Experimental human rhinovirus and enterovirus interspecies recombination

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Received 1 July 2011
Accepted 16 September 2011

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Human rhinoviruses (HRVs) and enteroviruses (HEVs), two important human pathogens, are non-enveloped, positive-sense RNA viruses of the genus Enterovirus within the family Picornaviridae. Intraspecies recombination is known as a driving force for enterovirus and, to a lesser extent, rhinovirus evolution. Interspecies recombination is much less frequent among circulating strains, and supporting evidence for such recombination is limited to ancestral events, as shown by recent phylogenetic analyses reporting ancient HRV-A/HRV-C, HEV-A/HEV-C and HEV-A/HEV-D recombination mainly at the 5′-untranslated region (5′ UTR)–polyprotein junction. In this study, chimeric genomes were artificially generated using the 5′ UTR from two different clinical HRV-C strains (HRV-Ca and HRV-Cc), an HRV-B strain (HRV-B37) and an HEV-A strain (HEV-A71), and the remaining part of the genome from an HRV-A strain (HRV-A16). Whilst the chimeric viruses were easily propagated in cell culture, the wild-type HRV-A16 retained a replication advantage, both individually and in competition experiments. Assessment of protein synthesis ability did not show a correlation between translation and replication efficiencies. These results reflect the interchangeability of the 5′ UTR, including its functional RNA structural elements implicated in both genome translation and replication among different enterovirus species. The 5′ UTR–polyprotein junction therefore represents a theoretic interspecies recombination breakpoint. This recombination potential is probably restricted by the need for co-infection opportunities and the requirement for the progeny chimera to outcompete the parental genomes’ fitness, explaining the rare occurrence of such events in vivo.

INTRODUCTION

Human rhinoviruses (HRVs) and human enteroviruses (HEVs) are non-enveloped, positive-sense RNA viruses and members of the genus Enterovirus, the largest genus in the family Picornaviridae. HRVs currently consist of 151 proposed types (Knowles, 2011; Simmonds et al., 2010), classified into three species (HRV-A, -B and -C), and represent the most common cause of respiratory tract infection in humans (Garbino et al., 2009; Mäkelä et al., 1998; Ruohola et al., 2009). HEVs encompass 107 types classified into four species (HEV-A, -B, -C and -D) and cause various mild to severe clinical manifestations, especially in children, such as hand, foot and mouth disease, meningoencephalitis, poliomyelitis and myocarditis (Sawyer, 2001).

HRVs and HEVs share a similar genomic organization, which consists of a 5′-untranslated region (5′ UTR), a single ORF, a 3′ UTR and a poly(A) tail. The ORF encodes a polyprotein that is co-translationally cleaved into four structural viral particle proteins (VP4, VP2, VP3 and VP1) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). The genomic RNA of these viruses also harbours a stem–loop structure called the cis-acting replication element (cre), which is essential for replication. The precise location of this cre stem–loop structure is constrained to the 2C region for all HEV species, whereas its position varies in the three HRV species and overlaps the 2A (HRV-A), VP1 (HRV-B) and VP2 (HRV-C) regions (Cordey et al., 2008).

The enterovirus 5′ UTR forms six distinct secondary-structure domains that can be divided into two functional units: a 5′ cloverleaf structure (CL) essential for replication (domain I) and an internal ribosome entry site (IRES)

A supplementary table and figure are available with the online version of this paper.
(domains II–VI) necessary for the cap-independent translation of the polyprotein (Rohll et al., 1994). The CL is further subdivided into four structural domains: stem A, stem–loop B, stem–loop C and stem–loop D. Stem–loop B is a known binding site for cellular proteins called poly(rC)-binding proteins (PCBs), and stem–loop D interacts with the viral 3C and 3CD proteases. The CL, cellular PCBs and viral 3CD are all implicated in the switch from translation to replication (Du et al., 2004; Perera et al., 2007). Although replication regulation has been attributed mainly to the CL, sequences located in the 3’ region of the poliovirus (PV) IRES may also be implicated in this process (Borman et al., 1994). Viral IRESs are classified into five types according to primary sequence, secondary structure, location of the initiation codon and activity in different cell types (Racaniello, 2007). HRVs and HEVs both share a loosely structured type I IRES, which is characterized by five key stems (domains II–VI) involved in protein binding and interaction with the 43S ribosomal subunit (Palmenberg et al., 2010).

