Features of the mammalian orthoreovirus Dearing L1 single-stranded RNA that direct packaging and serotype restriction

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INTRODUCTION

The family Reoviridae is only now revealing the mechanisms that it uses to replicate, assemble and preserve its segmented double-stranded (ds) RNA genome. The family contains more than 75 member species, all with genomes segmented double-stranded (ds) RNA genome. The family is used throughout to refer to the ss mRNA of the gene (l1 ssRNA); upper case denotes the ds form of the gene (L1 dsRNA).

METHODS

Viruses and cell lines. Mammalian orthoreovirus 1 Lang (MRV-1La), mammalian orthoreovirus 2 D5/Jones (MRV-2Jo) and mammalian orthoreovirus 3 Dearing (MRV-3De) were used (Ramer et al., 1977). Recombinant viruses containing the chloramphenicol acetyltransferase (CAT) gene were grown in L929 cells transformed with pDNA1-neo (Gunning et al., 1987), which contain the MRV-3De S2 (L-ST3.S2) (Roner & Roehr, 2006), the MRV-3De M1 cDNA (L-ST3.M1) (Roner & Steele, 2007) or the MRV-3De L1 cDNA under the control of the human β-actin promoter. L-ST3.L1 cells express protein L3, which is sufficient to rescue the temperature-sensitive (ts) mutant (tsD) (Ikegami & Gomatos, 1972; Coombs, 2006), an MRV-3De mutant with a ts mutation in the L1 genome segment. Lower case is used throughout to refer to the ss mRNA of the gene (l1 ssRNA); upper case denotes the ds form of the gene (L1 dsRNA).

MRV reverse-genetics system. The system was used as described previously (Roner et al., 1990, 2004; Roner & Joklik, 2001; Roner & Roehr, 2006; Roner & Steele, 2007). MRV-3De capped and methylated mRNA (referred to as ssRNA) was transcribed by cores (Skehel & Joklik, 1969). After transcription, the cores were pelleted at 10,000 g; the supernatant, which contained the ssRNA, was modified by the addition of 0.5% SDS and extracted three times with phenol/

A series of recombinant mammalian orthoreoviruses (mammalian orthoreovirus 3 Dearing, MRV-3De) were generated that express an MRV-3De l1–3–CAT fusion protein. Individual viruses contain L1CAT double-stranded (ds) RNAs that range in length from a minimum of 1020 bp to 4616 bp. The engineered dsRNAs were generated from in vitro-transcribed single-stranded (ss) RNAs and incorporated into infectious virus particles by using reverse genetics. In addition to defining the sequences required for these ssRNAs to be 'identified' as l1 ssRNAs, the individual nucleotides in these regions that 'mark' each ssRNA as originating from mammalian orthoreovirus 1 Lang (MRV-1La), mammalian orthoreovirus 2 D5/Jones (MRV-2Jo) or MRV-3De have been identified. A C at position 81 in the MRV-1La 5′ 129 nt sequence was able to be replaced with a U, as normally present in MRV-De; this toggled the activity of the MRV-1La ssRNA to that of an MRV-3De 5′ l1. RNA secondary-structure predictions for the 5′ 129 nt of both the biologically active MRV-3De l1 ssRNA and the U81–MRV-3De–restored MRV-1La 5′ ssRNA predicted a common structure.

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Introduction

The family Reoviridae is only now revealing the mechanisms that it uses to replicate, assemble and preserve its segmented double-stranded (ds) RNA genome. The family contains more than 75 member species, all with genomes containing 10, 11 or 12 dsRNA segments (Spence et al., 1984; Ramig & Ward, 1991; Urbano & Urbano, 1994; Patton & Spencer, 2000). A reverse-genetics system exists for two members, mammalian orthoreovirus (Roner & Joklik, 2001; Roner et al., 2004; Roner & Roehr, 2006; Kobayashi et al., 2007) and rotavirus (Komoto et al., 2006), and the foundation for a system exists for bluetongue virus (Boyce & Roy, 2007). The human viruses can be grouped into three serotypes that possess homologous genome-set segments. It is not only the sequences of the genome segments encoding the proteins that possess type-specific epitopes that differ significantly, but also the sequences of the other nine genome segments. In cells infected simultaneously with wild-type (wt) viruses of any two of the three serotypes, up to 15% of the progeny are intertypic reassortants that contain all possible combinations of parental genome segments. It turns out, however, that not all genome segments in reassortants are wt (Roner et al., 1995). We present our findings for the l1 single-stranded (ss) RNA and a series of chimeric l1–m1 and l1–s2 ssRNAs, offer a model for the molecular basis for serotype preference and genome selection during reassortment and demonstrate that the nucleotides used to identify individual genes and maintain serotypes are localized to the 5′ termini of the ssRNAs.

