In vivo generation of hybrids between different species of RNA phages

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Hybrids between different species or genera of the single-stranded RNA coliphages have not been found in nature. Here, it has been shown that viable hybrids between different phage species can easily be generated in the laboratory by in vivo recombination. cDNA of species I phage MS2 located on a plasmid and lacking part of its 5′ untranslated leader (5′ UTR) was complemented with another plasmid carrying the 5′ half of the genome of fr, a species I phage, or of KU1, a species II representative with low sequence similarity. When the two plasmids were present in the same cell there was spontaneous production of hybrid phages. Interestingly, these hybrids did not arise by a double or single crossover that would replace the missing MS2 sequences with those of fr or KU1. Rather, hybrids arose by attaching the complete 5′ UTR of fr or KU1 to the 5′ terminus of the defective MS2 phage. Several elements of the 5′ UTR then occurred twice, one from KU1 (or fr) and the other from MS2. These redundant elements are in most cases deleted upon evolution of the hybrids. As a result, the 5′ UTR of KU1 (or fr) then replaced that of MS2. It was earlier shown that this 5′ UTR could assume two alternating structures that facilitated transient translation of the proximal maturation gene. Apparently, this timer function of the 5′ UTR was exchangeable and could function independently of the rest of the genome. When hybrids were competed against wild-type, they were quickly outgrown, probably explaining their absence from natural isolates.

Introduction

The (+) strand RNA coliphages like MS2 have a 3.5 kb genome encoding four proteins: maturation, coat, lysis and replicase (Fig. 1A). We have previously constructed a full-size plasmid-borne cDNA copy of phage MS2. Cells transformed with this plasmid produced spontaneously about 10^{11} p.f.u./ml following overnight growth in liquid culture (Olsthoorn et al., 1994). It is not known exactly which events lead to phage formation. Presumably, transcripts containing MS2 RNA produce replicase, which then synthesizes properly sized (+) and (−) strands from oddly sized transcripts (Shaklee et al., 1988; Biebricher & Luce, 1992). The presence of a promoter upstream of the MS2 cDNA is not required. Apparently, there is enough spurious transcription to provide the necessary RNA (see also Taniguchi et al., 1978).

Even though the infectious plasmid is maintained in F− cells, which cannot be reinfected, the construct is not easy to handle and is always at risk of suffering deletions that inactivate the phage (Olsthoorn, 1996). In an attempt to attenuate the virulence of the cDNA construct, we divided the MS2 information between two compatible plasmids. Indeed, recombination via the provided genetic overlap still produced phages but at an approximately 100-fold reduced titre. This two-plasmid system is more stable and more convenient for introducing mutations into the phage genome.

Another important potential application is the generation of hybrid phages when overlapping information derived from two different phages is divided between two plasmids. The overlap can provide an abundance of potential crossover points from which only those that lead to viable phages will survive by natural selection.

There are many reports showing the exchange of segments between eukaryotic RNA viruses (Masuta et al., 1998; Aaziz & Tepfer, 1999; Worobey et al., 1999; Yuan et al., 1999; Canto...
Fig. 1. (A) Genetic map of RNA coliphage MS2. (B) and (C) Equilibrium structures for the 5’ untranslated leader of MS2 and KU1, respectively. The start codon of the A-protein gene and base differences between MS2 and fr are boxed. UCS, upstream complementary sequence; SD, Shine–Dalgarno sequence; LDI, long-distance interaction. (D) and (E) Metastable folding intermediates for MS2, fr and KU1 allowing translation of the maturation gene. The genetic map of KU1 and fr is identical to that of MS2. It is not certain whether KU1 starts with two or three G residues.
Fig. 2. Schematic representation of cDNA constructs used for formation of hybrids between fr or KU1 (acceptors) and MS2 (donor). The MS2 donor plasmids carry the full genome except for the deletions of variable length at the 5′ end, as indicated. Acceptor plasmids contain phage sequences from the 5′ end to the nt indicated. pMS\(\Delta\)BA, pMS\(\Delta\)SA and pMS\(\Delta\)NA produce 3, 0–1 and 1 times as much maturation protein as wild-type (Groeneveld et al., 1995). None of these plasmids produce phages.

