Non-random reassortment between the tripartite RNA genomes of La Crosse and snowshoe hare viruses

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The process of reassortment between the tripartite RNA genomes (segments designated L, M and S) of snowshoe hare and La Crosse bunyaviruses (Bunyaviridae) has been investigated by polymerase chain reaction analysis of > 250 progeny recovered at 72 h post-infection from dual wild-type virus infections involving high multiplicities (approximately 5) of each virus in a BHK-21 cell line. Statistical analysis of the data indicated that RNA segment reassortment was not random, and for these two viruses the data appeared to fit the hypothesis that there was a preference for homologous L–M and M–S associations among the progeny formed.

Introduction

Genome segment reassortment has been demonstrated for viruses representing most of the virus families that have genomes consisting of more than one segment of RNA, i.e. the Arenaviridae, Bunyaviridae, Birnaviridae, Orthomyxoviridae and Reoviridae. Within the Bunyaviridae, reassortment has been reported in the Bunyavirus genus between individual members of the California encephalitis serogroup (e.g. La Crosse (LAC), snowshoe hare (SSH), Tahyna (TAH), trivitattus, California encephalitis, Lumbo, etc.) (Bishop, 1985). In the same genus, reassortment has also been reported between members of the Bunyamwera serogroup (e.g. Batai, Bunyamwera and Maguari viruses), or group C viruses (Bishop, 1990). In the Phlebovirus genus of the Bunyaviridae, crosses of different strains of Rift Valley fever virus have produced reassortant progeny (Turell et al., 1990). However, no reassortment has been demonstrated between viruses representing different Bunyaviridae genera, or those representing different serogroups in the same genus (e.g. Bunyamwera and California encephalitis serogroups). The ability of two viruses to reassort their RNA segments is a measure of their genetic relatedness (Bishop, 1985).

Reassortants with all possible combinations of the genome segments of LAC and SSH viruses have been obtained in crosses of non-mutagenized LAC and TAH viruses (Janssen et al., 1986). In these studies, the genotypes of reassortant viruses were determined by RNA–RNA hybridization and S1 nuclease digestion after an initial screening of labelled viral proteins by SDS-PAGE. The method employed by Endres et al. (1991) to characterize LAC–TAH virus reassortants involved dot blot hybridizations using a LAC virus-specific cDNA probe. This method is convenient for the quick analysis of a large number of samples, as compared with the abovementioned procedures; however, the discriminatory power of the method depends on the degree of sequence homology between the parental RNA species and the specificity of the cDNA probes. Previous studies on the development of specific genetic probes to distinguish the S segments of LAC and SSH viruses have shown that long cDNA or RNA probes (634 nucleotides in length) hybridize equally well with either parent virus. The reasons are that the S segments of the two viruses are 88% identical and that the differences are unevenly distributed throughout the sequences (Akashi & Bishop, 1983).

In analyses of the process of genetic reassortment, evidence for non-random segregation of segments has been reported for bunyaviruses (Pringle et al., 1984), influenza viruses (Lubek et al., 1979), rotaviruses (Gombold & Ramig, 1986; Graham et al., 1987; Ramig & Ward, 1991; Ward et al., 1988) and reoviruses (for an example see Stott et al., 1987). The factors that cause non-random reassortment have not been elucidated.

In the present study, we have analysed the reassortment process of mixed infections of cultured cells using...
wild-type LAC and SSH bunyaviruses and the polymerase chain reaction (PCR) to process a large number of progeny viruses (> 250) derived from wild-type SSH and LAC virus infections in order to identify the parental origin of each RNA segment of the progeny. The data suggest that certain preferences exist for RNA segment associations, preferences that result in the non-random nature of the LAC–SSH virus reassortment process.

Methods

Viruses and cells. The origins and passage histories of wild-type SSH and LAC viruses have been described (Clewley et al., 1977). Viruses were grown and plaque-assayed at 36 °C in a cloned strain of BHK-21 cells.

