatus	12	4	6	0	2	
		Lumbo	9	2	7	0	0	
			8	1	7	0	0	Ozden and Hannoun
Uukuvirus		Uukuniemi	13	3	2	0	8	Gahmberg (1984)
		Total	210	68	124	2	16	

plication cycle and it can occur at high frequency in mixed infections of genetically compatible viruses. Nonetheless, Gentsch and Bishop (1976) and Ozden and Hannoun (1978) were able to demonstrate intergenic complementation between mutants belonging to different reassortant groups in the case of snowshoe hare virus and Lumbo virus, respectively, both of which belong to the California serogroup of bunyaviruses.

Intragenic complementation involving ts mutants of the same complementation group has been described by Iroegbu and Pringle (1981a,b). Individual pairs of mutants classified in reassortant group I of Maguari virus (Bunyamwera serogroup) were able to complement each other, suggesting that the protein encoded in the genome subunit corresponding to group I has a multifunctional role in viral biosynthesis. Complementation was not observed in pairwise mixed infections with any of the group II mutants of Maguari virus.

4. Absence of Intrasubunit Recombination

Although reassortment of genome subunits occurs universally in negative-stranded RNA viruses with segmented genomes, intramolecular recombination (i.e., the generation of covalently joined RNAs from heterologous molecules) has not been demonstrated unequivocally in any negative-stranded RNA virus irrespective of whether the genome is segmented or not. In eukaryotic viruses the sensitivity of detection is limited by the reversion frequency of the markers employed, usually ts mutants, which is often high. However, recombination has not been observed in prokaryotic RNA viruses where nonreverting markers were used.

It is likely that recombination does occur under rare circumstances, because defective interfering viruses have been generated during high-multiplicity passage of vesicular stomatitis virus and influenza A virus which are mosaics of sequences from different regions of the genome (O'Hara *et al.*, 1984; Nayak *et al.*, 1985). Other evidence suggestive of rare recombination events is the inversion of a region of the genome of the pneumovirus turkey rhinotracheitis virus (Ling *et al.*, 1992), and an insertion of 54 nucleotides derived from the 28 S ribosomal RNA of the host cell in the hemagglutinin of influenza A virus (Khatchikian *et al.*, 1989).

Bergmann *et al.* (1992) recovered several viruses after transfection of *in vitro* reconstituted ribonucleoprotein into influenza A virus-infected cells which could only have originated by a recombinational event. Five different recombinant viruses were obtained. Two contained a neuraminidase (NA) gene whose defective polyadenylation signal had been repaired by an intergenic recombination. Surprisingly the additional sequences in the rescued gene appeared to be derived from RNA contaminating the polymerase preparation in the transfection mix and not from the helper virus in the transfected cell. Although these two recombinants originated from separate transfection experiments, the overall frequency of success was low, indicating that even in this system recombination is a rare event. Two other recombinants were

NA gene mosaics, and one a mosaic containing variable amounts of sequence derived by multiple recombinational events from matrix, PB1, and NA genes derived also from the virus used to prepare the polymerase proteins for the tranfection mix. In some experiments DNA was excluded from the transfection protocol thereby confirming that recombination involved RNA molecules only. It was not possible, however, to discriminate between a copy-choice model or a breakage-joining mechanism, nor whether the recombination occurred during *in vitro* incubation or during amplification in the helper-infected cells.

5. Homologous Reassortment and Gene Assignment

Crosses of heterologous viruses belonging to the same serogroup have been employed to correlate reassortment groups with genome subunits. Genotyping of clones of progeny virus derived from mixed infections with heterologous parental viruses has been carried out by RNA fingerprinting and dot hybridization with subunit specfic probes. These techniques were used in conjunction with phenotypic analysis using specific antibodies and by determination of the mobilities of radiolabeled polypeptides by SDS/PAGE (Gentsch and Bishop, 1976; Gentsch *et al.*, 1977, 1979, 1980; Rozhon *et al.*, 1981; Bishop *et al.*, 1984; Pringle and Iroegbu, 1982; Pringle *et al.*, 1984a,b; Iroegbu and Pringle, 1981a,b; Endres *et al.*, 1989). The development of the polymerase chain reaction (PCR) has greatly facilitated the genotyping of progeny clones and increased the accuracy and resolving power of such experiments (Urquidi and Bishop, 1992). To enrich the isolation of reassortant clones it was usual in the early work to employ ts mutants as the parental viruses and to characterize the non-ts virus in the progeny. It soon became clear that the frequency of reassortment was sufficiently high to dispense with the need for enrichment and to make use of the ts character as an additional phenotypic marker. Overall the data obtained by the different methodologies were usually consistent although some anomalies have remained unresolved. It was observed generally that heterologous reassortment was restricted and not always reciprocal, the extent of the restriction decreasing as the serological distinctiveness of the parental viruses decreased.

It has become conventional in reassortment experiments to express the genotype of a virus as the three-letter abbreviation for each virus listed in the International Catalogue of Arboviruses (Karabatsos, 1985) to represent the parental origin of the L/M/S subunits. Thus, the genotype of wild-type snowshoe hare virus becomes SSH/SSH/SSH and that of La Crosse virus LAC/LAC/LAC. In the California serogroup, crosses of the closely related snowshoe hare virus and La Cross virus yielded non-ts reassortants designated SSH/LAC/LAC and SSH/LAC/SSH when the snowshoe hare parental virus was a ts mutant classified in group I, and the La Crosse virus parent was a ts mutant classified in group II. This result is consistent with expectation if the

group I ts mutation is located in the M RNA subunit and the group II mutant in the L RNA subunit. However, Gentsch *et al.* (1979) observed that the same two reassortants were obtained when non-ts wild-type parental viruses were employed and the remaining four expected reassortants (LAC/LAC/SSH, LAC/SSH/SSH/, SSH/SSH/LAC, LAC/SSH/LAC) were absent. No viable reassortants were isolated from the reciprocal cross of a group I La Crosse virus ts mutant and a group II snowshoe hare virus ts mutant, leaving the group assignment unconfirmed. However, by a series of ingenious backcrosses, Gentsch *et al.* (1979) were able to obtain the missing reassortants. First, ts mutants were induced in the SSH/LAC/SSH and SSH/LAC/LAC reassortants by random mutagenesis and identified as group I or group II mutants by genetic analysis. Each of these mutants was backcrossed in turn to the appropriate ts parental virus to yield further non-ts reassortants. The SSH/LAC/SSH group II reassortant backcrossed to the SSH/SSH/SSH group I grandparent yielded a new reassortant having the gene combination LAC/LAC/SSH. This new reassortant in its turn was mutagenized to obtain group I and group II ts mutants. Backcrossing of these viruses to the original SSH/SSH/SSH and LAC/LAC/LAC parental viruses yielded a non-ts reassortant virus with the fourth gene combination SSH/SSH/LAC in the progeny of the cross of a group II SSH/SSH/LAC virus and the original SSH/SSH/SSH group I mutant. The data generated in this complicated chain of experiments are consistent with the interpretation that the mutations classified in reassortment group I are located in the M RNA subunit, and those classfied in group II are located in the L RNA.

Several crosses yielded no reassortants, however, and two (LAC/SSH/SSH and LAC/SSH/LAC) of the six possible subunit combinations expected in these experiments were not recovered. These reassortants were isolated only later in experiments by Rozhon *et al.* (1981) in which the input virus was concentrated by polyethylene glycol precipitation to increase the input multiplicity. The interaction between parental virus appears to be an early event in the multiplication cycle (Iroegbu and Pringle, 1981a) and superinfection exclusion developed fairly soon after initial infection. Consequently, the relative multiplicities of infection of the parental viruses could have an important bearing on the outcome of any particular mixed infection.