Methods

**Bacterial strains and plasmids.** E. coli F\(^{-}\) strain M5219 (M72 trpA\(_{219}\) lacZ\(_{405}\) Sm\(_{1}\) /2∆bio\(_{325}\) cl\(_{452}\) ΔH\(_{1}\)) encoding the thermosensitive λ repressor (\(cI_{434}\)) and the transcriptional anti-termination factor N (Remaut et al., 1981), was employed as a host for plasmids carrying phage cDNA under the control of the thermoinducible \(P_L\) promoter of phage λ. E. coli F\(^{+}\) KA797 (F\(\lambda\) lacI\(_Q\) pro ara Δlac–pro thi) (Miller et al., 1977) was used to evolve RNA phages. The strains were grown on LC broth containing per litre 10 g bactotrypton, 5 g yeast extract, 8 g NaCl, 2 g MgSO\(_4\) 140 mg thymine and 1 ml 1 M Tris–HCl pH 7.6.

Table 1. Plasmid combinations used for generation of hybrid phages

<table>
<thead>
<tr>
<th>Plasmid combination</th>
<th>Donor</th>
<th>Acceptor</th>
<th>Titre (p.f.u./ml)*</th>
<th>Recombinant phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMS(\Delta)BA</td>
<td>pUCMS2</td>
<td></td>
<td>(3 \times 10^9)</td>
<td>wild-type MS2</td>
</tr>
<tr>
<td>pMS(\Delta)SA</td>
<td>pUCMS2</td>
<td></td>
<td>(2 \times 10^9)</td>
<td>wild-type MS2</td>
</tr>
<tr>
<td>pMS(\Delta)NA</td>
<td>pUCMS2</td>
<td></td>
<td>(2 \times 10^9)</td>
<td>wild-type MS2</td>
</tr>
<tr>
<td>pMS(\Delta)BA</td>
<td>pFR400</td>
<td></td>
<td>(2 \times 10^4)</td>
<td>frms hybrid (frms3)</td>
</tr>
<tr>
<td>pMS(\Delta)SA</td>
<td>pFR400</td>
<td></td>
<td>(2 \times 10^4)</td>
<td>frms hybrid (frms3)</td>
</tr>
<tr>
<td>pMS(\Delta)NA</td>
<td>pFR400</td>
<td></td>
<td>(1 \times 10^4)</td>
<td>frms hybrid (frms3)</td>
</tr>
<tr>
<td>pMS(\Delta)BA</td>
<td>pKU8</td>
<td></td>
<td>(2 \times 10^2)</td>
<td>kums hybrid (kums4)</td>
</tr>
<tr>
<td>pMS(\Delta)SA</td>
<td>pKU8</td>
<td></td>
<td>(2 \times 10^2)</td>
<td>kums hybrid (kums4)</td>
</tr>
<tr>
<td>pMS(\Delta)NA</td>
<td>pKU8</td>
<td></td>
<td>(2 \times 10^2)</td>
<td>kums hybrid (kums4)</td>
</tr>
</tbody>
</table>