Dual virus infections. Confluent monolayers of BHK-21 cells (approximately 1-2 × 10⁶ cells/dish) were simultaneously infected with 5 p.f.u./cell of either wild-type LAC or SSH, or 5 p.f.u./cell of both viruses. After 1 h at 36 °C, 150 µl of inoculum was removed and the cells were washed twice with PBS to remove unadsorbed virus. The infected cell monolayers were incubated for 2 h at 36 °C under 3 ml of Eagle's MEM (EMEM) and then washed three times with PBS to remove desorbed viruses. Incubation was continued in fresh medium at 36 °C and the supernatant was harvested at 72 h post-infection (p.i.). The virus titre rose by more than 100-fold over the course of the LAC–SSH virus reassortment process.

Plaque selection of reassortants. Supernatants of the single and dual virus infections were serially diluted and used to infect BHK-21 cell monolayers. Cultures were overlaid with 1% Sea-Plaque agarose, and plaques were visualized with neutral red. A total of 350 well separated plaques from the dual infections was picked at random, irrespective of size, on days 6 or 7 p.i. Twelve plaques of the progeny of each single (parental) virus infection were also picked. For protein analyses of parental and reassortant viruses, 40 plaque plugs were homogenized in EMEM and used to infect BHK-21 cell monolayers in 96-well plates. The supernatants of these cultures were harvested at 48 h p.i., and used to infect BHK-21 cells that had been grown in 24-well plates. Three days p.i. the supernatants were harvested and stored at −70 °C for use as stock virus.

RNA preparations. Individual plaque plugs were mixed with 400 µl of TES buffer (5 mM-Tris–HCl pH 7.4, 2.5 mM-EDTA, 0.5% SDS), and the mixtures were vortexed, frozen and thawed once and extracted with phenol–chloroform. RNA was precipitated by ethanol containing 2 µg of glycogen as a coprecipitant. RNA from virus present in 300 µl of the supernatant from the 24-well cultures was similarly extracted using the TES buffer (5 mM-Tris–HCl pH 7.4, 2.5 mM-EDTA, 0.5% SDS), and the radiolabelled RNA preparations were purified by ethanol precipitation and resuspended in H₂O.

cDNA synthesis. cDNA synthesis was dependent on the nature of the LAC–SSH virus reassortment process. The complete sequences of the S and M segments of LAC and SSH viruses (Akashi & Bishop, 1983; Bishop et al., 1982; Grady et al., 1987; Eshita & Bishop, 1984), and 200 of the 3′-terminal nucleotides of their L segments (Clerx-van-Haaster & Bishop, 1980) were compared using the ALIGN program (Staden, 1986) to locate differences between the aligned sequences of the two viruses, and to select and synthesize the appropriate oligonucleotide primers for PCR. Each of the selected primer sequences was then compared against the known genomic sequences of LAC and SSH viruses using the BESTFIT program (University of Wisconsin Genetics Computer Group Package) to identify alternative base-pairing regions, i.e. ones other than those corresponding to the primers. A list of the nine primers that were selected and used to determine the genotype of SSH/LAC reassortant viruses is given in Fig. 1. The S vc primer was identical to the corresponding sequences of both the LAC and SSH virus S segments. For the M segments, the primer represented the SSH vc sequence, having a one base mismatch with the equivalent LAC virus sequence (Fig. 1). For the L segments, the primer represented the LAC vc sequence, having a two base mismatch with the equivalent SSH virus sequence (Fig. 1). Neither mis-
Fig. 1. Oligonucleotide primers used for the selective amplification of LAC or SSH virus S, M or L RNA species. Boxed upper case sequences indicate the selected primers. The residue numbers correspond to the genomic sequences (Clerx-van-Haaster & Bishop, 1980; Bishop et al., 1982; Akashi & Bishop, 1983). Asterisks indicate nucleotide identities between the primer and heterologous (lower case) sequences. The orientation of the sequences is 5' to 3'. The fragment sizes represent those expected for the respective PCR products.
Discrimination of LAC and SSH virus RNA species by PCR