It could be concluded from these experiments, therefore, that the asymmetric and restricted patterns of gene segregation observed in these experiments were not related to any inherent incompatibility of the genome subunits of these two members of the California serogroup. The restrictions are more likely a consequence of other factors such as compartmentalization of replication within the cell, aberrant morphogenesis, variable cytopathogenicity, etc. A more systematic restriction was observed when more distantly related members of the California serogroup were crossed. Crosses of group II mutants of Tahyna virus with group I mutants of snowshoe hare virus or trivitattus virus, group I mutants of Tahyna virus with group II mutants of La Crosse virus, and group I mutants of trivitattus virus with group II mutants

of La Crosse virus, yielded reassortants whose genotypes were again consistent with assignment of the group I mutations to the M RNA and the group II mutations to the L RNA. There was a marked bias, however, for the L and S RNA segments to cosegregate; 58 of the 65 non-ts reassortants characterized derived their L and S RNA subunits from the same parent. The same tendency was observed in the cross of a group I La Crosse virus and a group II Tahyna virus, where 9 of 12 non-ts reassortants derived their L and S RNA from the same parent. However, two reassortant genotypes conflicting with the group I and II assignments were recovered in the progeny of this cross. This discrepancy was attributed to experimental error, but again certain combinations of parental viruses (Tahyna virus group I × trivitattus or snowshoe hare virus group II, and La Crosse virus group I × trivitattus virus group II) failed to yield the expected non-ts reassortant progeny virus.

In corresponding experiments involving characterization of non-ts progeny virus emanating from crosses of ts mutants of three Bunyamwera serogroup viruses, all six possible reassortant genotypes were recovered (Iroegbu and Pringle, 1981a,b). In contradistinction to the California serogroup data, however, analysis of the genotypes of the reassortants suggested that the group I and group II mutations of Batai, Bunyamwera, and Maguari viruses were located in the S RNA and M RNA subunits, respectively (Iroegbu and Pringle, 1981a,b; Pringle *et al.*, 1984a,b). As in the case of the California serogroup viruses, it was apparent that segregation did not occur at random except that in this case the M subunit appeared to segregate unrestricted and the L RNA and S RNA subunits showed linkage. This phenomenon was observed both when non-ts reassortants were selected from the progeny of crosses of ts mutants, and when the frequency of reassortment was sufficiently high to allow direct analysis of unselected progeny. Curiously in the former case, invariably only one of the two possible classes of non-ts progeny was obtained. The same bias was observed in unselected progeny also, and a total of 279 reassortants pooled from several experiments appeared to have derived their L and S RNA subunits from one parent only. A different situation pertained, however, when these reassortant viruses were in their turn used as the parental viruses. For example, all six possible reassortants were obtained at comparable frequencies in unselected progeny from a mixed infection of viruses of genotype BUN/MAG/BUN and BAT/BUN/BAT, i.e., containing subunits originating from three parents. This confirms that as in the case of the California serogroup viruses the restricted pattern of subunit segregation in some crosses is not the result of any inherent genetic incompatibility.

The molecular basis of these restricted segregations has not been elucidated, but the apparent linkage of the L and S RNA subunits which encode core and replicative functions suggests that the restriction acts at the gene product level. The Bunyamwera serogroup data suggest that once a homologous gene combination has been disrupted by reassortment, there is no preferred association of genes or gene products that has any selective advan-

tage. This suggests that after the disruption of adapted gene combinations, restrictions are relaxed and the bunyaviruses may exhibit the same propensity for rapid and abrupt changes in properties as the influenza A viruses.

A major discrepancy exists between the results obtained with the California serogroup viruses, where the larger of the two groups of ts mutants was assigned to the L RNA, and the Bunyamwera serogroup viruses, where the largest group was assigned to the S RNA. Analysis of the assignment of the single ts mutant of Maguari virus should have resolved the issue, but because the phenomenon of restricted segregation was again encountered in crossing ts mutants of heterologous viruses, it was not possible to assign the group III lesion to either the L or the S RNA subunit. Non-ts reassortants of genotype BUN/MAG/BUN were obtained from the cross of MAGts23(III) with a group II Bunyamwera virus parent, which excluded location of the group III lesion in the M RNA subunit. However, the non-ts reassortant required to unequivocally assign the group III mutation to the L or S RNA subunits was not obtained, because no non-ts progeny virus was obtained from the critical cross of the MAGts23(III) and the group II Bunyamwera virus parent (Pringle and Iroegbu, 1982). Further attempts were made to resolve this problem by analysis of reassortants in the unselected progeny of crosses of various ts mutants with a heterologous wild-type virus so that the ts mutation could be employed as a fourth marker (Murphy and Pringle, 1987; Hampson, 1987). Reassortants of genotype MAG/BUN/BUN/ts$^+$ and MAG/BUN/MAG/ts were obtained from crosses of several Maguari virus group I mutants with wild-type Bunyamwera virus, and reassortants BUN/MAG/MAG/ts and MAG/MAG/BUN/ts from crosses of several Maguari virus group II mutants with wild-type Bunyamwera virus. These results are consistent with the previous tentative assignment of the group I mutants to the S RNA and the group II mutants to the M RNA. The cross of MAGts23(III) with Bunyamwera virus wild type yielded several reassortants (MAG/BUN/BUN/ts, BUN/MAG/MAG/ts$^+$, MAG/MAG/BUN/ts, BUN/MAG/BUN/ts$^+$) that were consistent with the assignment of the group III lesion to the L RNA subunit. These later experiments with Bunyamwera serogroup viruses were complicated by the frequent occurrence of heterozygous and quasidiploid particles, which were sometimes difficult to detect since in mixed infections Maguari virus appeared to have a replicative advantage and incorrect assignments may have been made.

The physical nature of these heterozygous particles is unknown, but Talmon *et al.* (1987) have demonstrated by electron microscopy of vitrified specimens that the number of subunits in particles is variable. Furthermore, in the genetic analysis of progeny virus from crosses of ts mutants of rotaviruses, it has been established that the genetic background of the recipient can affect the phenotype conferred by the gene introduced by reassortment (Chen *et al.*, 1989), which introduces an additional complication in progeny analysis. The non-ts phenotype of some bunyavirus reassortants may be

determined by interactions between heterologous subunits and not solely by the absence of a ts lesion, and thereby may be responsible for the failure of gene assignment by this approach.

Nucleotide sequence analysis of the S RNA subunit of mutant MAGts23-(III) by a coupled reverse transcription/PCR method (Dunn *et al.*, 1994) revealed a transition at position 327 conferring a valine-to-alanine substitution in the N protein product and a phenylalanine-to-leucine change in the NSs protein (D. C. Pritlove and R. M. Elliott, unpublished data). This mutation at residue 327 is not present in the S RNA of mutant MAGts6(I) and mutant MAGts17(II), representing groups I and II, or in the S RNA of another 12 bunyaviruses (Dunn *et al.*, 1994). The alanine introduced into the N protein of MAGts23(III) is not present at this position in any other N protein sequence. The valine in wild-type Maguari virus is conserved in all of the other bunyavirus sequences, excepting for a substitution of isoleucine in three California serogroup viruses. The NSs protein sequence is less well conserved and the leucine substituted in MAGts23(III) also occurs in three California group viruses. These observations indicate with reasonable certainty that the group III mutation is located in the S RNA, and bring the Bunyamwera serogroup assignments into line with the California serogroup assignments. However, revertants of mutant MAGts23(III) have not been isolated and it could not be confirmed directly that the mutation at position 327 conferred temperature-sensitivity.