* Titres represent amount of phage after overnight growth in M5219 (F\(^{-}\)) cells. Once formed the hybrids grow to titres approximately as high as wild-type.
Fig. 3. Generation of KU1–MS2 hybrids (kumsBA). (A) The first two lines show the KU1 and MS2 regions present on the plasmids in the same cell. The boxes depict the various elements in the 5′ UTR. White boxes with black letters belong to MS2 and black boxes with white lettering to KU1. N, Northern hairpin; UCS, upstream complementary sequence; W, West arm; S, South arm; E, East arm; SD, Shine–Dalgarno sequence; fMet, start codon of the maturation gene. The dashed arrow shows the fusion site. The third line shows the first sequenced hybrid in schematic form (cycle 3). Line 4 shows schematically one of the evolved hybrids (kums4). (B) Proposed structures of the 5′ UTR in the initial hybrids. MS2 sequences are in normal letters, BCCG
Plasmids pFR741 and pFR400 are pUC9 derivatives with fr cDNA (1–741 or 1–400, respectively) positioned in reverse orientation behind the lac promoter. pKU8 is a pUC9 derivative carrying KU1 cDNA from 1 to 1572 (Groeneveld, 1997). pUCMS2 contains the MS2 sequence from 1 to 741 and has been described earlier (van Meerden et al., 2001). The plasmids carry the ampicillin-resistance marker.

Plasmid pMSABA, pMSASA and pMSANA (Groeneveld et al., 1995) carry the complete MS2 cDNA sequence with deletions from 1 to 112, 40 to 113 and 78 to 113, respectively. The MS2 cDNA, which is preceded by 11 G residues, is present just behind the thermoinducible P<sub>i</sub> promoter. The deletion mutants are derivatives of pMS2000. This plasmid contains the complete infectious MS2 cDNA and has been described by Olsdhoorn et al. (1994). pMS2000 and its derivatives confer kanamycin resistance.

### Results

#### Generating hybrid phages by in vivo recombination

In Fig. 2(B), we have shown three deletions introduced into the infectious MS2 cDNA clone. pMSABA miss the first 111 nucleotides, pMSASA carries a deletion from position 40 to 113 and pMSANA lacks the nucleotides 78 to 113. None of these constructs produced viable phages, presumably because transcription from the (−) strand is defective. However, when the missing wild-type sequence (1–741) was provided on a second plasmid (pUCMS2, Fig. 2A), wild-type MS2 was obtained at a high titre (≈ 10<sup>8</sup>–10<sup>9</sup> p.f.u./ml; Table 1, upper panel).

We have found that rescuing the defective MS2 phage can also be carried out with sequences from related phages, such as fr, a species I phage like MS2, or KU1, a phage belonging to species II. In these cases, considerably less phage is produced (Table 1, panels two and three), probably because such plasmid combinations provide less opportunity for homologous recombination and because not all crossovers may produce viable hybrids. However, once formed, the hybrids reach titres that are approximately the wild-type level.

Hybrid phages were visualized as plaques on a lawn of E. coli F<sup>+</sup> cells (cycle 2). They were sequenced after multiplication in liquid medium (cycle 3) and also after passaging for further infection cycles.

### Complementing plasmid pMSABA with pKU8

Plasmid pMSABA, lacking the first 111 nucleotides of MS2, was combined with pKU8 containing the first 1572 nucleotides from KU1. Six plaques, from two different transformations, were taken for sequence analysis. The results, presented in Fig. 3, will first be discussed in general terms. The first two lines of Fig. 3(A) (in a bracket) give a schematic presentation of the various structure elements present in the 5′ UTR of the donor and acceptor sequences. The zigzag line shows the crossover or fusion site. Line three (kumsBA,x) presents the general structure of the initial hybrids and line four (kums4), the structure after evolution. Fig. 3(B, C) shows the nucleotide sequence of the initial and evolved hybrids in the context of the predicted RNA secondary structure. The shaded parts in Fig. 3(B) were deleted upon passaging. Finally, Fig. 3(D) shows the linear sequences of the relevant sections of donor and acceptor plasmids. MS2 sequences are shown as black on white (Fig. 3A) and in normal letters (Fig. 3B–D), whereas KU1 sequences are white on black (Fig. 3A) and bold letters (Fig. 3B–D). Italic letters in Fig. 3(D) are derived from the vector.