The vc and vs oligonucleotide primers were designed to discriminate between homologous and heterologous templates in the PCR, and also to simplify the procedure for cDNA synthesis in the analysis of a large number of samples. The L, M and S vc primers were used to prime reverse transcription of the respective segments of both LAC and SSH virus RNA species, since overall these primers represent regions conserved between the genomes, including the sequences corresponding to the 3' extremities of the respective primers (Fig. 1). The cDNAs representing each segment of SSH and LAC viruses were synthesized simultaneously using the three vc primers in a single reverse transcription reaction mix. The specificity of the subsequent PCR amplifications for LAC or SSH virus RNA species using these derived reverse transcription products was determined by the six vs primers that were used (LAC vsL, vsM and vsS; SSH vsL, vsM and vsS). As illustrated in Fig. 1, these were chosen from regions less well conserved than those represented by the vc primers and had 3' end mismatches with the comparable sequence of the heterologous virus. In addition, the distance between the vc and the vs primers was designed to yield DNA fragments of different sizes for each RNA segment (Fig. 1; e.g. SSH virus L, 66 bp; LAC virus L, 119 bp; SSH virus M, 183 bp, etc.).

The efficacy and specificity of this method of determining the genotype of reassortant viruses were verified by performing independent L, M and S PCRs, using LAC and SSH virus cDNAs (obtained by reverse transcription as described in Methods), and similar products obtained from RNA derived from uninfected cells. Selective amplification of the parental virus

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**Fig. 2.** PCR products of LAC and SSH virus S, M and L RNAs. (a) The stained individual products, obtained using primers corresponding to the LAC and SSH virus S (lanes 1 and 2), M (lanes 3 and 4) or L (lanes 5 and 6) RNA species resolved in a 2% agarose gel. In lanes 7 to 9 are similar products obtained using S, M and L RNA from uninfected BHK-21 cells, and in lanes 10 and 11 are those obtained using viral RNA and the combined LAC or SSH virus primers. Size markers (1419 to 65 bp) are shown in lane 12. (b) The S RNA-specific, M RNA-specific and L RNA-specific PCR products of RNA from plaques representing the indicated LAC-SSH virus reassortants (large/medium/small genotypes derived from LAC and SSH viruses, e.g. SLL, SSH L RNA + LAC M and S RNAs), compared to the PCR products of LAC and SSH viruses. The sizes of the expected PCR products are indicated to the left of each panel.
sequences by their corresponding primer pairs in the presence of the heterologous vs primer was confirmed for all six RNA segments (Fig. 2). The expected amplification products were obtained using annealing temperatures of 55 °C in the PCR. This condition appeared to be adequate to prevent the heterologous primers forming stable hybrids. The PCR products from uninfected cells did not exhibit any of the sizes corresponding to those expected for the LAC or SSH virus sequences (Fig. 2).

Protein analyses of progeny clones representing the eight possible segment combinations confirmed the PCR results for the S and M segments (Fig. 3). The N protein (encoded by the S RNA segment) of LAC virus and that of SSH virus can readily be distinguished by SDS–PAGE

Table 1. Analysis of the frequency of reassortant viruses obtained from cells infected with wild-type SSH and LAC viruses