Methods

Viruses and cell lines. Mammalian orthoreovirus 1 Lang (MRV-1La), mammalian orthoreovirus 2 D5/Jones (MRV-2Jo) and mammalian orthoreovirus 3 Dearing (MRV-3De) were used (Ramer et al., 1977). Recombinant viruses containing the chloramphenicol acetyltransferase (CAT) gene were grown in L929 cells transformed with pH/AfAPr1-neo (Gunning et al., 1987), which contain the MRV-3De S2 (L-ST3.S2) (Roner & Roehr, 2006), the MRV-3De M1 cDNA (L-ST3.M1) (Roner & Steele, 2007) or the MRV-3De L1 cDNA under the control of the human β-actin promoter. L-ST3.L1 cells express protein L3, which is sufficient to rescue the temperature-sensitive (ts) mutant (tsD) (Ikegami & Gomatos, 1972; Coombs, 2006), an MRV-3De mutant with a ts mutation in the L1 genome segment. Lower case is used throughout to refer to the ss mRNA of the gene (l1 ssRNA); upper case denotes the ds form of the gene (L1 dsRNA).

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A supplementary table showing the location and identity of differences in MRV l1 ssRNAs in the 5′ 129 nt is available with the online version of this paper.
chloroform. The RNA was precipitated with polyethylene glycol, reextracted three times with phenol/chloroform and precipitated with 2.5 M ammonium acetate and ethanol. ssRNA prepared in this manner contained no residual infectious virus. For all lipofections, we used 10 μl rabbit reticulocyte lysate (Promega catalogue no. L4960) primed with 0.3–0.5 μg MRV-3De acceptor ssRNA and 0.1 μg of the indicated s2, m1 or l1 chimeric ssRNA [obtained from in vitro transcription using T7 RNA polymerase and the indicated cDNA template (Promega RibomAX-T7) and 12 units Rnasin Plus RNase inhibitor (Promega)] in 1 μl H2O. Translation was allowed to proceed for 1 h at 30 °C. After translation, an additional 0.3–0.5 μg MRV-3De ssRNA in 1 μl H2O was added and the mixture was added immediately to 0.5 ml minimal essential medium (MEM) containing 100 units penicillin ml−1, 100 μg streptomycin ml−1 and 50 μl Lipofectin or Lipofectamine (Invitrogen). This mixture was added immediately to PBS-washed monolayers of 10⁷ cells (L-ST3.L1, L-ST3.M1 or L-ST3.S2) in six-well multiplates. After 6 h, this mixture was replaced with 0.25 ml MEM containing 4 × 10⁷ p.f.u. MRV-2Jo and, 1 h later, with 1.75 ml MEM containing 5% fetal bovine serum. After 24 h, the cells were harvested, washed twice in MEM and sonicated in 2 ml MEM, and virus in the sonicates was titrated on monolayers of the same cell line. To avoid detection of the MRV-2Jo helper virus, plaques were counted and selected on day 5.

Removal of wt l1, m1 or s2 ssRNAs to create the accepter ssRNA sets. wt ssRNAs were removed from the mixture of ten ssRNA species as described previously (Roner et al., 2001). Briefly, the DNA oligonucleotide (10 pmol) complementary to nt 937–949 for s2, nt 521–532 for m1 or nt 1700–1681 for l1 was added to 2 pmol of each ssRNA. After hybridization, the mixture was treated with RNase H for 20 min. Degradation of the targeted ssRNAs was confirmed by gel electrophoresis of both the RNA and its translation products.

Engineering of MRV L1, M1 and S2 cDNAs used to generate the engineered ssRNAs. As described previously, we can incorporate an engineered s2 ssRNA into the MRV genome as a stable dsRNA (Roner & Joklik, 2001; Roner et al., 2004; Roner & Roehr, 2006), and an engineered m1 as well as s2/m1 chimeric ssRNAs (Roner & Steele, 2007). We used the same procedure to generate the L1CAT cDNA template. For the L1 cDNA template, 555 nt from the wt L1 gene precede the CAT gene, and 1152 nt from the wt gene follow the CAT template. Transcription by T7 RNA polymerase is terminated with the T7 terminator sequence, located 3' of the ssRNA. This template is transcribed by T7 RNA polymerase to yield an RNA transcript that possesses 5'- and 3'-terminal sequences as authentic L1 ssRNA. Transcription by T7 RNA polymerase is terminated with the T7 terminator sequence, located 3' of this construct. Transcription of this construct yielded an RNA that contained the 5' 555 nt of L1 RNA fused in frame to the CAT mRNA sequence, followed by the 3'-terminal 1152 nt of L1 RNA. This was achieved by inserting the hepatitis delta virus (HDV) ribozyme sequence in such a way that, when the ribozyme underwent autocleavage, it left a terminal C at the 3' terminus: -UCAU. Recloning and subsequent sequencing and cleavage analysis confirmed the authenticity of the 5'- and 3'-terminal sequences. This cDNA template, pL1CAT2459, is summarized in Fig. 1.