HRVs and HEVs are characterized by an important diversity of types, and previously unknown strains are constantly being reported (Arden et al., 2006; McErlean et al., 2007; Smura et al., 2007; Tapparel et al., 2009a; Yozwiak et al., 2010). The main explanations for such an important variability are the high error rate of the viral RNA-dependent RNA polymerase and recombination (Domingo & Holland, 1997). Intraspecies recombination events have been extensively described for HEVs and represent an evolutive force for this virus group (Lukashev, 2005; Santti et al., 1999). The enterovirus recombination breakpoints are most frequently reported around the 5’ (VP4) and 3’ (2AB) ends of the P1 region, whilst they are almost absent in the capsid VP2–VP3–VP1 region (Lukashev et al., 2005; Simmonds & Welch, 2006). Evidence for recombination is more limited among rhinoviruses. Few recombination events have been reported for circulating HRV strains (Tapparel et al., 2009b), with breakpoints located at the 3’ end of the 5’ UTR and the 5’ end of the 3C gene. However, analysis of the full-length sequences of all known HRV serotypes suggests that some serotypes have actually resulted from ancient intraspecies recombination (Palmenberg et al., 2009).

Natural interspecies recombination among HEVs may have occurred in the past. Strains resulting from putative 5’ UTR exchange between HEV-C and HEV-A as well as between HEV-D and HEV-A have been reported recently (Smura et al., 2007). Similarly, Yozwiak and co-workers described a novel HEV-C (EV-109) whose 5’ UTR was presumably acquired through recombination with an HEV-A member (Yozwiak et al., 2010). Likewise, recombination between the 5’ UTR of HRV-A and the polyprotein of HRV-C was proposed as the mechanism responsible for the generation of the HRV-Ca subgroup exhibiting HRV-A-like 5’ UTR sequences (Huang et al., 2009; McIntyre et al., 2010). The remaining HRV-C strains, called HRV-Cc, have 5’ UTR sequences that segregate from those from HRV-A, HRV-B and HRV-Ca members. Putative interspecies recombination breakpoints in the 5’ UTR have been mapped for HRV-Ca strains: one around position 481, the second around position 565 in the polypyrimidine tract and the third around position 523 within stem–loop 5 of the IRES (McIntyre et al., 2010). In the majority of sequences analysed, recombination presumably occurred in either one of the last two recombination hotspots, which are located in highly conserved sequence stretches. These two particular locations may therefore represent preferred sites for other interspecies 5’ UTR recombination within members of the genus Enterovirus. Furthermore, some HRV-C strains harbour short HRV-A sequences in their 2A region (Huang et al., 2009; McIntyre et al., 2010). Finally, based on full-genome phylogenetic analysis, we have proposed that ancient recombination events between HRV-A and HEV members gave rise to the HRV-B species (Tapparel et al., 2007). However, based on sequence homology, all the above proposed natural interspecies HEV and HRV recombination events probably occurred between ancestors of the current HEV and HRV circulating strains.

Constructed viable interspecies HRV and HEV recombinants have also been reported in the literature. Examples include a chimeric PV1/HRV-14 construct in which the 5’ UTR was derived from PV-1 and the remainder of the genome from HRV-14 (Todd et al., 1997), a chimeric HRV-2/PV-1 in which the PV-1 IRES is replaced by an HRV-2 IRES (Gromeier et al., 1996), and a chimeric coxsackievirus 4 (CV-B4)/PV-3 consisting of a PV-3 genome in which the 5’ UTR is derived from CV-B4 (Rohll et al., 1994).

This study aimed to explore further the recombination potential of different enterovirus species at the 5’ UTR–polypeptide junction, and therefore the compatibility between these species at that level. For this purpose, we assessed the viability of chimeras with 5’ UTRs originating from different species (HRV-Ca, HRV-Cc, HRV-B and HEV-A) fused to a common HRV-A (HRV-A16) backbone. Each of these recombinant genomes gave rise to infectious viral particles. We also tested and compared the replication, translation and competition abilities of these 5’ UTR chimeric viruses.

**RESULTS**

**Chimera constructs**

The four chimeric genomes represented in Fig. 1 were constructed using a plasmid encoding the full-length HRV-A16 genome in which the entire 5’ UTR was replaced by the 5’ UTR originating from an HRV-Ca, HRV-Cc, HRV-B37 or HEV-A71 strain (see Methods). The resulting plasmids were then used to generate virions by transfection of *in vitro*-transcribed RNA into HeLa Ohio cells.