Additional evidence in support of this assignment comes from two other directions. By Northern blotting it has been shown that mutant ts23(III) is replication-defective but transcription-competent, which suggests that the defect lies in S RNA gene products rather than the L RNA-derived polymerase (Pritlove and Elliott, unpublished data). Dunn *et al.* (1995) have developed an *in vitro* chloramphenicol acetyl transferase (CAT) activity rescue system that is dependent on added L and S RNA gene products. In this system they have shown that the S RNA of mutants MAGts6(I) or MAGts17(II) will act in conjunction with Bunyamwera virus L RNA to rescue CAT activity at both 33 and 38°C, whereas MAGts23(III) S RNA will only rescue at 33°C (Elliott *et al.*, unpublished data). This is further strong evidence in favor of the location of the group III lesion in the S RNA.

Thus, the group I mutants represent mutations in the L RNA and group II mutants represent mutations in the M RNA. This assignment is more in accord with the frequency of isolation of the mutants and the genome target size. It is also the expectation from studies of mutants of other negative-strand RNA viruses that L protein mutants should be in excess (Pringle, 1987, 1991a,b). However, until the L and S subunits of each mutant have been sequenced, these revised assignments are not entirely conclusive. It remains to be determined why the Bunyamwera serogroup progeny analyses were so misleading, and why ts mutations were so rarely recovered in the S RNA. The overlapping encoding of information in the S RNA may account for the latter phenomenon.

C. Heterologous Reassortment and the Definition of Gene Pools

Reassortment of subunits occurs with varying facility between viruses that belong to the same serogroup. Heterologous crosses of viruses belonging to the Bunyamwera, California, and Simbu serogroups have been carried out in the laboratory to explore the extent of the ability to reassort genome subunits. The recovery of reassortants was forced by employing pairs of ts parental viruses and selecting non-ts progeny virus. The phenotype and/or genotype of progeny clones were determined by several procedures. Since the specificity of neutralization of bunyaviruses is determined by the M RNA (Gentsch *et al.*, 1980; Iroegbu and Pringle, 1981a,b; Millican and Porterfield, 1982; Murphy and Pringle, 1987), it is possible to screen for reassortment rapidly by crossing any virus for which a specific neutralizing antiserum is available with a standard group I (or group III) ts mutant which will donate a non-ts M RNA subunit. Reassortants can be detected by screening the progeny virus at the restrictive temperature in the presence of the neutralizing antibody, a procedure that should eliminate all parental virus and allow growth of two of the six possible reassortants (Table IV).

The results of such experiments are summarized graphically in Fig. 2. In the absence of an appropriate ts parental virus, the following procedure can be used to detect reassortants provided that a neutralizing antiserum against the non-ts virus is available. All six viruses of the California serogroup that have been tested were able to exchange genome subunits. On the other hand, only five of the eight Bunyamwera serogroup viruses studied were able to exchange subunits. Therefore, in addition to the restricted segregations ob-

TABLE IV. An Example of Rapid Screening for Reassortment between Heterologous Bunyaviruses

	Genotype[a]	Growth at restrictive temperature	Growth at restrictive temperature in presence of anti-NOR serum
Parental	BUN*/BUN/BUN	−	−
	NOR/NOR/NOR	+	−
Progeny	BUN*/BUN/BUN	−	−
	NOR/NOR/NOR	+	−
	BUN*/NOR/NOR	−	−
	NOR/BUN/BUN	+	+
	BUN*/BUN/NOR	−	−
	NOR/NOR/BUN	+	−
	BUN*/NOR/BUN	−	−
	NOR/BUN/NOR	+	+

[a]BUN = Bunyamwera virus L, M, and S RNA subunits.
BUN* = A Bunyamwera virus L RNA subunit carrying a temperature-sensitive mutation.
NOR = Northway virus L, M., and S RNA subunits.

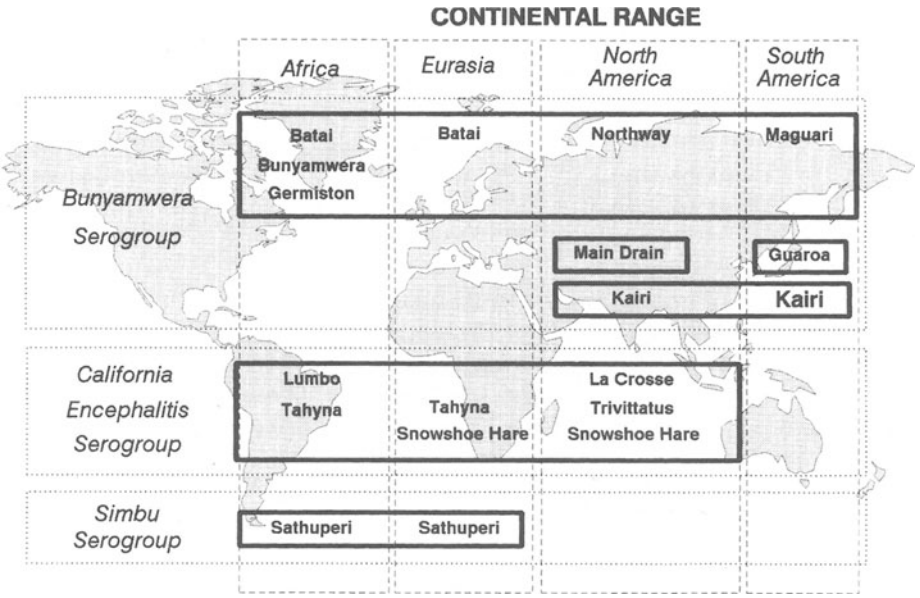

FIGURE 2. Patterns of genome subunit exchange among viruses of the Bunyamwera, California, and Simbu serogroups of the genus *Bunyavirus*. The heavy lines enclose viruses that are able to exchange genome segments by reassortment in cultured cells. Viruses in different boxes are unable to exchange genome subunits and appear to be genetically isolated. Serogroups are indicated horizontally and known geographical distribution vertically.

served in the repirocal ts mutant crosses described previously, some viruses within the same serogroup are less able to exchange genome subunits and are in effect genetically isolated. Some viruses, such as Main Drain and Kairi viruses, seem to be totally isolated from the remainder. The pattern of restriction within the Bunyamwera serogroup corresponds to the serological relationships of the viruses (Hunt and Calisher, 1979); the greater the serological divergence, the greater the restriction in subunit exchange. Guaroa virus, although included in the Bunyamwera serogroup in Fig. 2, has been ranked as a bridging virus between the Bunyamwera and California serogroups (Beaty and Calisher, 1991). In complement fixation tests, Guaroa virus is closer to the Bunyamwera serogroup viruses, whereas it reacts similarly to the California serogroup viruses in neutralization tests (Whitman and Shope, 1962). ts mutants of Guaroa virus have been isolated independently by Gentsch *et al.* (1980) and Pringle (1991c) and employed to detect heterologous reassortment. However, there was no evidence of subunit exchange between Guaroa virus and members of either the Bunyamwera serogroup or the California group, and Guaroa virus appears to be genetically isolated from both serogroups. Dunn *et al.* (1994) concluded from a comparison of the S RNA sequences of seven viruses of the Bunyamwera serogroup that Main Drain

virus, Kairi virus, and Guaroa virus were less closely related than the other four (Cache Valley, Northway, Maguari, and Batai viruses), reflecting their compatibility in reassortment experiments. Germiston virus, however, does not conform to this generalization. They concluded in addition that Guaroa virus represented a reassortant virus derived from two ancestral parental viruses now included in separate serogroups.