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Observed frequency (%)†</th>
<th>Expected frequency†</th>
<th>$\chi^2$</th>
<th>Significance (P)</th>
<th>RNA segment</th>
<th>Relative observed frequency†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLL</td>
<td>81 (32)</td>
<td>60</td>
<td>9.32</td>
<td>&lt;0.005</td>
<td>L</td>
<td>0.68</td>
</tr>
<tr>
<td>LLS</td>
<td>39 (12)</td>
<td>39</td>
<td>2.23</td>
<td>&gt;0.1</td>
<td>M</td>
<td>0.57</td>
</tr>
<tr>
<td>LSS</td>
<td>46 (14)</td>
<td>46</td>
<td>2.94</td>
<td>&gt;0.05</td>
<td>S</td>
<td>0.61</td>
</tr>
<tr>
<td>LSL</td>
<td>29 (11)</td>
<td>29</td>
<td>0.05</td>
<td>&gt;0.8</td>
<td>S</td>
<td>0.57</td>
</tr>
<tr>
<td>LSS</td>
<td>28 (11)</td>
<td>28</td>
<td>1.61</td>
<td>&gt;0.2</td>
<td>S</td>
<td>0.57</td>
</tr>
<tr>
<td>SLL</td>
<td>13 (5)</td>
<td>13</td>
<td>1.57</td>
<td>&gt;0.2</td>
<td>M</td>
<td>0.57</td>
</tr>
<tr>
<td>SSL</td>
<td>21 (7)</td>
<td>21</td>
<td>0.99</td>
<td>&gt;0.3</td>
<td>L</td>
<td>0.68</td>
</tr>
<tr>
<td>SSS</td>
<td>14 (11)</td>
<td>14</td>
<td>18.13</td>
<td>&lt;0.001</td>
<td>L</td>
<td>0.68</td>
</tr>
<tr>
<td>Total</td>
<td>255</td>
<td>255</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† The relative observed frequencies of the L, M and S RNA segments were used to calculate the expected frequencies.

Fig. 3. Analysis of the protein produced by parental LAC and SSH viruses and eight progeny genotypes (see legend to Fig. 2 for nomenclature) identified by PCR. Differences between the M RNA-encoded G1 proteins and the S RNA-encoded N proteins are highlighted (open arrowheads, LAC virus; closed arrowheads, SSH virus). BHK-21 cells were infected with each virus or mock-infected (lane M), and labelled with $^{35}$S]methionine at 24 h p.i. Polypeptides were resolved by SDS–PAGE in a 17% gel with an acrylamide:bisacrylamide ratio of 300:1.
(Gentsch et al., 1977a). Under the conditions of SDS-PAGE used, it was found that the G1 protein (encoded by the M RNA segment) of LAC virus and reassortants containing the LAC virus M RNA reproducibly migrated slightly faster than that of SSH virus or the reassortants containing the SSH virus M RNA. This constitutes an additional phenotypic difference between the two viruses with regard to the glycoprotein products of their M segments (see Fuller & Bishop, 1982).

**Analysis of the progeny of dual infections**

BHK cells were infected with 5 p.f.u./cell of each parental virus to produce a high probability of every cell becoming infected with both viruses. Inocula and desorbed viruses were removed during the initial phases of the course of infection.

To determine whether reassortment was a random process, the sample to be examined had to be representative of the total population, and large enough to perform an analysis of statistical significance. With the purpose of obtaining a random sample, and taking into account that a single growth period for LAC and SSH viruses is under 24 h (Gentsch et al., 1977a), the mixed infection was allowed to progress until 72 h p.i., providing ample opportunity for all forms of reassortant to be incorporated into the progeny sample. The same consideration applied to the selection of clones from the plaque assays. The first plaques could be detected by 3 days p.i.; further development of new plaques was monitored until 7 days p.i. when, as expected, a greater heterogeneity of plaque size was observed (smaller plaque types took longer to develop and be recognized). A total of 350 well separated plaques were picked on day 6 or 7 p.i., regardless of their size, and processed as described in Methods.