The pL1CAT2459 construct was transcribed in vitro by using T7 RNA polymerase and the transcript was capped by using m' GpppG (Promega) to yield s2-CAT mRNA. It was translated in vitro by using a rabbit reticulocyte lysate system (Promega) and the lysate was found to contain CAT activity (CAT-ELISA; Boehringer Mannheim). This ssRNA was used to supplement the acceptor ssRNA sets and lipofected into cells to generate virus.

Mutagenesis of 5’ and 3’ l1 sequences flanking the CAT gene in construct pL1CAT2459. Sequential deletion and mutagenesis of the 5’ and 3’ L1CAT-flanking sequences were carried out by using GeneEditor (Promega catalogue no. Q9280). As directed by the manufacturer, we annealed the selection oligonucleotide to our pL1CAT2459 dsDNA template at the same time as a mutagenic oligonucleotide. The mutagenic oligonucleotides that we selected were all ≥50 nt in length, i.e. 25 nt matching the L1 and/or CAT nucleotide sequence, depending upon the location of the sequence that we wished to retain, and 25 nt matching the HDV ribozyme nucleotide sequence. By using 25 perfectly matched nucleotides on each side of the mismatched sequence that we wished to loop out and remove, we were able to remove nucleotides from the original pL1CAT2459 sequence. We initially made large 100 or 200 nt deletions and narrowed down the biologically active sequence by using single nucleotide deletions. The results are summarized in Table 1 and Fig. 2. Additionally, we expanded the 3’ sequence to include the complete L1 3’ sequence minus the 5’ 129 nt, creating pL1CAT4616 (outlined in Fig. 3).

Construction of chimeric cDNA templates. As outlined in Table 2, the cDNA template ⁵L1-CAT-L1⁻⁵ is identical to the pL1CAT1020 template, the ⁵M1-CAT-M1⁻⁵ DNA is identical to the pMICAT1048
Virus titration/determination of CAT activity. Monolayers of lipofected and infected L-ST3.L1, L-ST3.M1 or L-ST3.S2 cells were incubated at 37 °C for 5 days. Neutral red was added 24 h before counting plaques (Roner & Joklik, 2001; Roner et al., 2004; Roner & Roehr, 2006). CAT activity in cell lysates was assayed by using a CAT ELISA (Boehringer Mannheim). CAT activity is low, as it is expressed as a l3–CAT, m2–CAT or s2–CAT fusion protein. The engineered dsRNAs of all recombinant viruses were sequenced to confirm the presence of the indicated genome segment and its exact nucleotide sequence.

Detection of MRV l1, m1 and s2 ssRNAs in vivo. Twelve hours following lipofection of L929, L-ST3.L1, L-ST3.M1 or L-ST3.S2 cells, total RNA was extracted from cell monolayers by using a Perfect RNA Eukaryotic Mini kit (Eppendorf) and the manufacturer’s protocols. The ssRNA was electrophoresed in a formaldehyde denaturing gel by using a NorthernMax kit (Ambion). Following the manufacturer’s protocol (Ambion), the ssRNA was transferred to a BrightStar-Plus positively charged nylon membrane and UV cross-linked. Hybridization and detection were carried out at 40 °C according to}

### Table 1. Minimal flanking sequences of the l1CAT ssRNA

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<th>dsRNA detected</th>
<th>Engineered RNA incorporated into infectious virus</th>
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the manufacturer’s directions by using ULTRAhyb buffer and $^{32}$P-labelled oligonucleotides. For detecting the MRV-3De l1 ssRNA, the oligonucleotide (L1.5) 5'-CAAACACGTCATTCGATTGATCAGTG-ATA-3', complementary to nt 100–72 near the 5' end and 28% mismatched with the undetected MRV-2Jo l1 ssRNA, was used. For the MRV-3De m1 ssRNA, oligonucleotide (M1.5) 5'-GAACGACATTCGCGTCAGCCCCAGCGTCTACTCCAAACG-3', complementary to nt 120–81 and 42% mismatched with the undetected MRV-2Jo m1 ssRNA, and for the MRV-3De s2 ssRNA, oligonucleotide (S2.5) 5'-CAAACACGACGTTCAGCCCAGCGTCTACTCCAAACG-3', complementary to nt 55–95 and 19.5% mismatched with the undetected MRV-2Jo s2 ssRNA, were used. For detecting the l1 CAT, m1 CAT or s2 CAT ssRNA, the oligonucleotide (CAT.1) 5'-TTTACGATGCCATTGGGATATATCGGTGGTATATCC-3', complementary to the CAT gene, was used. The membrane was exposed to X-ray film.

**Detection of MRV L1CAT, M1CAT or S2CAT dsRNA in vivo.**

Twelve hours following lipofection of L-ST3.L1, L-ST3.M1 or L-ST3.S2 cells with the indicated ssRNAs and protein translation mixture and infection with MRV-2Jo helper virus, total cell monolayers were harvested. The dsRNA was electrophoresed in SDS-PAGE gels (7.5% acrylamide) for 2650 V h as described by Moody & Joklik (1989). Following the protocol used for the ssRNA gels, the dsRNA was transferred to a BrightStar-Plus positively charged nylon membrane (Ambion) and UV cross-linked. Hybridization and detection were carried out at 40 °C according to the manufacturer’s directions (Ambion), using ULTRAhyb buffer and
32P-labelled oligonucleotides. For detecting the L1CAT, m1CAT or s2CAT ssRNA, the oligonucleotide CAT.1 (Roner et al., 2004), complementary to the CAT gene, was used. The membrane was exposed to X-ray film.