As can be seen from Fig. 3(A), the first event in the formation of hybrids was coupling of the complete 5′ UTR of KU1, sometimes including the start codon (kumsBA1 and kumsBA4), but other times not, to the 5′ end (UCCU) of the defective MS2 sequence (kumsBA,x; cycle 3, Fig. 3A, B). In these initial hybrids there were several elements that occurred twice, such as the SD sequence. In two plaques, the start codon...
was also present twice. In Fig. 3(B), these extra nucleotides are shown in the context of the predicted RNA structure for five plaques (kumsBA.1, kums2, kumsBA.3, kumsBA.4 and kumsBA.2). Generally, the same nucleotides at the 5' terminus of the defective MS2 sequence (UCCU) became attached to the KU1 sequence around the KU1 start codon. When these initial hybrids were passaged, some of them (kumsBA.3 and kumsBA.4) lost the redundant nucleotides present in the predicted stem-loop (shaded in Fig. 3B), to yield the tailored hybrid kums4 in which the fusion point was between the KU1 East arm and the SD box of MS2 (Fig. 3C). This deletion may be the result of aberrant homologous recombination favoured by the common GGAGG sequence (boxed in Fig. 3D). In the resulting cloverleaf structure, the SD of MS2 took the place of the KU1 SD sequence. In the three other plaques, the redundant nucleotides were still present, for kumsBA.2 even after 20
cycles. The extra arm is apparently a small burden and its removal may take more time. Note that part of the extra hairpin in kumsBA.2 was a duplication of the nearby KU1 sequence (italics).

The sixth plaque we examined was only sequenced after cycle 5 and 10. At cycle 5 all recombination events had apparently been completed for this plaque as there were no changes in the next five cycles (cycle 10). The sequence of this plaque and the putative structure of its RNA is shown as kums1 in Fig. 5. As for kums4, the final fusion between the two phages was between the SD sequence of MS2 and the East arm of KU1, though at a slightly different point resulting in a hybrid two nucleotides shorter than kums4.

**Complementing pMSΔBA with pFR400**

We also combined the same MS2 deletion with plasmid pFR400, carrying the fr sequence from 1 to 400. The results are shown in Fig. 4.
Fig. 6. Generation of fr−MS2 hybrids (frmsSA). Eight plaques were examined and found to be identical. The boxed GGG residues in (B) depict a common sequence in MS2 (5′ end) and fr (nt 118). For further details see legends to Figs 3 and 4.

Two plaques were sequenced and found to be identical. Surprisingly, despite the much higher sequence identity between fr and MS2 (Fig. 1B), the pathway of hybrid formation was the same as when coinfected with pKU8. First, the complete 5′ UTR of fr up to the start codon was appended to the 5′ end of the deleted MS2 clone (Fig. 4A, B). In fact, at the fusion or crossover point we found a stretch of nucleotides derived from the vector plus some whose origin was unknown (Fig. 4B). Then, upon further evolution, the redundant nucleotides present as a hairpin structure were removed to yield the frms3 hybrid in which the fusion was again between the SD box of MS2 and the East arm of fr (Fig. 4C). This deletion is possibly favoured by homologous recombination over the shared CUAGG sequence (boxed in Fig. 4C, D). It is interesting to note that frms3 could have been obtained directly from the two plasmids by a single crossover at the common CUAGG sequence, but this was not the pathway that was followed.

Complementing pMSΔSA with pKU8 and pFR741

We then used pMSΔSA, lacking the three arms of the cloverleaf, for hybrid generation using pKU8 (Fig. 5A). The
Generation of hybrid RNA phages

Fig. 7. Generation of KU1–MS2 hybrids (kumsNA). Eight plaques were analysed and all were identical. See legends to Figs 3 and 4 for details. The sequence of the first hybrid kumsNA.3 is shown in (B). The deletion leading to kums1 is overlined and may be facilitated by the shared sequence GGAGG (boxed) although the A116 of MS2 is not expected to be there in that case. The predicted RNA structure of this hybrid is not shown.