The genotypes of the randomly selected clones were determined by performing separate PCRs for the L, M and S segments. This procedure was adopted because when all nine primers were incorporated into a single reaction mixture, aberrant products representing non-specific amplification occasionally occurred, hindering immediate interpretation of the results. The parental origin of progeny viruses with a single form of S, M and L RNA species was established for 255 (73%) of all the plaques tested. The remaining 27% was accounted for by: 3% (12 plaques) in which no PCR products were obtained for any of the three segments (probably due to false identification of a virus plaque, or insufficient RNA recovered for the reverse transcription reaction); 14% (50 plaques) in which no products were obtained for one of the segments; 1% (two plaques) in which no products were obtained for two segments (presumably due to insufficient reverse transcription reaction product representing the missing segment); and 9% (31 plaques) in which PCR products were obtained corresponding to one segment from each parental virus (or, less frequently, more than one segment), indicating the presence of more than one type of virus in the original plaque, or the existence of diploid progeny viruses. These viruses were not analysed further in this study.

The co-infection progeny included all eight possible segment combinations with the frequency distribution and percentages shown in Table 1. The question of whether these observed frequencies differed from those that may be expected assuming a free segregation of segments was next examined by calculating the relative frequencies of the individual SSH and LAC virus L, M and S segments among the progeny, and then computing the values that should be obtained from these observed frequencies (i.e. expected frequency, Table 1).

Although the design of the experiment was such that all cells should have been infected with similar numbers of each parental virus, there was no guarantee that in any experiment this would occur. Second, in the synthesis of progeny from the available gene pools, if a segment of one virus type was more proficient at replication than the corresponding segment of the other virus, then even if there had been identical m.o.i., the numbers of available RNA species of that segment type from which progeny could be derived would differ for the two viruses. This phenotype could vary for different segments (e.g. if the LAC virus L RNA replicated more proficiently than the SSH virus L RNA, but SSH virus M RNA replicated more proficiently than LAC virus M RNA, etc.).

**Fig. 4. Analyses of determinants of SSH and LAC virus reassortment.** The full and derived models considered in the analysis of the interaction effects between the RNA species of SSH virus and LAC virus in the reassortment process are shown. A, L RNA; B, M RNA; C, S RNA. The frequency distribution shown in Table 1 was arranged into a three-way table, in which each segment was considered as a factor with two categories per factor (i.e. LAC or SSH virus). The analysis was performed using GLIM with a link function, log and a Poisson error. Quantities shown along the arrows correspond to changes in the S statistic (described in Results) when an indicated term (A, B, C, or A, B, etc.) is removed from a prior model. Values in parentheses are the degrees of freedom associated with S. The confidence levels (P) refer to the significance of the omitted terms. Confidence levels of lower than 0.1% (P < 0.001) are considered highly significant (i.e. the probability of the pertinent term being negligible is very small, so it cannot be removed without affecting the goodness of fit very significantly), a > 5% confidence level is considered not significant (i.e. removal of the term does not significantly affect the goodness of fit). Four of the derivatory results for the successive removal of terms are illustrated in the lower boxes. Genotypes (genot) refer to the L, M and S RNA species of the parental and reassortant viruses. The observed (obs) and predicted (fitted) frequencies of these genotypes and the residual values, which relate to the goodness-of-fit from the analyses, are listed for four of the models. See the text for discussion of the double boxed models.
factors could also influence the net production of progeny (e.g. the effects of gene products on heterologous templates, etc.).

The analysis of the progeny from the wild-type LAC and SSH virus co-infection indicated that there was a preponderance of all three LAC virus segments among the progeny (depending on the segment, between 1.3- to 2.1-fold more LAC virus than SSH virus segments, see Table 1). In view of the observation that LAC virus is somewhat more efficient than SSH virus in infection, as shown by the time of c.p.e. appearance and virus yield (unpublished data), an attempt was made to pre-infect BHK-21 cells with SSH virus 4 h before the addition of LAC virus. Of 20 progeny analysed, no LAC virus was shown by the time of c.p.e. appearance and virus yield (unpublished data), an attempt was made to pre-infect BHK-21 cells with SSH virus 4 h before the addition of LAC virus. Of 20 progeny analysed, no LAC virus was identified indicating that prior SSH virus infection in some way blocked the ability of LAC virus to superinfect the cells. Other means of increasing the SSH virus gene pool in cells in comparison to that of LAC virus were not investigated.