A comparison of the 5’ UTR sequence homologies of the viral genomes used in this study is shown in Table 1. The
HRV-Ca and HEV-A71 5’ UTRs displayed the highest (77%) and lowest (64%) sequence similarities, respectively, compared with the HRV-A16 5’ UTR. As expected, HRV-A and HRV-Ca did not segregate on a 5’ UTR-based sequence phylogenetic tree (see Supplementary Fig. S1a, available in JGV Online), reflecting the previously mentioned 5’ UTR transfer from the HRV-A species to the HRV-Ca subspecies. Whilst dendrogram trees based on RNA structure mainly reflected the grouping of known species, some species were completely misplaced with regard to the sequence-based trees (Supplementary Fig. S1b). Although we cannot exclude the limitations of current single-sequence ab initio structure prediction programs, they may indicate the high similarity of structure elements among species, which is not visible from a sequence-based comparison. Of note, as for HRV-A and HRV-Ca, HEV species did not segregate correctly based on their 5’ UTR sequence.

The chimeric derivatives replicate less efficiently than the non-chimeric parental HRV-A16

The replication ability of each chimera was compared with that of HRV-A16 at 2 h, 48 h and three passages after transfection of standardized amounts of in vitro-transcribed RNA. This experiment was performed in duplicate. This comparison was made using a quantitative real-time PCR assay specific for the HRV-A16 3D region, as shown in Fig. 1. The 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) housekeeping gene was used for normalization. Of note, this replication ability assessment was based on the assumption that the target RNA measured reflected viral genome amplification and thus replication.

Although the amount of RNA measured at 2 h post-transfection was similar for the different constructs, notable differences in replication efficiency were observed after three passages, as shown in Fig. 2(a). At this time point, the non-chimeric HRV-A16 had the highest viral RNA load, followed by the HRV-Cc/A16 and HEV-A71/A16 chimeras. The lowest viral RNA load was observed for the HRV-Ca/A16 and HRV-B37/A16 chimeras. The same trend was observed by immunofluorescence with an anti-HRV-A16 antibody performed on HeLa Ohio cells at 48 h post-transfection in duplicate. Bioimaging quantification showed that 28, 24, 23, 18 and 13% of cells transfected with HRV-A16, HEV-A71/A16, HRV-Cc/A16, HRV-Ca/A16 and HRV-B37/A16 RNA, respectively, were immunofluorescence positive (results not shown).

HRV-A16 outcompetes the chimeras in co-transfection assays, except when present at a lower concentration

To assess whether the replication advantage observed for the parental HRV-A16 virus was biologically significant, we designed a competition experiment in which normalized

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<tr>
<th>Construct</th>
<th>5’ UTR HRV-A16</th>
<th>5’ UTR HRV-Ca</th>
<th>5’ UTR HRV-B37</th>
<th>5’ UTR HRV-Cc</th>
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<tr>
<td>5’ UTR HE-A71</td>
<td>64</td>
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<tr>
<td>5’ UTR HRV-A16</td>
<td>77</td>
<td>68</td>
<td>68</td>
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<tr>
<td>5’ UTR HRV-Ca</td>
<td>68</td>
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<td>71</td>
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<tr>
<td>5’ UTR HRV-B37</td>
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amounts of the four chimeric and the non-chimeric HRV-A16 RNAs were co-transfected in triplicate. A strong cytopathic effect was observed in each sample at 3 days post-transfection. Cell supernatants were passaged three times. Viral cDNAs obtained from the first- and third-passage supernatants were PCR amplified over approximately 1 kb of the 5' UTR–VP2 region with primers exactly matching each of the five derivatives to avoid any PCR bias. PCR performed on the supernatant collected 48 h after transfection (defined as the first passage; Fig. 2b, lane p1) and after the third passage (Fig. 2b, lane p3) yielded two fragments of different sizes and intensities. These PCR products were subcloned and sequenced. For the first passage, eight of ten clones harboured the HRV-A16 5' UTR sequence, whilst the remaining two contained the HEV-A71 5' UTR sequence. This ratio was 9 : 1 after two additional passages. Notably, sequencing of the gel-purified upper band four passages after co-transfection into HeLa Ohio cells with equivalent amounts of the four chimeric RNAs and the non-chimeric HRV-A16 RNA (b) or equivalent amounts of each chimeric RNA and a tenfold lower amount of HRV-A16 RNA (c). The identities of the 5' UTRs determined by sequencing are indicated (arrows). These experiments were carried out in triplicate. L, 1 kb ladder.