Extensive heterologous crosses of viruses belonging to the Bunyamwera, California, and Simbu serogroups have failed to find any evidence of exchange of genome subunits between serogroups. These experiments were carried out under a variety of conditions and in different host cells, including both vertebrate and *Aedes albopictus* C6/36 cells (Elliott *et al.*, 1984). Consequently, it is likely that the different serogroups of viruses in the genus *Bunyavirus* represent discrete gene pools. Genetic variation in the family *Bunyaviridae* is the result of both genetic drift and ecological isolation, and by gene capture from heterologous viruses. The opportunity for gene exchange in viruses other than the hantaviruses is probably greatest during the arthropod-borne phase of the multiplication cycle, since more than one blood meal may be taken by an individual female mosquito. It is unlikely that immune selection is a driving force in the evolution of these viruses, given the predominant role of arthropods in the life cycles of most members of the *Bunyaviridae*. Geographical range does not seem to be a major factor in determining the genetic divergence of bunyaviruses. Figure 2 shows that viruses such as Maguari virus, which is restricted to South America, can exchange subunits with viruses originating from different continents. Conversely, viruses occupying the same geographical range (e.g., Main Drain, Kairi, and Northway) seem unable to interact. Our knowledge of the natural distribution of bunyaviruses is rudimentary, however, and the geographical isolation of different bunyaviruses may be more apparent than real. [Figure 2 has been modified from an earlier version (Pringle, 1991c) to take account of more recent information.] The pattern of genetic interaction in the *Bunyaviridae* is very different from that of the orthomyxoviruses, where it is likely that reassortment is possible between all influenza A virus, although this has not been rigorously tested.

D. Nonrandomness of Reassortment

Nonrandom reassortment of genome subunits has been described for influenza A virus (Lubeck *et al.*, 1979), bluetongue virus (Stott *et al.*, 1987), and rotavirus (Gombold and Ramig, 1986; Graham *et al.*, 1987; Ward *et al.*, 1988; Ramig and Ward, 1991), as well as for viruses of the Bunyamwera serogroup (Pringle *et al.*, 1984a,b). Urquidi and Bishop (1992) have now carried out a more critical analysis of the process of reassortment of subunits using two closely related members of the California serogroup. Monolayers of BHK-21 cells were infected with an inoculum of 5 pfu per cell of wild-type

La Crosse and snowshoe hare viruses to ensure a high probability of mixed infection. Progeny virus harvested after 72 hr incubation at 36°C was analyzed employing the polymerase chain reaction (PCR) with a set of nine primers designed to identify the parental origin of the six subunits segregating in this cross by their relative electrophoretic mobility in 2% agarose gel. This approach enabled a larger sample of progeny virus to be characterized with greater accuracy than in any previous experiment. A total of 350 plaques were isolated from monolayers incubated for a period of 7 days to ensure that small plaque-forming virus was not missed, and the parental origin of the subunits determined. The results of the analysis of the 255 clones with the proper complement of S, M, and L RNA species are given in Table V. This accounts for 73% of the progeny; the remainder not included in the analysis consisted of 52 clones (15%) where there was no amplification of one (or rarely two) of the three RNA species, 31 isolates (9%) with PCR products derived from both parents representing either diploid virus or uncloned virus, and a residue of 3% which were PCR-negative. All eight possible genotypes were identified in the progeny with the frequency distribution given in Table V. The difficulty experienced previously by Gentsch *et al.* (1979) in identifying reassortants of genotype LAC/SSH/SSH and LAC/SSH/LAC may relate to the fact that in the earlier experiments ts mutants were used as the parental viruses. These observations reemphasize the caveat that the phenotypes of parental ts mutant viruses may influence the outcome of reassortment (Chen *et al.*, 1989).

The expected frequencies in Table V are calculated from the relative frequencies of the individual L, M, and S RNA subunits on the assumption that there are no constraints on subunit segregation. There is a highly significant difference between the observed and expected frequencies ($\chi^2 = 32.41$; $p<0.001$), and an overrepresentation (43%) of the two parental genotypes. Since the progeny virus titer exceeded the input virus titer 100-fold, this

TABLE V. Nonrandom Reassortment in a Cross of La Crosse and Snowshoe Hare Viruses[a]

Genotype	Observed (%)	Expected[b] (%)	χ^2	Significance
LAC/LAC/LAC	81 (32%)	60 (24%)	9.32	<0.005
SSH/SSH/SSH	29 (11%)	14 (6%)	18.13	<0.001
LAC/SSH/SSH	28 (11%)	29 (11%)	0.05	>0.8
SSH/LAC/LAC	22 (9%)	28 (11%)	1.61	>0.2
LAC/LAC/SSH	30 (12%)	39 (15%)	2.23	>0.1
SSH/SSH/LAC	17 (7%)	21 (8%)	0.99	>0.3
LAC/SSH/LAC	35 (14%)	46 (18%)	2.94	>0.05
SSH/LAC/SSH	13 (5%)	18 (7%)	1.57	>0.2
Total	255	255	32.41	<0.001

[a]Data from Urquidi and Bishop (1992).
[b]Calculated from the observed relative frequencies of the genome segments: LAC L = 174/255 = 0.68; LAC M = 146/255 = 0.57; LAC S = 155/255 = 0.61; SSH L = 81/255 = 0.32; SSH M = 109/255 = 0.43; SSH S = 100/255 = 0.39.

excess of parental virus must represent a preferential association of homologous subunits in virus replication and not the reisolation of residual inoculum. The interaction between segment pairs was examined using a log-linear statistical model (Sokal and Rohlf, 1981), and the results of this analysis confirmed the existence of positive associations between certain pairs of subunits. Homologous L–M and M–S associations were preferred in this particular cross. A positive association between homologous subunits has also been described in a less rigorous analysis of progeny from crosses of viruses of the Bunyamwera serogroup (Pringle *et al.*, 1984a,b). In this case the preferential association was between the L and S RNA subunits.