**RESULTS**

**Generation of an L1CAT MRV**

By using the cDNA template pL1CAT2459 (Fig. 1), we have transcribed an ssRNA and incorporated it successfully into a stable MRV (Fig. 1). The L1CAT viruses represent approximately 8% of the viruses generated following lipofection of the L1CAT ssRNA, together with a set of nine wt serotype 3, strain Dearing (ST3D) ssRNAs. The remaining 92% contain the wt ST3D L1 gene. The source of the wt L1 gene is the l1 ssRNA, which is not removed following cleavage treatment with an L1 antisense oligo and RNase H treatment. The yield of S2CAT virus by using this procedure is approximately 15% (Roner et al., 2004; Roner & Roehr, 2006) and, for the generation of an M1CAT virus, the yield is 10% (Roner & Steele, 2007). The primary reasons for these differences in efficiency are associated with the removal of the wt ssRNAs by using an oligo and RNase H treatment. Removal of the s2 ssRNA is more efficient than removal of the m1 ssRNA, which is itself more efficient than removal of the l1 ssRNA, using the same methods. This is probably due to intrinsic properties of the oligos selected and, possibly, ssRNA secondary structures at or near the oligo-binding sites. As was done for the oligo used to remove the s2 ssRNA, the oligos selected for the m1 and l1 ssRNAs were based on efficient cleavage of the targeted ssRNAs, with only minimal cleavage of the remaining nine ssRNAs. The efficiency in generating an L1CAT and M1CAT MRV is still high enough to allow isolation of all engineered viruses.

The l1 ssRNA produced from the cDNA template pL1CAT2459 is 2459 bp in length. As shown in the autoradiogram in Fig. 1, when this ssRNA is replicated to dsRNA and incorporated into a virus, it has a migration rate similar to those of the ST3D M1/M2 dsRNAs at 2304 and 2203 bp. Sequencing the L1CAT2459 dsRNA confirms that the RNA is as engineered, with a length of 2459 bp.

By using the methods that we described for the s2 ssRNA (Roner et al., 2004; Roner & Roehr, 2006), we have used the cDNA pL1CAT2459 to find the minimal 5' and 3' flanking sequences. We were able to reduce the L1CAT ssRNA from 2459 to 1020 nt and to produce an MRV containing this short l1 ssRNA. In Fig. 1, the genome segments of this virus (ST3D with L1CAT1020) are shown. This virus was produced by reducing the 5' L1 of pM1CAT2459 from 555 to 129 nt, and the 3' length from 1152 to 139 nt. These 200 nt were sequenced by using Moloney murine leukemia virus reverse transcriptase (RT) (Promega) and a complementary primer, and the 3' ends were first poly(A)-tailed by using yeast poly(A) polymerase (TaKaRa Mirus Bio), then sequenced by using RT and an oligo(T) primer, as described previously (Wiener et al., 1989; Roner & Joklik, 2001; Roner et al., 2004). Following purification, all recombinant viruses were propagated and the engineered dsRNA genome segments were sequenced directly by using RT, as described previously (Wiener et al., 1989).
deletions are summarized in Table 1 and the in vivo activity of the created ssRNAs is shown in Fig. 2.

Generation of an L1CAT MRV with an L1 gene increased from 3854 to 4616 nt

We were interested in the possibility of increasing the genome size of MRV. To explore this possibility, we engineered an L1CAT MRV, L1CAT4616, that contained the minimal 5’ 129 nt that we had identified and a 3’ region consisting of the remaining 3735 nt of the wt MRV-3De L1 gene. This cDNA template yields an ssRNA of 4616 nt upon transcription. This cDNA and virus are summarized in Fig. 3 and included in Table 1, and the in vivo activity of the created ssRNAs is shown in Fig. 2.

Activity of ST3 chimeric ssRNAs, using single gene knockout acceptor RNA sets

We created chimeric ssRNAs by using the minimum and extended 5’ and 3’ sequences identified in this report for the l1 ssRNA in combination with 5’ and 3’ sequences from either the s2 or m1 ssRNAs, all flanking the CAT gene. The chimeric ssRNAs $^{5}$L1-CAT-M1$^{3}$, $^{5}$L1-CAT-S2$^{3}$, $^{5}$M1-CAT-L1$^{3}$ and $^{5}$S2-CAT-L1$^{3}$ are only slightly different in length from the starting ssRNA, $^{5}$L1-CAT-L1$^{3}$.