The highly significant difference between the observed and expected distributions of LAC and SSH viruses and their reassortants (Table 1; \( \chi^2 = 32.41; P < 0.001 \)) was attributable to the over-representation of both parental genotypes in the sample (43% of the total progeny). This was established by individual \( \chi^2 \) tests for each genotype, arranging their respective values with the rest of the distribution in 2 \( \times \) 2 tables. In view of the protocols employed and the fact that the progeny virus titres were approximately \( 1 \times 10^9/ml \), whereas the inoculum was \( 1 \times 10^7 \) p.f.u. in 150 \( \mu l \), the high proportion of LAC and SSH virus genotypes in the progeny could not have come from desorbed virus. Previous experiments with similar protocols (Vezza et al., 1979) have shown that desorbed virus represents < 5% of adsorbed virus. Therefore, from the results it can be concluded that there was a preference for homologous virus L, M and S RNA associations among the progeny.

To ascertain whether preferential associations between segments of the same parental origin (i.e., the variables) were responsible for the observed genotype frequencies, the effects of all possible interactions between segments were analysed with a log-linear model (Sokal & Rohlf, 1981). This model describes the structure of the variable under investigation in terms of the linear sum of the effects played by other variables (Fig. 4, double boxed A.B + B.C + A.C). Since the change of the scaled deviance (0.2596 with 1 degree of freedom) was not significant (\( P > 0.5 \)), it was concluded that joint associations between all three segments were not important, therefore the A.B.C term could be dropped from the model.

The subsequent analysis of the effects of the omission of each of the two-way factor interaction terms (A.B, B.C or A.C) revealed the existence of partial associations of different magnitudes between pairs of factors, as indicated by the change of S and residual values of the respective models shown in Fig. 4. The consequence of the removal of the B.C interaction term (i.e. segments M and S) led to an unacceptable decrease in the goodness of fit, as illustrated by the starred residual values for the model A.B + A.C, and by the significance test (Fig. 4, \( P < 0.005 \)). Therefore this can be interpreted as the importance and necessity to include the B.C interaction effect in the description of the total population of progeny viruses.

The least significant association was found to be the one between the L and S segments (A.C term). The expected values in the model that considered only the A.B and B.C interactions (Fig. 4, double boxed) fitted reasonably well with those observed, with only small divergences evident for the SSL and SSS genotypes (starred residual values). The model fitted in the absence of the A.B interaction term provided an intermediate goodness of fit between the A.B + A.C and the A.B + B.C models. Further removal of interaction terms (Fig.
4) are equivalent to simultaneous removal of two terms from the A.B + B.C + A.C model. The results did not fit the data at all well. Thus it can be concluded that, in addition to the B.C term, at least one of the other two-way factor interaction terms must be included in a model that adequately describes the data. The models fulfilling this requirement are highlighted with a double box in Fig. 4.

With regard to the significance of the A.B and A.C terms, it was found that neither reached the 1% confidence level. Small values of such confidence levels are desired when several hypotheses are tested for the same distribution in order to avoid a large probability of a type I error (i.e., rejection of a true null hypothesis, Sokal & Rohlf, 1981). It was concluded that either factor could be independently dropped from the model, taking into account that the effects of the A.B term play a more important role in the definition of the linear structure.

The observed high frequency of progeny viruses with LLL and SSS genetic composition (32% and 11% respectively) thus is explained by the double effect of the preferential homologous association of pairs of segments. This effect is particularly evident in the case of the SSH virus genotype. Although the representation of the SSH virus L, M and S segments in the sample predicted the smallest value for the SSS combination (14; Table 1), its observed frequency (29) was actually greater than those of the LSS, SLL, SLS and SSL genotypes. Likewise, the predominance of LLL progeny can be explained by the combination of a greater availability of LAC virus genetic material and the preferential association of the LAC virus L–M and M–S segments.