Iroegbu and Pringle (1981) observed that reassortment of subunits could occur in cultured invertebrate cells multiply infected with Bunyamwera serogroup viruses perhaps more readily than in vertebrate cells, and Beaty *et al.* (1981a,b 1985) demonstrated that reassortment occurred *in vivo* when mosquitoes were infected experimentally with more than one virus of the California serogroup. All six reassortants were also found in the progeny from a mixed infection of vertebrate cells with unmutagenized stocks of La Crosse and Tahyna viruses (Janssen *et al.*, 1986; Endres *et al.*, 1991), and also *in vivo* in mosquitoes (Chandler *et al.*, 1991). The genotypes of 708 isolates obtained from *Aedes triseriatus* mosquitoes 23 days after inoculation were analyzed by Chandler *et al.* (1991) by molecular hybridization using three gene probes specific for the LAC L, M, and S RNA. High-frequency reassortment was observed and all six reassortants were identified. Analysis of these data by the method of Urquidi and Bishop (1992), however, indicates that the observed frequencies of the eight genotypes diverge significantly from the expected frequencies. This may be related to the less definitive method used in establishing genotype, but probably reflects selective effects occurring during the prolonged period of multiplication in the insect host. These experiments show, however, that reassortment can occur *in vivo* at high frequency in an arthropod vector. Preferential association of subunits during reassortment appears to be the rule in bunyaviruses, and perhaps in all other single-component segmented genome viruses. Nonrandomness is presumed to represent constraints on packaging and morphogenetic events, and the different associations observed with different viruses (or even the same viruses under different conditions) may reflect diverse replicative abilities, nonequivalence of the components of an inoculum, and deviation from equimolarity of RNA subunits at different stages of morphogenesis. It is well known that the subunit content of most virus preparations departs somewhat from equimolarity (Elliott, 1990). Interference may be another factor. Heterologous interference was observed between different members of the Bunyamwera serogroup in dual infection experiments, such that Maguari virus appeared to suppress replication of Bunyamwera virus or Batai virus (Murphy and Pringle, 1987). Urquidi and Bishop (1992) attempted to compensate for a perceived replicative advantage of La Crosse virus by delaying superinfection of snowshoe hare virus-infected cells by 4 hr. Under these conditions they observed complete interference with La Crosse virus replica-

tion and only viruses of genotype SSH/SSH/SSH were recovered in the progeny. Interference to reassortment has also been described in the case of oral infection of mosquitoes with two ts mutants of La Crosse virus (Beaty *et al.*, 1985), in mixed infections with two variants of Rift Valley fever virus (Turell *et al.*, 1990), and also in the midge *Culicoides variipennis* asynchronously infected with different serotypes of bluetongue virus of the *Reoviridae* (El-Hussein *et al.*, 1989).

E. The Role of Reassortment in Arthropods in the Evolution of the *Bunyaviridae*

Evolutionary trends within the genus *Bunyavirus* and within the *Bunyaviridae* can be recognized by their relationships to their arthropod vectors. Viruses of each genus, with the exception of the vectorless hantaviruses, have preferential relationships with one or two arthropod families only, and within genera each serogroup is preferentially associated with arthropod species belonging to one or two genera only (Labuda, 1991). Based on isolations from nature, 8 of the 16 bunyavirus serogroups are associated with mosquitoes of the genus *Culex*, two with mosquitoes of the genus *Aedes*, three with mosquitoes of the genus *Anopheles*, and one serogroup each with *Aedeomyia* mosquitoes, *Culicoides* midges, and *Hyalomma ticks*.

Chandler *et al.* (1990) have reported that replication and reassortment were enhanced in the ovaries of female mosquitoes and that the newly generated reassortants were transmitted transovarially to about 10% of their progeny. These new reassortants in their turn could be transmitted to a susceptible vertebrate host. These observations indicate that reassortment in the arthropod vector may be the driving force in the evolution of bunyaviruses in nature. *Aedes triseriatus* mosquitoes, for example, feed on a variety of hosts which increases the chance of multiple infection with different viruses since many California serogroup viruses co-circulate in the same area sharing the same vector.

Presumptive reassortant bunyaviruses have been isolated from trapped mosquitoes. Ushijima *et al.* (1981) reported that oligonucleotide fingerprints of the L and S RNA of Shark River virus and Pahayokee virus, two members of the Patois serogroup isolated from *Culex melanoconium*, were virtually identical. Their M RNA subunits on the other hand were quite distinct. These observations were interpreted to mean that these isolates of Shark River virus and Pahayokee virus were natural reassortants which had acquired their M RNA subunit from different unknown donor viruses. This interpretation is reinforced by the observations of El-Said *et al.* (1979) and Klimas *et al.* (1981) who studied the natural variation of La Crosse viruses isolated from different ecological situations and found that the L and S RNAs of these California serogroup viruses were not more conserved than the M RNA, indicating the genetic homogeneity of these viruses. The pattern of

variation for all three subunits was continuous and more analogous to the progressive mutational drift observed in isolates of influenza A virus during the interpandemic phase.

The role of genetic drift in the evolution of bunyaviruses has not been studied systematically, and its relative importance has not been assessed. Klimas *et al.* (1981) found that two isolates of La Crosse virus had virtually identical oligonucleotide fingerprints, although originating from samples of human brain tissue taken 18 years apart. Likewise, 12 cycles of transovarial transmission of a phlebovirus did not induce significant changes in the oligonucleotide fingerprints of the L, M, and S RNA subunits (Bilsel *et al.*, 1988). Hewlett *et al.* (1992) have compared in terms of oligonucleotide fingerprint profiles and plaque-reduction neutralization titers the variability of field isolates of La Crosse virus and snowshoe hare virus with laboratory-derived stocks with different *in vitro* passage histories. They observed that the field isolates differed significantly, whereas the laboratory-passaged viruses exhibited little change. Battles and Dalrymple (1988) estimated the genetic variability of Rift Valley fever virus by sequencing of a region of the M gene thought to encode antigenic determinants of importance in protection. Twenty-two isolates originating from several different host species over a period of 34 years were compared and it was considered that most isolates were very similar to a reference strain at both the nucleotide (0–4.5%) and deduced amino acid sequence (0–2.4%) levels. On this rather limited and circumstantial evidence, it would appear that subunit reassortment may be the more potent force in the evolution of the *Bunyaviridae*.

By contrast, reassortment of genome subunits does not seem to play a major role in the evolution of the hantaviruses where there is no arthropod vector in the transmission cycle. Phylogenetic analyses of the nucleotide sequences of the M and S RNA subunits of the Puumala/Prospect Hill subgroup of hantaviruses suggest that the M and S RNA subunits may have evolved in parallel (Hjelle *et al.*, 1994; Spiropoulou *et al.*, 1994). It was concluded that there was no evidence to support a hypothesis that reassortment of genome subunits was responsible for the emergence of Sin Nombre virus, a new hantavirus associated with an outbreak of a previously unrecognized fatal respiratory disease in the southwestern United States in May, 1993.

Bunyaviruses are closely adapted to particular vectors (Kramer *et al.*, 1993), and the efficiency of transmission of bunyaviruses by different susceptible vectors is variable. For example, Schopen *et al.* (1991) found that the California serogroup virus La Crosse virus was transmitted transovarially by 53% of *Aedes triseriatus*, its natural vector, and by only 22% of *Culiseta inornata* mosquitoes. By contrast, the closely related snowshoe hare virus was transmitted transovarially by 89% of *C. inornata* mosquitoes, the presumptive natural vector, and by only 29% of *A. triseriatus*. Experiments monitoring the transovarial transmission of reassortant viruses showed that the La Crosse M RNA subunit was the genetic determinant favoring efficient transovarial transmission in *A. triseriatus* mosquitoes.

V. SUBUNIT REASSORTMENT IN OTHER SEGMENTED GENOME VIRUSES

Reassortment of genome subunits has been observed in the majority of viruses possessing segmented genomes, and the phenomenon is particularly well-documented for the orthomyxoviruses and the reoviruses. It is probable that reassortment of genome subunits between viruses adapted to growth in different host organisms contributes to the evolution of theses viruses in the natural environment and that it provides a mechanism for rapid adaptation to changing circumstances. Reassortment is particularly obvious where, as in the influenza A viruses, there are several reservoir host species, and is not apparent where no reservoir species exist as in the case of the influenza B viruses. The influenza C viruses present in the human and pig populations may have diverged sufficiently to have become genetically incompatible, but this has not been verified by laboratory experiment. Intrastrain reassortment of genome subunits has been described for most of the multicomponent plant viruses.