Two additional chimeric ssRNAs were created to explore genome size. The ssRNAs transcribed from cDNA templates $^{5}$M1-CAT-L1$^{+}$ and $^{5}$S2-CAT-L1$^{+}$ contain the extended 3’ 3735 nt, preceding the CAT gene. The ssRNAs generated are much larger than the predicted m1 and s2 wt ssRNAs. The structure of the ssRNAs generated in this report, as well as those of four ssRNAs from previous work, included for comparison, are outlined in Table 2. Fig. 4 summarizes the activity of these ssRNAs.

We found that the chimeric ssRNAs $^{5}$L1-CAT-M1$^{3}$ and $^{5}$L1-CAT-S2$^{3}$ were incorporated into an infectious MRV in place of the L1 dsRNA, but never in place of the M1 or S2 dsRNAs, as was reported for the M1/S2 chimeric ssRNAs (Roner & Steele, 2007). The chimeric ssRNA $^{5}$M1-CAT-L1$^{3}$ was incorporated into an infectious MRV in place of the M1 dsRNA and the $^{5}$S2-CAT-L1$^{3}$ in place of the S2 dsRNA, but neither in place of the L1 dsRNAs, again as has been reported for the M1/S2 chimeric ssRNAs (Roner & Steele, 2007).

The remaining two chimeric ssRNAs, $^{5}$M1-CAT-L1$^{+}$ and $^{5}$S2-CAT-L1$^{+}$, were expected to follow the same rules that we have discovered and to be replicated to dsRNA and incorporated into viruses according to the origin of their 5’ leader sequences. The $^{5}$L1-CAT-L1$^{+}$ ssRNA, which, at 4616 nt, is 762 nt longer than the wt L1 at 3854 nt, and the $^{5}$M1-CAT-L1$^{+}$ at 4611 nt, i.e. more than twice the length of the wt M1 at 2304 nt, are both biologically inactive in our system. The $^{5}$M1-CAT-L1$^{+}$ ssRNA is not replicated to dsRNA or incorporated into virus. The same was found for $^{5}$S2-CAT-L1$^{+}$, which, at 3831 nt, is almost three times the length of the wt S2 at 1331 nt. The $^{5}$L1-CAT-L1$^{+}$ ssRNA demonstrates that some flexibility exists for increasing the size of individual MRV ssRNAs and their genomic dsRNAs, but size limitations do appear to exist.

Activity of ST3\ST2\ST1 chimeric ssRNAs

Having demonstrated that sequences present at the 5’ end of a MRV ssRNA are used to identify a genome segment,
we wished to explore whether this identification also extended to serotypes. To accomplish this, we replaced the 5′ and 3′ 129 and 139 nt of the 5′L1-CAT-L13′ cDNA template with either 129 nt (5′L1) or 139 nt (3′L1) present in the l1 ssRNAs of either MRV-1La or MRV-2Jo. The results are summarized in Table 3 and the in vivo activity of the ssRNAs is shown in Fig. 4. The ssRNAs with the substituted 3′ sequences, 5′L1ST3-CAT-L1ST1′ and 5′L1ST2-CAT-L1ST1′, are active and identified as ST3 l1 ssRNAs. The ssRNAs with the substituted 5′ sequences, 5′L1ST1-CAT-L1ST3′ and 5′L1ST2-CAT-L1ST3′, are inactive and no dsRNA or virus has ever been detected.

**Presence of ‘serotype signals’ in MRV ssRNAs**

We wished to explore the molecular basis for the inactivity of the chimeric ssRNAs 5′L1ST1-CAT-L1ST3′ and 5′L1ST2-CAT-L1ST3′. An examination of the 5′ 129 nt of the l1

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**Fig. 4.** (a) Incorporation of chimeric MRV-3De ssRNA into reovirus. ssRNAs were generated from the chimeric ssRNAs shown in Table 2. The ssRNA (in the top panel) and the CAT dsRNA (in the middle panel) are shown, using Northern blots analysing RNA extracted from cells lipofected 12 h earlier. The bottom panel is an autoradiogram following SDS-PAGE generated by in vivo labelling with 32P of the dsRNA genome segments of an isolated progeny virus. (b) Incorporation of chimeric MRV-3De, MRV-2Jo and MRV-1La ssRNA into reovirus. Detection of virus-generation intermediates using the ssRNAs generated from the chimeric ssRNAs shown in Table 3. The ssRNA (in the top panel) and the CAT dsRNA (in the middle panel) are shown, using Northern blots analysing RNA extracted from cells lipofected 12 h earlier. The bottom panel is an autoradiogram following SDS-PAGE generated by in vivo labelling with 32P of the dsRNA genome segments of an isolated progeny virus. The table indicates the nucleotide differences in the 5′ 129 nt between MRV-3De and MRV-1La l1 ssRNAs.
ssRNAs of MRV-1La, MRV-2Jo and MRV-3De revealed three differences between MRV-3De and MRV-1La and 20 differences between MRV-3De and MRV-2Jo (see Supplementary Table S1, available in JGV Online) (Wiener & Joklik, 1989). We explored the impact of replacing, individually and in groups of two, the MRV-1La nucleotides of the ssRNA L1ST1(U81)-CAT-L1ST3 with those present in the MRV-3De ssRNA. The results are summarized in Table 3 and the in vivo activity of the created ssRNAs is shown in Fig. 4. We discovered that the presence of a C at position 81 in the ST1 5’–129 nt sequence is associated with the inactivity of this ssRNA, and replacement with a U, as present in MRV-3De, restores the activity so that the ssRNA L1ST1(U81)-CAT-L1ST3 is readily replicated to dsRNA and incorporated into an MRV-3De virus. Replacement of either of the other two nucleotides, L1ST1(G46)-CAT-L1ST3 or L1ST1(G60)-CAT-L1ST3, or any combination of two replacements, L1ST1(G46,G60)-CAT-L1ST3, L1ST1(G60,U81)-CAT-L1ST3 or L1ST1(G46,G60,U81)-CAT-L1ST3, all failed to restore activity, i.e. to identify the ssRNA as MRV-3De.