Discussion

Previous studies have demonstrated reassortment between bunyaviruses (Gentsch et al., 1977b, 1979, 1980; Rozhon et al., 1981; Pringle et al., 1984; Janssen et al., 1986; Chandler et al., 1990, 1991; Endres et al., 1991) using direct and inferred genotype determinations. The PCR method that we adopted to analyse a large number of reassortant viruses was based upon the exploitation of a few nucleotide differences between SSH and LAC viruses. The specificity of the PCR was provided by the vs oligonucleotide primers. Since the distance between the two primers was different for each segment of LAC and SSH virus, the size of the amplification products was used to indicate the viral genotypes. The accuracy of the method was confirmed in part by the protein determinations for the LAC and SSH virus S and M RNA segments. All possible combinations of L, M and S RNA subunits were obtained in the progeny. When selected viruses were re-plaqued, none of the reassortants (SSL, LLS, etc.) exhibited a significant difference either in plaque size or plaque development that could have biased the selection of progeny. The recovery of LSS and LSL genotypes contrasts with the difficulties encountered by Gentsch et al. (1979) in identifying these viruses. The reason may be due to the phenotypes of the particular ts mutants they used to obtain those reassortants (Gentsch et al., 1979). With regard to the efficiency of the method, 52 of the 338 clones tested (15%) could only be partially genotyped, owing to the absence of PCR products for at least one of the segments. In most cases (45 of 52), no result was obtained for the S segment (in three cases the M segment was untyped, in two cases the L segment was untyped and in two cases two segments were untyped). This is surprising in view of the fact that the S segments are usually the most abundant species in LAC and SSH virus populations (Gentsch et al., 1977a). We have subsequently found that elimination of the secondary structure in RNA by means of methylmercury treatment increases the yield and length of cDNAs in reverse transcription reactions. Another procedure to reduce the numbers of unclassified viruses further would be to prepare virus stocks for each plaque isolate prior to analysis. Of the 45 viruses for which the S RNA was untyped, in 35 the L and M species came from one parental type (LLX or SSX). This is only slightly higher than the frequencies given in Table 1; however, the low numbers preclude an evaluation of the significance. Because the lack of complete data was most likely to be due to technical reasons, and no evidence was obtained for a genetic or phenotypic cause (e.g., plaque development), this group of viruses was discounted from the statistical analyses.

Interestingly, 9% (31) of the 350 plaques analysed gave evidence for the presence of the corresponding segment of both LAC and SSH viruses (usually only the S segment). Since particular care was employed to recover well separated plaques and stain them at 6 or 7 days p.i., this number is high. A trivial cause would be plaques derived from infections involving two different progeny viruses. One possibility is that they are diploid viruses, i.e., progeny that encapsidated more than one copy of a particular segment (e.g., S). Replaquing the viruses would be a means to establish the phenomenon if evidence was obtained for the segregation of viral genotypes. The presence of diploid viruses involving two copies of S might also explain the difficulties reported in the recovery of spontaneous ts mutants representing the S segment of the California encephalitis serogroup of bunyaviruses (Rozhon et al., 1981). Similarly a clone obtained from a cross between Maguari and Bunyamwera viruses has been reported to possess the N protein from both parents (Iroegbu & Pringle, 1981).

The statistical analysis of the progeny of the mixed
infection indicated that the process of reassortment was not random. The representation of LAC virus RNA species was larger than that of the corresponding SSH virus segments, as revealed by the calculated relative frequencies for each segment. This may be a reflection of the better replication properties of LAC virus in terms of virus titres and c.p.e. induction in BHK cells. When, in an attempt to minimize these differences in the co-infection experiments, LAC virus was inoculated 4 h after the infection of BHK cells with SSH virus, all the progeny analysed had the SSH virus genotype (details not shown). Similar interference to reassortment has been described previously when invertebrate hosts are orally infected with two ts mutants of LAC virus (Beaty et al., 1985) and in infections involving two strains of Rift Valley fever virus (Turell et al., 1990). Co-infection, or superinfection of mosquitoes with SSH virus 1 h after LAC virus infection, results in reassortant progeny (Chandler et al., 1990). Our data from cultured vertebrate cells agree with the view that the opportunity for reassortment may depend on the time of initiation of parent virus replication.