The titre of this plasmid combination was about the same as the previous MS2/KU1 combination (Table 1). Six plaques were analysed and all were identical. Strikingly, the pathway of hybrid generation was the same as before. The complete 5′ UTR of KU1, including the start codon, was appended to the 5′ end of the defective MS2 sequence (Fig. 5A, C; kumsSA.3). Upon evolution of this hybrid, the redundant elements, SD (KU1), fMet (KU1), N (MS2) and UCS (MS2), were deleted resulting in kums1, a hybrid also found above as the end result of a different plasmid combination (Fig. 5B). As before in the pMS∆BA series, there was the choice of removing the SD sequence and start codon from either KU1 or MS2. It was even possible to combine the SD of KU1 with the start codon of MS2, by deleting fMet (KU1) and N, UCS and SD elements of MS2. We always observed that the SD sequence that originally belonged to the maturation gene was maintained, even though this created an LDI that derived its constituent sequences from different phages. We do not know why this should be so.

We have also complemented the pMSASA plasmid with fr sequences (pFR741) (Fig. 6). Again, generation of hybrids proceeded along the same lines as with pKU8. That is, first, the 5′ UTR of fr up to and including the East arm was appended to the very 5′ end of pMSASA (Fig. 6). Subsequently, the redundant MS2 elements (N and UCS) were removed giving rise to frms3 (Fig. 4), a hybrid we have obtained before as the result of another plasmid combination. The deletion may be facilitated by the common UAGG sequence (boxed in Fig. 6C).

Complementing pMSΔNA with pKU8 and pFR741

Finally, we have combined pMSΔNA from which only the South and East hairpins are missing, with pKU8 (Fig. 7A). As before, we noted in the recombinant hybrids that the first step towards making a viable phage was the positioning of the 5′ UTR of the acceptor in front of the defective MS2 sequence (Fig. 7A, B). In subsequent evolutionary steps the superfluous sequences were removed [SD (KU1), fMet (KU1), N, UCS and W, all from MS2] leading again to kums1 (Fig. 5). The SD and fMet sequences of KU1 were deleted but those of MS2 were maintained as before. Hybrid kums1 was found before as the result of complementing pMS ΔSA with pKU8.

When pMSΔNA was rescued with pFR741, the pathway to hybrid formation was essentially the same. The 5′ UTR of fr up to and including the East arm was linked to the 5′ end of the defective MS2 genome (Fig. 8). During this process or shortly after, the West arm was already lost. Further evolution caused the deletion of the redundant N and UCS elements leading to frms3 (Fig. 4), a hybrid also emerging from other
plasmid combinations. Interestingly, in this case the deletion of the redundant N and UCS elements cannot be attributed to a short repeat (last line, Fig. 8B).

**Discussion**

In this paper we have described the generation of hybrid RNA phages by *in vivo* recombination using a two-plasmid system. Plasmids encoding various deletion mutants of MS2 cDNA were coinfected with a second plasmid carrying the deleted portion in a generous overlap but from a different phage. The deletions involved three different parts of the 5′ UTR of RNA phage MS2 and the missing information was supplied by either fr, a rather close relative also belonging to species I, or by KU1, a more distantly related species II phage (Groeneveld et al., 1996). The interesting conclusion was that viable hybrid phages do arise. Once formed their titre is approximately as high as wild-type. However, the number of solutions was very low. Disregarding those hybrids that had not yet got rid of their insert (Fig. 3B), we found only two solutions for the KU1–MS2 combination (kums1 and kums4) and one solution for the fr–MS2 combination (frms3). Given the relatively large overlap present between the complementary genomes, especially in the pMSΔNA case (Figs 7 and 8), this indicates that there are restrictions that limit the number of different viable hybrids. Although this study did not reveal what these restrictions might be, our previous work has suggested that these phages are very sensitive to changes in the proper RNA folding (Olsthoorn et al., 1994; Klovins et al., 1997, 1998; van Meerten et al., 1999). For a large number of potential fusion points the RNA structure may be compromised because fr/KU1 sequences may not be able to form the same base pairs as the original wild-type and this could basically restrict hybrid formation to cases where RNA structure modules are exchanged, as we observed in this work; the cloverleaf of MS2 was replaced by that of fr or KU1 (see below).