Among arenaviruses, reassortment has been demonstrated between ts mutants of the same strain and between different strains of the two arenaviruses Pichinde virus and lymphochoriomeningitis (LCM) virus. There was evidence of restriction of reassortment, as in the bunyaviruses, but gene assignments could be made without difficulty. The L and the S RNAs of arenaviruses encode two gene products in ambisense configuration, and ts mutants mapped to the S RNA subunit, but not those mapped to the L subunit, could be classified further into two complementation groups. Pichinde virus and LCM did not exchange genome segments under *in vitro* conditions and the prevalence of reassortment of arenaviruses in the natural environment is unknown. Pichinde virus and LCM virus belong to different serogroups, however, and there is some evidence of reassortment between Lassa fever and Mopeia viruses which belong to the same serogroup.

The three serotypes of human reovirus exchange genome subunits freely during mixed infection and reassortment has been employed with great success in analysis of the genetic determination of pathogenesis and virulence in these viruses. Little is known, however, regarding the role of reassortment in the evolution of these viruses. Natural reassortants of rotaviruses may be of frequent occurrence, and reassortants have been recovered from mice infected with ts mutant parental viruses (Ramig and Gombold, 1991). Genetic reassortment between simian rotavirus SA11 and rhesus rotavirus occurred at high frequency in suckling mice and the frequency of reassortment could be manipulated by passive immunization of the host (Gombold and Ramig, 1989). In contrast to the bunyaviruses, in cultured cells at least superinfecting rotaviruses are not excluded from participating in subunit exchange for at least 24 hr after infection with the virus initiating infection (Ramig, 1990). By contrast, superinfection exclusion occurred after 4 hr in cultured Vero cells infected with different serotypes of bluetongue

virus (Ramig *et al.*, 1989). Reassortment was nonrandom whether the multiplicity of infection of the parental rotaviruses was equal or not. Asynchronous infections of the midge *Culicoides variipennis* by bluetongue virus, however, indicated that *in vivo* interference to superinfection did not occur until day 5 after initial infection (El-Hussein *et al.*, 1989). The fact that these vectors frequently seek a second blood meal suggests that there is frequent opportunity for reassortment and that this may play an important role in the evolution of bluetongue virus.

Evidence for gene capture from viruses present in alternate hosts is abundant. The K8 strain of human rotavirus has been identified as a reassortant derived from parental viruses belonging to the Wa and Au-1 serogroups (Nakagomi *et al.*, 1992a; Isegawa *et al.*, 1992). The Au-1 virus has a VP4-coding segment encoding a feline rotavirus-like VP4, suggesting that genetic interaction between feline and human rotaviruses is possible. Nakagomi *et al.* (1992b) have also reported evidence for presumed reassortment of an Au-1 human rotavirus with a bovine rotavirus in nature. Three epidemiologically distinct rotaviruses of the G8 serogroup have been characterized by Browning *et al.* (1992). These viruses appear to have originated from interaction between bovine and human rotaviruses and by reassortment with a rotavirus of a third, as yet unknown, host species. Finnish isolates of the G8 serogroup closely resembled bovine isolates of the G8 serogroup and may possibly have a zoonotic origin or be derived from the live bovine rotavirus used to vaccinate Finnish children. Similarly, reassortment has been recorded in field isolates of the orbivirus, bluetongue virus (De Mattos *et al.*, 1991). Sequence analysis of the S1 segment of bluetongue virus, encoding the VP7 protein carrying the major neutralizing antigen, in conjunction with hybridization data for the M3 segment, encoding the VP5 protein, suggested that the five serotypes of bluetongue virus present in North America were derived from three separate gene pools, and bluetongue virus serotype 17 had evolved by a combination of both reassortment and genetic drift (Kowalik and Li, 1991). Reassortment in the *Reoviridae* may be restricted to serologically related viruses. Genome subunit reassortment in the Kemerovo serogroup viruses appears to be restricted to taxonomically related viruses and does not correlate with geographical origin (Moss *et al.*, 1988; Brown *et al.*, 1989a,b). Brown *et al.* (1991) failed to demonstrate genetic interactions between viruses of the Orungo and Lebombo serogroups, although gene reassortment was possible between viruses of the same serogroup. Mascarenhas *et al.* (1989) have described atypical human serotype 2 rotavirus strains that appear to be reassortants in which a subgroup II rotavirus had exchanged its serotype-specific gene for the equivalent gene of a subgroup I rotavirus.

Genetic reassortment plays a major role in the evolution of the influenza A viruses and is epidemiologically important in human disease as a consequence of the generation of new pandemic forms of the virus. Avian viruses provide a reservoir of antigenic variation which can be introduced into the human virus gene pool. Much evidence suggests that the pig provides a

"mixing vessel" where avian and human viruses can interact yielding progeny with new antigens and constellations of genes facilitating spread to human and other animal hosts (Gammelin *et al.*, 1989; Castrucci *et al.*, 1993). In South China the domestic duck may serve as the intermediate host carrying viruses from feral ducks to the domestic pig (Yasuda *et al.*, 1991). In North America, on the other hand, the population of viruses in swine appeared to be homogeneous, while by contrast a high degree of reassortment was observed in the domestic turkey population (Wright *et al.*, 1992). While reassortant viruses appear to have been responsible for the major influenza pandemics in the human population since the beginning of the 20th century, a recent study of avianlike viruses associated with high mortality in horses in China suggests that on occasion avian viruses can spread directly to mammals without the intervention of reassortment (Guo *et al.*, 1992). Conversely, although there is no evidence of antigenic change in current human H1N1 influenza A viruses, genetic drift and subunit reassortment are occurring undiminished (Xu *et al.*, 1993).

Genome subunit reassortment in all segmented genome viruses appears to exhibit the same general characteristics with only minor variation between families:

1. Reassortment is restricted to taxonomically related groups. In the family *Orthomyxoviridae* the boundaries are defined by species, and by serogroups within species in the other families. Genetic compatibility as measured by ability to reassort genome subunits may be a useful approach to equating taxons in different virus families.
2. Reassortment is rarely random. The outcome of a mixed infection is influenced by the genetic compatibility of the parental viruses, input multiplicity, and timing of superinfection. In mammals and birds the outcome may be affected by the immune status of the host. Preferential association of subunits has been observed wherever comprehensive progeny analysis has been carried out.
3. Transmission by vectors and multiplication in alternate vertebrate hosts are common features of viruses with segmented genomes.
4. In all segmented genome viruses there is potential for rapid adaptation to new hosts, abrupt appearance of viruses with new antigenic characteristics, and sudden extensions of host range and virulence.