The theoretical consideration that free reassortment of genome subunits would result in 25% parental genotypes and 75% reassortants would only be valid if the intracellular gene pools of each parent were represented by equimolar quantities of the respective RNA segments at all stages of virus morphogenesis (i.e. when RNA segments were selected for packaging). Even with similar m.o.i., it is obvious that the likelihood of achieving exactly equivalent gene pools is small. A statistical analysis of the progeny therefore must be based on the observed frequencies of RNA segments among the progeny, and analysed on the basis of the predictions derived from those frequencies. On the basis of the recoveries of the SSH and LAC virus L, M and S species, the sum of the expected frequencies for all types of reassortant in the event of complete independence for every segment should be 71%. The observed frequency was 57% (Table 1). To determine the extent of preferential interactions, the observed values were classified according to their L, M and S genotypes in a three-way contingency table, where the three segments were considered as factors, with two categories (LAC/SSH) per factor. A log-linear model was used to test the significance of the interactions between segments (see Sokal & Rohlf, 1981). The results of the analysis revealed the existence of positive associations between certain pairs of segments.

A low frequency of reassortant genotypes has been reported from co-infections of LAC and TAH viruses (Janssen et al., 1986), and in crosses between two of their reassortants (Endres et al., 1991). Positive association between homologous subunits has also been observed by Pringle et al. (1984) in crosses of Batai, Bunyamwera and Maguari viruses, but in these cases it occurred between the L and S RNA subunits. Non-random segregation of genome segments has been observed in vivo in reassortment studies with bluetongue virus (Stott et al., 1987), and rotaviruses (Gombold & Ramig, 1986), as well as in vitro for rotaviruses after multiple passage (Graham et al., 1987; Ward et al., 1988). The latter studies are not directly comparable to the study reported here. We restricted our analysis to the progeny of a single co-infection in order to study the interaction of RNA subunits during the assembly process. In addition, we tried to minimize the effects of differences in the growth properties of progeny viruses by analysing the progeny at 72 h p.i. Under similar conditions, homologous associations of parental segments have been described for influenza A virus reassortants (Lubeck et al., 1979).

The mechanism for packaging the correct set of segmented RNA virus genomic subunits is not well understood. The way in which RNA is packaged in the non-segmented RNA genome viruses is also ill-defined. Since for such viruses the ratio of individual RNA segments inside the cell is often very different from that in the population of virus particles (for bunyavirus, segments S > M > L), a specific selective mechanism has been postulated for the inclusion of the complete genetic complement to form, on average, an infectious virus particle. Virus morphogenesis must be considered as a stochastic process, in which a variety of factors inherent to a virus affect the result. Understanding the mechanisms underlying this process has to be based upon the study of the most probable ones. A mechanism in which virions have more than a single complement of the various genome segments has been invoked for packaging the segmented nucleocapsids of influenza virus. In part, this model is based on the observed high particle: p.f.u. ratios of the influenza viruses (Compans et al., 1970). Provided enough RNA is packaged in virions, the probability of including all types of species to form an infectious particle that yields infectious progeny is increased. Relevant to this model are the observations referred to previously concerning diploidy and the recent report by Enami et al. (1991) on the engineering of an influenza virus containing nine different RNA segments. The pleomorphic character of most enveloped viruses would facilitate the inclusion of extra species. LAC virions, examined under conditions that preserve their native structure, have been shown to vary in size, suggesting that they may vary in their content of RNA segments (Talmon et al., 1987). Even if amounts greater than those equivalent to a single complement of the segments are incorporated into some virions, packaging is probably governed by other laws, such as preferred segment–segment interactions. Such laws
influence the outcome of reassortment of viruses in the same gene pool.

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References


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