Morphivirus viruses form a closely related, serologically cross-reacting group in the family Paramyxoviridae, but have different host ranges. They include: measles virus (MV), rinderpest virus (RPV), peste-des-petits-ruminants (PPRV), canine and phocine distemper viruses (CDV and PDV) and cetacean morbillivirus.

Morphivirus genomes range in size from 15 690 for CDV to 15 948 nt for PPRV (Bailey et al., 2005). They start with a 52 nt 3′-leader region followed by a conserved 5′-CTT-3′ (in the positive sense) intergenic region and six genes encoding the nucleocapsid (N), phospho- (P) and large (L) proteins of MV, canine distemper virus (CDV) or RPV in order to determine whether the proteins of different viruses can function together. Homogeneous sets consisting of N, P and L plasmds derived from one virus were able to generate reporter gene expression from either mini-genomic construct. Heterogeneous sets of proteins from different viruses were not functional, with the exception that a low level of activity was obtained when MV N and P protein were combined with RPV L protein in the rescue of the MV mini-genomic construct, or CDV N was combined with RPV P and L in the rescue of the RPV mini-genome. However, only homogeneous sets of plasmids were able to rescue infectious virus from full-length anti-genome-expressing plasmids.

Chloramphenicol acetyltransferase (CAT)-expressing negative-sense mini-genomic constructs of measles virus (MV) and rinderpest virus (RPV) were rescued by standard technology with helper plasmids expressing the nucleocapsid (N), phospho- (P) and large (L) proteins of MV, canine distemper virus (CDV) or RPV in order to determine whether the proteins of different viruses can function together. Homogeneous sets consisting of N, P and L plasmds derived from one virus were able to generate reporter gene expression from either mini-genomic construct. Heterogeneous sets of proteins from different viruses were not functional, with the exception that a low level of activity was obtained when MV N and P protein were combined with RPV L protein in the rescue of the MV mini-genomic construct, or CDV N was combined with RPV P and L in the rescue of the RPV mini-genome. However, only homogeneous sets of plasmids were able to rescue infectious virus from full-length anti-genome-expressing plasmids.
and 5’ non-coding genome termini, were kind gifts from Professor M. A. Billeter (Institute for Molecular Biology, Zürich, Switzerland). A similar plasmid to p107MV(−) : CAT was made for RPV (Fig. 1). The CDV and RPV plasmids expressing N, P and L proteins were generated in our laboratories (Gassen et al., 2000; Baron & Barrett, 1997). In order to assess which combinations of the N, P and L proteins were able to co-operate in the mini-genome rescue assay, expression of the CAT reporter gene of p107MV(−) : CAT and p(−)RPV DICAT mini-genomic constructs was measured with all possible combinations of N, P and L proteins derived from RPV, CDV and MV (Fig. 2). CAT activity was assessed at 20 h post-transfection by measuring the amount of [3H]acetylated chloramphenicol (Gassen et al., 2000). Experiments were repeated three times and standard deviations were calculated. For negative controls, the L plasmid was omitted. CAT activity was also measured using an ELISA system (Baron & Barrett, 1997) and, although the assay was less sensitive and had a smaller dynamic range, the results (data not shown) were entirely consistent.

Homogeneous mixtures of plasmids (i.e. all from the same virus) were able to drive expression of the reporter genes from both MV- and RPV-based mini-genomic constructs, but heterogeneous sets were generally not functional. In some experiments, the p(−)RPV DICAT plasmid occasionally generated low (∼10 %) levels of CAT activity with some combinations such as the N protein of CDV and the P and L proteins of RPV (combination C + R + R), as reported in Fig. 2(b). However, it was not routinely possible to confirm this low-level activity in further separate experiments. When the mini-genomic construct p107MV(−) : CAT was used in combination with plasmids expressing the MV N and P proteins and the RPV L protein (e.g. M + M + R), CAT activity was consistently detected. This represented, respectively, 41 or 23 % of the control activity with the homogeneous sets of MV or RPV proteins (Fig. 2a). However, it was noted that the variation between triplicate determinations of these low activities was much larger than in the homogeneous sets of plasmids, i.e. M + M + M and R + R + R. We have observed that CAT activity is obtained over a very large concentration range of L plasmid (D. D. Brown and others, unpublished data). Hence, we reanalysed the activity generated by the M + M + R combination, not only at the standard concentration of L plasmid (400 ng) per transfection well but also at 10 ng and 1 μg plasmid. The results were 4 ± 1 % of control activity (100 % is the activity generated by the R + R + R set) at 10 ng plasmid; 27 ± 21 % at 400 ng plasmid and 46 ± 40 % at 1 μg plasmid. This confirmed both the substantial and as yet unexplained variation between triplicates specific to the assays using the M + R + R set and increasing activity with increasing L plasmid concentration.