**Pathway of hybrid formation**

The path along which hybrids were generated is outlined in its simplest form in Fig. 9. It was basically the same for complementation with fr or KU1, in spite of the fact that sequence identity of MS2 with fr is high (~90%) whereas it is very low with KU1. The pathway also seemed insensitive to the size and position of the deletion. We always found as a first step that the 5′ UTR of the acceptor plasmid became attached to the 5′ end of the defective phage (the donor). It did not seem to matter very much where exactly the junction with the acceptor sequence was formed. Mostly, it included the fMet
start codon of the acceptor's maturation gene, but sometimes
the connection was made between the East arm and SD
sequence of the acceptor. It was interesting that the acceptor 5' UTR was nearly always attached to the first nucleotide of the
MS2 sequence. In pMSASA and pMSANA this is the original
5' end (GGG). The likely purpose of this first step seems to be
to create a replicable (-) strand. Addition of the 5' UTR was
not needed to rescue A-protein synthesis because it is known
that the three MS2 deletion mutants used here can still make
this protein (see Fig. 2 legend), even though the timer function
has been lost (at least in pMSASA and pMSABA). Because
uncontrolled production of maturation protein is disadvan-
tageous but not lethal (Poot et al., 1997), we have ascribed
lethality of the initial MS2 deletion constructs to failure to
transcribe the (-) strand.

After replication had been rescued, the hybrids began to
improve their genome by removing the superfluous sequences.
Often, this process seemed to be aided by short repeats at the
edges of the stretch to be deleted (CUAGG and GGAGG; see
below), but such repeats cannot always be found (frmsNA.3).
Frequently, the sequence that was deleted was present as a
hairpin structure, e.g. frmsBA.3. It is not clear whether this
is a coincidence or whether the hairpin physically facilitated
the deletion. This deletion usually occurred within a few
passages, but sometimes we were still left with extra
nucleotides in the leader after 20 infection cycles. We suppose
that eventually the surplus nucleotides will be removed.

If we had not measured the early stages of hybrid formation,
but only the final outcome after passing, one could wrongly
have concluded that hybrids such as frms3, kums1 and kums4
were the result of a simple RNA or DNA crossover at regions
of sequence identity such as CUAGG (frms3) and GGAGG
(kums1 and kums4). However, the existence of the early
hybrids shows that such a crossover is not the most likely
event. Instead, the unexpected attachment of the acceptor 5' UTR to the very 5' end of the defective donor RNA from
pMSANA and pMSASA turns out to be the more probable
reaction. These new RNA combinations are unlikely to be
driven by nucleotide identity between donor and acceptor

sequence because there is none at the initial fusion points. If
recombination occurred at the DNA level it would be difficult
to rationalize what preference there could be to cross over at
the first nucleotide of the phage in the absence of any sequence
identity. Therefore, our observations suggest that the linkage
between donor and acceptor sequence occurs at the RNA level.
If so, the mechanism of this acquisition could be as follows. It
is known that MS2 and Qβ replicase can generate the proper 5'
and 3' termini from cloned cDNA copies (Taniguchi et al.,
1978; Olsthoorn et al., 1994) and from oversized phage RNA
transcripts transfected in spheroplasts (Shaklee et al., 1988).
Therefore, we would expect MS2 replicase to be able to make
the (-) strand carrying the proper 3' terminus with the
deletion present in pMSANA and pMSASA. At this point we
can see two possibilities. The (-) strand is released by the
enzyme and the free 3' hydroxyl attacks a phosphodiester
bond of a transcript derived from the acceptor plasmid. Natural
selection will subsequently preserve only those recombinants
that can produce phages. This recombination by transesterifi-
cation was shown to exist for Qβ replicase by Chetverin et al.
(1997). However, there is one problem. As part of the
termination reaction, the replicase usually adds a 3' A residue
to the strand it has just produced and we see no U preceding
the GGG sequence that marks the start of the donor RNA.
The second possibility is that when the replicase has
polymerized the end of the (-) strand of the donor RNA chain
it does not terminate but switches to the acceptor template.
This is precisely what has been found for hybrids between
defective interfering (DI) RNA derived from tomato bushy
stunt virus (TBSV) and cucumber necrosis virus (CNV) RNA
(White & Morris, 1995). Also in that study, the fusion occurred
between the 3' terminal nucleotide of the donor transcript and
variable positions in the acceptor molecule. This mechanism
was termed 'run off recombination' (White & Morris, 1995)
and the fact that we find it in almost all of our hybrids is in
agreement with its reported efficiency. We note that this
mechanism does not predict the presence of the terminally
added A residue, because the produced chain is still attached
to the replicase and has not been terminated.
### Functionality of the hybrids