**Predicted secondary structures associated with activity of the chimeric ssRNAs**

The software program RNAStructure (version 4.5) (Mathews et al., 2004) was used to predict the secondary structure of the chimeric ssRNAs. As can be seen in Fig. 5, the termini of the active ssRNAs, L1ST3-CAT-L1ST3 and L1ST1(U81)-CAT-L1ST3, are very similar and are predicted to possess three nearly identical loops in the region of nt 40–80. As can be seen for the structures predicted for the remaining five inactive ssRNAs, these loops are not expected to be present.

**Table 3.** Activity of MRV-3De, MRV-2Jo and MRV-1La chimeric l1 ssRNAs

<table>
<thead>
<tr>
<th>Template</th>
<th>Length of ssRNA (nt)</th>
<th>CAT</th>
<th>Total length of engineered ssRNA (nt)</th>
<th>Engineered ssRNA incorporated as dsRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’</td>
<td>3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST1</td>
<td>ST2</td>
<td>ST3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1ST3-CAT-L1ST3</td>
<td>129</td>
<td>139</td>
<td>752</td>
<td>1020</td>
</tr>
<tr>
<td>L1ST3-CAT-L1ST3</td>
<td>129</td>
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<td>139</td>
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<td>1020</td>
</tr>
</tbody>
</table>

**DISCUSSION**

This work expands the MRV reverse-genetics system to include genes from each of the three genome size classes. The 5’ and 3’ regions required to direct a large (l1) ssRNA to be incorporated into the MRV genome have now been identified. With this accomplished, the MRV genome can, without delay, be engineered to accept 4348 bp of foreign information in place of the L1 gene, 2008 bp in place of the M1 gene and 1137 bp in place of the S2 gene. The yield of engineered MRV produced from in vitro-generated ssRNAs decreases as the length of the ssRNA increases. During replacement of the S2 gene, 15% of the progeny viruses contained the engineered S2 genome segment. The remaining 85% contained the wt S2 genome segment, the source of which is the wt s2 ssRNA that is not completely removed during RNase H treatment. Following replacement of the M1 gene, the yield of engineered viruses is reduced to 10% and, for the L1 gene, the yield is reduced further to 8%.

With the current reverse-genetics system, the bottleneck for increasing these efficiencies lies with the degradation and subsequent removal of a wt ssRNA from the natural set of ten ssRNAs. This process is required to generate a set of nine ssRNAs into which the in vitro-generated ssRNA is readily incorporated. Two problems exist during this process: first, the oligo(s) selected for this process must be present at a ratio of approximately 10:1 to degrade the targeted ssRNA. Second, some of the targeted ssRNAs remains under these conditions, but, as the ratio is increased, non-targeted ssRNAs begin to become targets and are degraded. The conditions are a compromise between the amount of oligo used and the impact on non-target RNA.

We found that the lengths of the 5’ ends of the MRV ssRNAs required both for replication to dsRNA and for
subsequent incorporation into progeny virus are somewhat linked to the length of the gene that they originally flanked. The l1 ssRNA is 3854 nt long, with 5' and 3' flanking regions of 129 and 139 nt, respectively. The s2 ssRNA, at 1331 nt, has the shortest 5' and 3' required sequences, at 96 and 98 nt, respectively. The m1 ssRNA provides mixed results: the 5' required sequence of 124 nt is intermediate between the l1 and s2 ssRNAs, but the 3' required sequence, at 173 nt, is the longest required sequence that we found among all three ssRNAs. An l1 129 nt 5' sequence was able to direct an ssRNA (5'-L1-CAT-L1-3') to incorporation into a progeny virus as an L1 dsRNA. Similarly, an m1 124 nt 5' sequence was able to direct an ssRNA (5'-M1-CAT-S2-3') of a total of 974 nt to incorporation into a progeny virus as an M1 dsRNA. Finally, an s2 96 nt 5' sequence was able to direct an ssRNA (5'-S2-CAT-L1-3') of a total of 987 nt to incorporation into a progeny virus as an S2 dsRNA. The three engineered ssRNAs vary in length by only 13 nt, i.e. 987, 979 or 974 nt, with the longest (5'-S2-CAT-L1-3') flanked by the shortest 5' sequence; quality, not quantity, is used to identify the MRV ssRNAs.