The rescue of infectious virus from full-length cDNAs was carried out essentially as described by Baron & Barrett (1997) for RPV and by Gassen et al. (2000) for MV and CDV using the MVA-T7 system. Complementation analysis was performed for rescue of infectious virus with combinations of N, P and L protein-encoding plasmids. MVA-T7-infected HeLa cells for rescue of MV and CDV, and MVA-T7-infected Vero cells for RPV rescue, were transfected with the full-length anti-genomic plasmids and mixtures of the plasmids encoding the N, P and L proteins. There is no objective quantitative measure available for the rescue of infectious morbilliviruses. The numbers of primary syncitia in each of the six-well plates in which the transfections are carried out are too small and variable between various transfections to use them or titres of recovered p.f.u. as an accurate and reliable measure. Hence, the results were expressed by reporting the number of wells per six-well tray that gave primary syncitia and generated infectious virus after further passage (Table 1). The results confirmed that homogeneous combinations of plasmids allowed rescue of infectious MV, RPV and CDV. Whether the differences in the efficiency with which various sets of plasmids were able to rescue their homologous virus (e.g. rescue of CDV by C + C + C) compared with rescue of other morbilliviruses (e.g. rescue of RPV by C + C + C) are significant would require further experimentation. Even the heterogeneous sets with the highest activity in mini-genome rescue did not rescue infectious virus in six attempts.

Mini-genomic constructs of bovine respiratory syncytial virus replicate in cells infected with bovine, ovine or human respiratory syncytial viruses (Yunus et al., 1999). In contrast, human parainfluenza 3 (HPIV3) mini-genomes were not rescued by infection with bovine PIV3 (Dimock & Collins, 1993). Similar to the studies reported here, it has been shown that mini-genomic analogues of Sendai virus can be rescued by co-transfection with plasmids expressing proteins of HPIV3 (Pelet et al., 1996), but complementation analyses have not been reported.

CDV is very closely related to PDV. MV and RPV are highly related morbilliviruses with the second-highest levels of pair identity after CDV and PDV (Taubenberger et al., 2000). CDV diverges significantly from MV and RPV. However, differences in levels of similarity appear not to be significant in terms of the functional complementation analysis as, with one or possibly two exceptions, none of the heterologous mixtures of plasmids gave rise to significant levels of CAT expression. The failure to generate significant CAT activity in almost all heterogeneous sets involving proteins from two viruses made it unlikely that combinations in which all proteins were derived from different viruses would be functional in rescue.

Mini-genome assays measure the combined outcomes of (i) encapsidation of the T7 RNA polymerase transcript from the mini-genomic plasmid, (ii) subsequent replication of the mini-genomic analogue and (iii) its transcription as well as (iv) the translation of the mRNA into active enzyme. Successful replication is required for the generation of CAT activity in our assays, as it is not possible to rescue CAT...
Fig. 1. Construction of the monocistronic mini-genomic plasmid p(−)RPVDICAT. (a) The plasmid p(−)RPVDIEGFP(BB)DsRed1 was digested with NheI and MluI (marked in red) to remove the sequences encoding the two reporter genes and produce a linear vector. NCT, Non-coding terminus. (b) The CAT sequence was amplified from p107MV(−):CAT using the primers priCAT/NheI(+)+ and priCAT/MluI(-). Restriction sites engineered into the ends of the primers are boxed and marked in red and CAT ORF sequences are shown in yellow. (c) Structure of plasmid p(−)RPVDICAT. (d) Sequence analysis of p(−)RPVDICAT using priRPV/NarI(+)+ (5′-CCAGCGCCGGCCACAGGGCTGGGACCACATGGGCGGCGGACAGCGGCAAGCTGGGTGTAAG-3′) and priRPV/SapI(−) (5′-CGAGGAAAGGAAAGCTGAATACGACTCACTATAACCGAAGCTGGGGG-3′). The NheI site and MluI site used for cloning are boxed and the CAT initiation codon is underlined in green. The RPV 5′- and 3′-NCT and CAT sequences are marked with arrows and the CAT termination codon is underlined in red. (e) Amino acid sequence resulting from translation of the mutagenized CAT ORF shown at the NH2 and COOH termini and comparison with wild-type CAT. Mutagenized residues are marked in blue. Asterisks, end of EGFP at the TTA stop codon.
activity from MV or CDV plasmids that do not conform to the rule of six (Gassen et al., 2000) or have a mutation in the B box of the anti-genome promoter for replication (L. J. Rennick, W. P. Duprex & B. K. Rima, unpublished results). Thus, the assay reflects the combined replication and transcription efficiency and does not measure the contribution of each process separately. Hence, these data do not formally rule out the possibility that heterologous sets of plasmids may be able to replicate but not transcribe negative-sense mini-genome RNAs.