VI. HOST–VIRUS INTERACTIONS

A. Persistent Infection, Autointerference, and Deletion Mutations

Deletion mutations located predominantly in the viral polymerase genes are associated with the phenomena of autointerference and persistence in all RNA viruses. Undiluted passage of Bunyamwera virus in BHK-21 cells (Kacsak and Lyons, 1978) or BS-C-1 cells (Iroegbu and Pringle, unpublished

data) was associated with progressive diminution in yield of infectious virus. Other persistent infections of vertebrate cells have been established by propagation of Dugbe virus (genus *Nairovirus*) in pig kidney cells (David-West and Porterfield, 1974), and Toscana virus (genus *Phlebovirus*) in Vero cells (Verani *et al.*, 1984). In BHK-21 cells this phenomenon was associated with the appearance of S RNA-containing particles. Cunningham and Szilagyi (1987) have reported the presence of truncated L RNA in cytoplasmic extracts of BHK-21 cells infected with Germiston virus (Bunyamwera serogroup). These molecules resemble the polymerase gene deletions characteristic of the defective interfering (DI) particles which are associated with autointerference in other negative-stranded RNA viruses. Patel and Elliott (1992) detected novel polypeptides in mouse cells infected with small plaque variants of Bunyamwera virus. Four of the five variants studied interfered with the multiplication of standard virus. Subgenomic L RNAs were detected in the infected cells suggesting that interference might be mediated by truncated L proteins. Host cell components appeared to be involved in the generation of the DI virus, since subgenomic L RNAs were abundant in mouse cell-passaged virus and not apparent in BHK-21 cell-passaged virus. Similarly, DI RNA molecules derived from genomic L RNA have been observed also during sequential passage of tomato spotted wilt virus (TSWV) by mechanical inoculation of *Nicotiana rustica* plants at high multiplicity of infection. These DI molecules were single internal deletions of 60–80% of the L RNA (Resende *et al.*, 1992). Curiously, four different DI molecules retained open reading frames encoding the amino acid sequence of the carboxy-terminal region of the L protein, suggesting that translation of these proteins might interfere with the polymerase function of the intact L protein and thereby mediate inhibition of replication.

Multiplication of bunyaviruses in cultured arthropod cells is not normally accompanied by cytopathic effect (Kacsak and Lyons, 1978; Newton *et al.*, 1981; Elliott and Wilkie, 1986). Elliott and Wilkie reported that there was an overrepresentation of the S RNA subunit of Bunyamwera virus in persistently infected C6/36 *Aedes albopictus* cells and the identification of subgenomic L RNA suggested the presence of DI particles. By contrast, Rossier *et al.* (1988) failed to detect subgenomic RNAs in La Crosse virus-infected C6/36 cells and proposed that the benign nature of the interaction was a consequence of translational control mediated by the N protein. This hypothesis was supported by Hacker *et al.* (1990) who found that encapsidation of S RNA by N protein prevented its translation. The absence of subgenomic RNA in the inoculum virus (Rossier *et al.*, 1988; Hacker *et al.*, 1990) indicates that DI particles of the classic type were not responsible for establishing these persistent infections. Scallan and Elliott (1992) reinvestigated the course of persistent infection of C6/36 cells by Bunyamwera virus and confirmed that there was an overrepresentation of S RNA in C6/36 cells compared to BHK-21 cells. They found no evidence of DI particle-like RNA in the early phase of propagation of the infected cells, but after prolonged

propagation of the persistently infected cultures several subgenomic L RNAs were detected. These defective RNAs were not packaged into virions and there was no direct relationship between the presence of these particles and virus titer. Nor did the amount of defective RNA present correlate with resistance to superinfection with homologous virus. Consequently, the role of DI particles in the maintenance of persistence in arthropod cells is dubious. A preliminary analysis of the viral RNAs present in individual clones of cells from C6/36 cells by Scallan and Elliott (1992) revealed great heterogeneity and they suggest that cellular factors may play an important role in the maintenance of persistence.

Deletion mutations located in the M and S RNA subunits have not been described. Non-ts revertants of the Maguari virus group II mutant tsMAG8, however, exhibited gross shifts in the electrophoretic mobility of the G1 protein (Murphy and Pringle, 1987). These revertants grew normally and they have not been investigated to determine whether sequences have been deleted. The accelerated mobility phenotype of the G1 protein was considered tentatively to be the result of exposure of a cryptic proteolytic cleavage site (Hampson, 1987).

B. Determinants of Neurovirulence and Attenuation

The biology of the family *Bunyaviridae* is dominated by the M RNA since this subunit of the genome encodes the genes concerned in many of the most important interactions of the virus with its hosts. Virulence, host range, tissue tropism, transmissibility, neutralization, hemagglutination, and membrane fusion are the principal phenotypic properties that have been attributed to the M RNA subunit gene products. Characterization of heterologous reassortants has played a leading role in recognition of the predominance of the M RNA in determining the disease-producing potential of bunyaviruses.

1. Virus Virulence in Vertebrates

The virulence of bunyaviruses often diminishes rapidly during serial passage in cultured cells. For example, field isolates of Tahyna virus were generally virulent in suckling mice, but propagation in cultured cells resulted in the rapid appearance of strains with differing *in vivo* virulence and *in vitro* cytopathogenicity. Tahyna virus passaged serially in cultured mosquito cells at 20°C replicated less well in vertebrate cells at 37°C and exhibited reduced virulence for mice by any route of inoculation (Malkova and Marhoul, 1976). Conversely, a laboratory strain remained neurovirulent but exhibited reduced peripheral virulence as a consequence of serial passage in mouse brain (Janssen *et al.*, 1984). Clearly, bunyaviruses can adapt rapidly to

changed environmental circumstances without the necessity for reassortment of preexisting variation.

Experiments with California serogroup viruses in laboratory animals have shown that pathogenicity is determined by the M RNA (Shope *et al.*, 1981). Viruses of differing virulence for mice were crossed and disease-producing ability segregated according to the parental origin of the M RNA subunit in the progeny virus. Tahyna virus was avirulent by intracerebral or subcutaneous inoculation into 4-week-old mice, whereas La Crosse and snowshoe hare viruses and reassortants carrying LAC or SSH M RNA were virulent. Exceptionally, LAC/LAC/LAC and LAC/LAC/SSH reassortants obtained from a cross of tsLAC16(I) and a group II ts mutant of genotype SSH/LAC/SSH were significantly less virulent following intracerebral inoculation into mice. Analysis of further reassortants revealed that the L RNA of mutant tsLAC16(I) carried an additional mutation that could modify virulence (Rozhon *et al.*, 1981).

These findings were verified independently by Janssen *et al.* (1986) who further showed that the S RNA as well as the L RNA could modify virulence. Reassortants derived from the cross of an avirulent strain of Tahyna virus and a virulent La Crosse virus confirmed that in the California serogroup viruses the M RNA is the major determinant of peripheral virulence. Peripheral virulence mimics the natural route of transmission from insect vector to vertebrate host and is defined as susceptibility to infection by subcutaneous inoculation. It was apparent also, however, that the origin of the L and S RNAs influenced the level of peripheral virulence displayed by these reassortants. This is in accord with the general expectation that a complex phenotype such as pathogenicity is likely to have polygenic determinants. In fact, Gonzalez-Scarano *et al.* (1988) were able to partition the virulence phenotype into four independently varying components (neuroinvasiveness and neurovirulence in mice, and oral transmissibility and intrathoracic susceptibility in mosquitoes), establishing the essentially polygenic nature of the virulence phenotype (see Chapter 9). Griot *et al.* (1993) examined the genetic determination of extraneural replication of California serogroup viruses using two attenuated reassortants with attenuating mutations in the L and M RNA subunits, respectively. An inoculum of 1000 pfu of these attenuated viruses failed to replicate peripherally and did not reach the brain. Consequently, no visible disease resulted. An inoculum of 10^6 pfu, on the other hand, produced a transient viremia sufficient to enable virus to reach the brain and induce fatal disease. Reassortants generated from crosses of these viruses showed that the L and M RNA determinants acted independently and additively, since reassortants carrying both attenuating determinants were more attenuated than the parental viruses. Conversely, reassortants carrying neither determinant were more invasive than either parent.