MRV appears much less sensitive to reductions in the overall length of individual genes than it is to increases in length. It is possible to reduce the length of the L1 from 3854 bp, the M1 from 2304 bp and the S2 from 1331 bp to approximately 980 bp (to 979, 974 and 987 bp, respectively). Attempts to increase the length of these genes were not as successful. The size of the L1 dsRNA was increased successfully from 3854 to 4616 bp (5'-L1-CAT-L1+3'). This resulted in an increase in total genome size of 762 bp.

Fig. 5. Predicted secondary structures of the l1 ssRNAs of MRV-3De and MRV-1La–MRV-3De substituted 5' 129 nt. The predicted RNAstructure fold of the terminal 129 nt for 5'-L1ST3-CAT-L1ST3-3' is shown at the top, with the biologically important region that we altered with site-directed mutagenesis enlarged and enclosed in a solid circle. For the remaining ssRNAs, only the same biologically important regions of the 129 nt folds are shown. Regions of active chimeric ssRNAs are enclosed by solid circles and regions of inactive chimeric ssRNAs by dotted circles.
Attempts to increase the M1 from 2304 to 4611 bp (5'M1-CAT-L1 + 3') and the S2 from 1331 to 3831 bp (5'S2-CAT-L1 + 3'), which would have required increases of 2307 and 2500 bp in the total size of each virus genome, were not successful. It seems reasonable that the volume of the MRV core particle is close to capacity when filled with the normal complement of ten dsRNAs and that viable viruses can be generated with less genetic material, but increases are restricted by the space available in the core particle. We were able to add 762 bp, but not 2307 bp. MRV cores are 70 nm in diameter and contain approximately 23 551 bp dsRNA (Tosteson et al., 1993; Damodaran et al., 2002; Chandran et al., 2003; Xu et al., 2004; Jiang & Coombs, 2005). Rotavirus cores are 60 nm, enclosing 18 556 bp (Mansell et al., 1994; Tanaka & Roy, 1994; Chen et al., 1999; Monnier et al., 2006) and bluetongue virus cores are 100 nm with 19 219 bp enclosed (Grimes et al., 1998; Stuart et al., 1998). The approximate concentration of dsRNA in each of these virus cores is 722 mg ml⁻¹ for MRV, 657 mg ml⁻¹ for rotavirus and 410 mg ml⁻¹ for bluetongue virus. Rotovirus is known to accept at least an extra 1000 nt. MRV is the most densely packed of these three dsRNA viruses and would probably be able to accept the lowest amount of extra dsRNA.

The chimeric ssRNAs 5'L1-CAT-M15', 5'L1-CAT-S25', 5'M1-CAT-L15' and 5'S2-CAT-L15' are only slightly different in length from the starting ssRNA, 5'L1-CAT-L15'. An engineered ssRNA with a 5' sequence identical to that of the wt 11 ssRNA, supported by a 3' sequence from either the 11, M1 or S2 ssRNA, is incorporated into a virus as an L1 dsRNA. The chimeric ssRNAs 5'L1-CAT-M15' and 5'L1-CAT-S25' were incorporated into an infectious MRV in place of the L1 dsRNA, but never in place of the M1 or S2 dsRNA. In contrast, the chimeric ssRNA 5'M1-CAT-L15' was incorporated into an infectious MRV in place of the M1 dsRNA and the 5'S2-CAT-L15' in place of the S2 dsRNA, but neither in place of the L1 dsRNA. The CAT chimeric viruses produce yields identical to those of the wt viruses, with no difference seen in plaque size (Roner & Steele, 2007). The possibility still exists that interactions between the 5'- and 3'-terminal sequences of these ssRNAs are important for virus replication. Our findings suggest that the 5' sequence acts independently of the 3' sequence in determining the identity of each ssRNA. An interaction between the 5' and 3' sequences has been demonstrated in rotavirus (Patton et al., 1996; Wenz et al., 1996; Barro et al., 2001; Chen et al., 2001; Patton, 2001; Tortorici et al., 2006), bluetongue virus (Markotte et al., 2004) and ϕ6 (Zhang et al., 1994; Dennehy & Turner, 2004; Poranen & Tuma, 2004). Similarly to those found in ϕ6, we have demonstrated that the MRV 11, M1 and S2 ssRNAs also contain essential regions within the 5'- and 3'-terminal sequences, although, in MRV, these regions lie within the coding sequences.