Evidently, the foreign sequence (fr or KU1) must be able to perform in the new context and it is interesting to see if this can be rationalized on the basis of what is known about the functioning of the 5′ UTR. Let us consider the hybrids kums1 and kums4, in which the KU1 cloverleaf structure replaced that of MS2 [the titre of the plasmid combination was 10^6 p.f.u./ml, but after passing the titre of the evolved hybrid phage increased to approximately wild-type level (10^{11} p.f.u./ml)].

A major function of the cloverleaf is to ensure transient translation of the maturation gene by the metastable structure shown in Fig. 1(D, E). This metastable intermediate could still be formed in kums1 and kums4. In fact, we now had the KU1 metastable structure in the MS2 context. The long-distance interaction between the SD sequence and its upstream complement (UCS) was actually of a hybrid character since the UCS was a KU1 sequence, whereas MS2 provided the SD box. As in both phages the SD box contained the sequence GGAGG, the LDI could be formed in kums1 and kums4, though the stability of the interaction may have been slightly different from wild-type. In summary, in the hybrids one structure module has been replaced by another. Still, it could be argued that an even better kums hybrid might be made if the structure module has been replaced by another. Still, it could be argued that an even better kums hybrid might be made if the deletion that matures e.g. kumsNA.3 (Fig. 7) was shifted so as to keep the SD box from KU1 but discard the MS2 SD box. Then an LDI with only KU1 sequences would be present. It may be that we do not find this because there is no sequence identity here from which the deletion may profit. Alternatively, for unknown reasons, it may be that the SD box cannot be uncoupled from the coding region.

Another function of the 5′ UTR is that it encodes the 3′ end of the (−) strand, which must interact with the MS2 replicase. Almost nothing is known about this subject. We can only conclude that MS2 replicase can function with a KU1 3′ end in the (−) strand.

### Hybrids cannot compete with wild-type

The family of Leoviridae has two genera: the Leviviruses such as MS2 and KU1 and the Alloleviviruses like Qβ and SP. In each genus there are two species, each having its distinct sequence with limited similarity to the other one. Within the species, all characterized strains cluster in a narrow zone around the master sequence (> 95% identity) (Inokuchi et al., 1986, 1988; Adhin, 1989; Beekwilder, 1996; Olsthoorn, 1996; Groeneveld, 1997). Hybrids between species have never been found. This is possibly a consequence of the fact that sampling was mostly performed in city sewage systems, which represent enormously large pools of RNA phages with continuous input (Furuse, 1987). Therefore, it is not difficult to imagine that competition in such an environment is strong, and that aberrant phages, such as hybrids, will quickly be outgrown by wild-type. Indeed, when we mixed hybrid kums4 in a 10:1 ratio with wild-type MS2 and the mixture was passaged, it took only five infection cycles before the hybrid was completely outgrown by wild-type and could no longer be detected by RT–PCR. When mixed in a 100:1 ratio this hybrid was no longer detectable at cycle 10. Eukaryotic viruses, on the other hand, may develop in relative isolation within a single organism, where hybrids or other aberrant viruses may multiply and evolve in the absence of strong competition.

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### References


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