The lack of a reliable in vitro transcription system for any of the morbilliviruses makes it impossible to assess the activities of viral proteins directly other than in the assays used here. The lack of functional complementation between the N, P and L proteins suggests that they are tightly tuned, functional components in each of the viruses. In contrast, their interaction with genomic and anti-genomic promoters is unrestricted, since the MV, CDV and RPV helper plasmids are all able to rescue mini-genomic as well as full-length constructs of all three viruses. Bankamp et al. (2002) showed that L proteins from two attenuated viruses directed the production of up to eight times more reporter protein from a MV mini-genome than three wild-type L proteins and that this did not depend on whether the promoter sequences in the mini-genomes were derived from a wild-type or a vaccine strain.

Table 1. Rescue of infectious virus from plasmids p(+)MV, p(+)CDV and p(+)RPV by different combinations of N, P and L proteins

Results are expressed as the fraction of the total number of monolayers (test wells) positive for virus rescue in a transfection assay. ND, Not determined.

<table>
<thead>
<tr>
<th>Source of plasmids (N + P + L)</th>
<th>Rescued virus</th>
<th>(N + P + L)</th>
<th>(N + P + L)</th>
<th>(N + P + L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV + MV + MV</td>
<td>p(+)MV</td>
<td>11/12</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>MV + MV, no L</td>
<td>p(+)CDV</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CDV + CDV + CDV</td>
<td>p(+)RPV</td>
<td>6/6</td>
<td>6/6</td>
<td>2/6</td>
</tr>
<tr>
<td>CDV + CDV, no L</td>
<td></td>
<td>0/6</td>
<td>0/3</td>
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<td>6/6</td>
</tr>
<tr>
<td>RPV + RPV, no L</td>
<td></td>
<td>0/6</td>
<td>ND</td>
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<tr>
<td>MV + MV + RPV</td>
<td></td>
<td>0/6</td>
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</tbody>
</table>

Fig. 2. Functional assessment of morbillivirus N, P and L expression plasmids in a CAT assay using p107MV(−): CAT and p(−)RPVDICAT. HeLa cells were infected with MVA-T7 and transfected with the mini-genomic constructs and the three helper plasmids. The means and standard deviations of three independent transfection experiments are given. (a) Histogram representing the c.p.m. resulting from CAT assays set up using lysates from cells transfected with p107MV(−): CAT and all possible combinations of MV, CDVOP and RPV N, P and L expression plasmids. (b) Histogram representing the c.p.m. resulting from CAT assays set up using lysates from cells transfected with p(−)RPVDICAT and all possible combinations of MV, CDVOP and RPV N, P and L expression plasmids. M, MV Edmonston strain; R, RPV; C, CDVOP; −, no plasmid included.
Both mini-genomic reporter and full-length genomic constructs were rescued with homogeneous sets of proteins. The M + M + R combination was the exception as it showed low levels of activity in the rescue of the RPV mini-genome but did not rescue infectious virus in six attempts. There is functionally a significant difference between the two types of rescue events. Mini-genomic reporter rescue depends on a continual supply of proteins generated from the helper plasmids, whereas the rescue of genomic constructs only requires an initiation event by the helper plasmids. In the latter, once a single RNA transcript is encapsidated, replicated and transcribed, the system generates a self-sustaining RNP. Its replication may be more sensitive to dominant-negative effects of plasmid-derived homologous viral proteins due to, for example, the generation of non-physiological levels of N protein, which may compete with the N protein generated from the rescued genome for binding to the P, L and M (and V and C) proteins and interfere with the balance of replication and transcription. Hence, rescue of the full-length genome of one morbillivirus may be more efficient using a set of N, P and L plasmids from another morbillivirus than by its own complement. We have limited evidence to support this as in MV rescue primary syncytia were generated earlier and in higher numbers with the R + R + R plasmids than with the M + M + M set. It is difficult to model outcomes since a large number of protein–protein interactions are involved, including: oligomerization of P to make P3 (or P4); interaction of P3 with N0 (the soluble form of N not in the RNP) to solubilize N0–P3; interaction of N0–P3 with RNA to make RNP; interactions between P3 and L to make P3–L and for the latter to attach to the RNP (Karlin et al., 2003). At present we do not have an in vitro assay systems for quantitative evaluation of these interactions.

In summary, we have shown that morbillviruses, either as whole genome or mini-genomic reporter gene constructs, are rescuable by homogeneous sets of plasmid-generated helper proteins from other morbillviruses, but that heterogeneous sets, with one exception, do not perform well.

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**References**