We have generated the cdNA templates and transcribed the ssRNAs corresponding to 5'M1-ORF-S25' or 5'S2-ORF-M15', with the ORF being from the M1 or S2 genes. We have never recovered a virus from our attempts. We offer two explanations: (i) the resulting fusion proteins, μ2-μ2 or μ2-σ2, may be inactive and not support virus growth; (ii) the redundant M1–M1 or S2–S2 sequence at the 5' end of these ssRNAs or the mixed S2–M1 or M1–S2 5' sequences of these ssRNAs may interfere with each other and prevent assortment/replication. We have mutated the ATG start codons to prevent the production of fusion proteins, but again, no viruses were produced. Mutation of the AUG start codons modifies the 5' leader sequences and this probably destroys the signals of the ssRNAs.

It is not possible to introduce MRV-3De genome segments into the MRV-1La genome by injecting either the ss or the ds forms of single MRV-3De genome segments into cells infected with MRV-1La by transfecting or lipofecting them, or by transcribing them intracellularly from appropriate plasmids (unpublished results). In contrast, the yields of virus in cells infected with mixtures of MRV particles belonging to two different serotypes contain roughly 15% reassortants that contain the genome segments of the two parents in an approximately Poisson distribution. Interestingly, when cores of the two virus serotypes are lipofected into cells (that is, under conditions when they possess the same specific infective as infectious virus particles), 10–15% of the progeny are again reassortants, but now they are all monoreassortants (Moody & Joklik, 1989). The overwhelming importance of sequence recognition in genome-segment reassortment, i.e. in the introduction of heterologous genome segments into genome-segment sets, is illustrated by examination of the sequences of the heterologous genome segments that are actually incorporated. The reassortants that were examined in this analysis were the result of MRV-3De × MRV-1La crosses (Roner et al., 1995). For the insertion of MRV-3De genome segments into the MRV-1La genome, the incoming MRV-3De genome segments are all mutants of their wt MRV-3De sequence. In each case, the effect of the mutation is to change a residue where the MRV-1La and MRV-3De genome segments differ to that of the MRV-1La genome segment – i.e. the effect is to render the incoming MRV-3De genome segment more similar to the MRV-1La genome segment that it replaces. To examine this, we created the chimeric ssRNAs 5'L15,1ST1-CAT-L15,1ST3 3' and 5'L1ST2-CAT-L1ST3 3'. Based on work that we have described with intact viruses (Roner et al., 1995), we predicted that these ssRNAs would not be incorporated efficiently into an MRV-3De set of nine ssRNAs in place of the deleted 11 ssRNA. Upon screening more than 2000 viruses, generated from four individual lipofections, we were unable to recover a virus containing our engineered L1 RNA. An examination of the 5' 129 nt of the L1 ssRNAs of MRV-1La, MRV-2Jo and MRV-3De revealed three differences between MRV-3De and MRV-1La and 20 differences between MRV-3De and MRV-2Jo (Wiener & Joklik, 1989). We were able to replace a C at position 81 in the ST1 5' 129 nt sequence with a U, as present in MRV-3De, and to restore the activity of the ssRNA...
In an effort to explore the molecular basis for these findings, we used the software RNAstructure to predict the secondary structure of the active and inactive serotype chimeric ssRNAs. Use of the program Mfold produces similar results, as does folding the entire 11 sequences and the 1ICAT chimeric ssRNAs. Secondary-structure predictions for the 5' 129 nt revealed similar structures for the active MRV-3De 11 ssRNA and the U81-MRV-3De-restored MRV-1La 5' ssRNA. The remaining five ssRNAs are predicted to possess a different structure and are inactive. We present the predicted ST3 and restored ST1 5' ssRNA and the U81-MRV-3De-1 ssRNA. Secondary-structure predictions for the 5' 129 nt revealed similar structures for the active MRV-3De 11 ssRNA and the U81-MRV-3De-restored MRV-1La 5' ssRNA. The remaining five ssRNAs are predicted to possess a different structure and are inactive. We present the predicted ST3 and restored ST1 5' ssRNA as the molecular basis for MRV-1La and MRV-3De 11 ssRNA assortment. We have demonstrated that the C at position 81 of the ST1 5' MRV-3De 1 ssRNA assortment. We have demonstrated inactive. We present the predicted ST3 and restored ST1 5' ssRNA and the U81-MRV-3De-1 ssRNA. Secondary-structure predictions for the 5' 129 nt revealed similar structures for the active MRV-3De 11 ssRNA and the U81-MRV-3De-restored MRV-1La 5' ssRNA. The remaining five ssRNAs are predicted to possess a different structure and are inactive. We present the predicted ST3 and restored ST1 5' ssRNA as the molecular basis for MRV-1La and MRV-3De 11 ssRNA assortment. We have demonstrated that the C at position 81 of the ST1 5' 129 nt is important in 'marking' this ssRNA as ST1, and we have presented evidence that secondary structure may be involved in this recognition. In summary, the current MRV system will allow researchers to engineer any MRV gene within the limits of required 5' and 3' sequence elements and allow individual genes to be mutated to explore protein function. Although we have used this genetics system exclusively to explore genome encapsidation in MRV, the 11 gene can be mutated readily by using this system to create non-lethal mutations that would not require a supporting cell line and lethal mutations requiring support, both powerful viruses with which to study 23 protein function.

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REFERENCES