Attenuation of alphaviruses has been achieved by selection for rapid penetration of BHK-21 cells (Olmsted *et al.*, 1984). Following this precedent, Endres *et al.* (1989) succeeded in isolating a novel neuroattenuated variant by

serial passage of a TAH/LAC/LAC reassortant in BHK-21 cells. The neuroattenuated phenotype (failure of intracerebral inoculation to produce disease in adult mice) of this variant is unique among California serogroup viruses. This variant also exhibited decreased neuroinvasiveness following subcutaneous inoculation of suckling mice, replicated less well in neuroblastoma cells than neurovirulent viruses, and was temperature-sensitive in BHK-21 cells at 38.9°C. Non-ts revertants simultaneously regained neurovirulence and ability to replicate efficiently in neuroblastoma cells. Thus, neuroblastoma cells provide a promising *in vitro* system for analysis of the phenomenon of neurovirulence in mice.

In another study of mouse virulence, Bishop *et al.* (1984) isolated reassortants from a cross of two parental strains of Caraparu virus (serogroup C) with different disease-producing properties and concluded that M RNA subunit gene products determined virulence in this serogroup also.

2. Mechanisms of Transmission in Invertebrates

Homologous reassortment has been demonstrated in mosquitoes dually infected with group I and group II ts mutants of La Crosse virus, either by simultaneous or by interrupted feeding, and by intrathoracic injection (Beaty and Bishop, 1989). Likewise, heterologous reassortants have been recovered from mosquitoes infected with different viruses of the California serogroup (Beaty *et al.*, 1981). Putative natural reassortants have been recovered also from mosquitoes in the wild. Transmission of bunyaviruses from an infected mosquito to a vertebrate host becomes possible only after virus reaches the salivary glands, a process that requires 7–14 days from ingestion of the virus. Characterization of heterologous reassortants has shown that dissemination of virus from the midgut to other tissues is under the control of the M RNA (Beaty *et al.*, 1982). Separate domains of the M RNA determine ability to overcome the midgut barrier on the one hand, and ability to multiply in thoracic organs on the other (Gonzalez-Scarano *et al.*, 1988). Since the M RNA encodes the viral membrane glycoproteins, it is likely that these tissue tropisms in the mosquito are receptor-mediated. The midgut also imposes a barrier to the dissemination of TSWV in adult thrips (Ullman *et al.*, 1992). The vector must ingest the virus in the larval stage to enable the virus to reach the salivary glands.

3. Vaccine Development

Experimental inactivated and live virus vaccines have been developed to protect humans and animals from the ravages of Rift Valley fever virus (genus *Phlebovirus*). The live virus vaccines have been developed by empirical routes (Smithburn, 1949; Moussa *et al.*, 1982) and by random mutagenization (Caplen *et al.*, 1985; Rossi and Turell, 1988). The latter involved propagation of a wild-type virus in BHK-21 cells in the continuous presence of 200 μg/ml

5-fluorouracil. Single plaques were picked after each cycle of multiplication to initiate the next infection. Virus passaged by this regimen became rapidly attenuated for mice, whereas virus passaged in the absence of the mutagen did not. Retrospective reassortment experiments suggested that attenuating mutations had accumulated in all segments. Takehara *et al.* (1989) sequenced the M RNA subunits of wild-type parent and attenuated derivative and found that 12 nucleotide substitutions had occurred resulting in seven amino acid changes and creation of a new upstream AUG start site. The identity of the critical attenuating mutations could not be determined, however, because the virulent parental strain differed by 7 nucleotide substitutions and three coding changes from another virulent strain isolated simultaneously during the same epidemic, and because the L and S RNAs were not sequenced.

C. Genetic Determination of Host Resistance in Plants

Host resistance to tospovirus infection has been studied extensively in the interests of crop protection. Classical genetic approaches have been employed to identify sources of resistant germ plasma for use in plant breeding. The genetic determination of resistance to TSWV appears to be highly specific and genetically complex. Boiteux *et al.* (1993) evaluated 70 species and cultivars of *Capiscum* and observed different levels of resistance under field conditions of infection. As a result of controlled greenhouse trials, a complex pattern of resistance was observed and it was concluded that several genetic mechanisms were involved in TSWV resistance. For example, two lines of *C. chinense.* which were virtually immune to one isolate of TSWV, were susceptible to another isolate of the same virus from a different region of Brazil. Kumar and Irulappan (1992) compared the resistance of tomato plants (*Lycopersicom peruvianum* var. *humifusum*, *L. hirsutum* f. *glabratum*, *L. hirsutum*) to TSWV under field and greenhouse conditions. Fifteen crosses involving five susceptible parents and three wild species revealed that resistance was controlled by a small number of recessive genes. On the other hand, Stevens *et al.* (1991) identified a single dominant gene in the "Stevens" cultivar of *L. peruvianum* which provided solid resistance to several isolates of TSWV. Similarly, the combined resistance to both TSWV and *Thielaviopsis basicola* of the Polalta cultivar, derived from wild *Nicotiana alata*, was inherited as a dominant characteristic that could be introduced into several susceptible Oriental tobacco cultivars (Yancheva, 1990).

The production of transgenic plants is an alternative approach to producing host resistance which has potential for crop protection. Transgenic "Samsun" cultivars of tobacco expressing the complete NP gene of TSWV have been produced which were resistant to superinfection with TSWV (Mackenzie and Ellis, 1992). These experiments with a bunyavirus confirmed that protection of host plants could be obtained by expression of homologous coat protein genes of membrane-enveloped, multicomponent, negative-stranded

viruses, as well as isometric positive-stranded viruses. Pang *et al.* (1992) investigated the cross-resistance spectrum of tobacco plants transgenic for the NP gene of a strain of TSWV belonging to the L serogroup. Resistance to the homologous TSWV strain was almost universal in the transgenic plants and a fairly broad spectrum of resistance to heterologous strains of the L serogroup was observed even where NP gene expression was low. Plants that expressed high levels of NP were resistant also to an isolate belonging to serogroup I. However, all of the transgenic plants remained suceptible to a Brazilian strain of TSWV belonging to another serogroup. These results indicate that a fairly broad spectrum of resistance can be obtained by expression of the coat protein gene of TSWV in a susceptible host plant, but there are limits to the extent of cross-protection obtainable by this means. Subsequently, it was shown that protection against the homologous isolate and closely related isolates in plants expressing low levels of the N gene was related to the presence of N gene RNA, whereas protection against the homologous isolate and isolates of the distantly related impatiens necrotic spot virus in plants expressing high levels of the N gene was related to accumulation of N protein (Pang *et al.*, 1994). Thus, different mechanisms appear to be involved in protection against infection by tospoviruses that share different levels of N gene sequence identity.

VII. PROSPECTS

Future progress in the study of the genetics of bunyaviruses will depend greatly on exploitation of the reverse genetic approach whereby genes are manipulated in the form of cDNA and subsequently reintroduced into infectious viral genomes. The problems inherent in the application of reverse genetics to viruses with negative-stranded RNA genomes have been solved for the orthomyxoviruses (Enami *et al.*, 1990). Although this methodology is not directly transferable from one negative-strand virus group to another, the feasibility of the reverse genetic approach with negative-stranded RNA viruses has been established. The technical problems specific to the bunyaviruses are close to solution (Dunn *et al.*, 1995), and we are now on the threshold of a new phase in the investigation of genetic and evolutionary processes.

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