The experiment was repeated with a tenfold dilution of HRV-A16 RNA, as shown in Fig. 2(c). Again, the PCR product was subcloned and sequenced. For the first passage, five clones harboured the HEV-A71 5' UTR sequence, three harboured the HRV-B37 5' UTR sequence,
and one harboured the HRV-Cc 5’ UTR sequence. This mixed population suggested that, after one passage, HEV-A71 presented a slight replication advantage over the other chimeras and the diluted HRV-A16. This advantage became important after four passages, as eight clones contained the HEV-A71 5’ UTR sequence and two contained the HRV-Cc 5’ UTR sequence. These results showed that, when HRV-A16 was present at a lower concentration, it was unable to outcompete the recombinant viruses. In addition, these data tended to confirm that, aside from the non-chimeric HRV-A16, HEV-A71/A16 and HRV-Cc/A16 replicated the most efficiently.

**There is no correlation between replication and translation efficiencies**

The translation abilities of the chimeras and parental HRV-A16 were assessed using a cell infection assay. For each virus, a 50% cell-culture infective dose (CCID<sub>50</sub>) of 10<sup>5</sup> ml<sup>-1</sup> was used to inoculate HeLa Ohio cells. To minimize the impact of replication on translation efficiency, a Western blot was performed on cell lysates at early time points (2, 6 and 9 h) after infection, and the amount of protein was compared with the amount of normalized intracellular positive-strand RNA (the template for translation) measured by quantitative real-time PCR (Fig. 3 and data not shown). RNA levels measured at 2 and 6 h post-infection (p.i.) were comparable among the five viruses, and increased amounts were observed only after 9 h, suggesting that replication started between 6 and 9 h for all derivatives. Furthermore, amplification of the positive-strand RNA matrix appeared to be necessary for protein detection as no viral proteins were visible by Western blotting before 9 h (data not shown). Both Western blot and RNA quantification results were reproducible with biological replicates.

Overall, viral protein and positive-strand RNA amounts measured at 9 h p.i. correlated well except for the HRV-Cc/A16 and HRV-Ca/A16 chimeras. HRV-Cc/A16 displayed low levels of protein synthesis, despite intermediate positive-strand RNA levels at 9 h p.i. (Fig. 3) and high replication levels afterwards (Fig. 2a). However, HRV-Ca/A16 chimera translation ability was equivalent to that of HEV-A71/A16 and HRV-A16 (Fig. 3a), whilst its replication potential was notably lower at later time points (Fig. 2a).

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**Fig. 3.** Comparative translation efficiencies of the chimeric genomes and the non-chimeric HRV-A16. (a) Western blot analysis at 9 h p.i. of HeLa Ohio cell lysates with an anti-HRV-16 VP2 mAb (which also detects the VP0 precursor) and an anti-actin antibody. Protein identities are indicated on the right. (b) For each derivative, the level of positive-sense RNA was quantified by real-time RT-PCR and normalized to a housekeeping gene at 2, 6 and 9 h p.i. The viral positive-strand RNA level is expressed relative to HRV-A16 RNA measured at 2 h p.i. (input RNA) and set as a reference (see Methods for calculation). RNA samples monitored at 9 h p.i. were extracted from the same cell lysates as those used for Western blotting (a). Results are shown as means ± SD derived from two separate experiments.
The growth phenotype of the HEV-A71/A16 chimera is defined by its polyprotein rather than its 5′ UTR sequence

HRVs and HEVs exhibit a different cell tropism and optimal growth temperature in vivo. To assess whether the 5′ UTR region might play a role in these different phenotypes, we compared the growth ability of the HEV-A71/A16 chimera with the non-chimeric HEV-A71 and HRV-A16 in different cell types and at different temperatures. When the chimeric RNA was transfected into HeLa Ohio cells, propagation of the resulting virus was similar to that of HRV-A16 and much stronger than that of HEV-A71 (data not shown). In addition, as for HRV-A16, the optimal growth temperature was 33 °C. When transfected into Vero cells, a cell line typically used for HEV culture, there was no visible cytopathic effect for the HEV-A71/16 chimera or for HRV-A16 at 33 or 37 °C, whereas the HEV-A71 virus replicated efficiently (data not shown). Overall, the viral culture phenotype of the chimera containing the HEV-A71 5′ UTR and HRV-A16 polyprotein was similar to that of the parental rhinovirus.

DISCUSSION

Recombination is a well-described driving force for picornavirus evolution. This phenomenon is reported more frequently for HEVs than for HRVs (Lukashev, 2005; McIntyre et al., 2010; Santti et al., 1999) and occurs mainly within members of the same species. Interspecies recombination events have rarely been reported and, based on sequence similarity, these recombinations have been described mainly as ancient events, possibly at the origin of new HEV types, subspecies or species (Huang et al., 2009; McIntyre et al., 2010; Smura et al., 2007; Tapparel et al., 2007; Yozwiak et al., 2010).

The aim of our study was to test the interspecies recombination potential at the 5′ UTR–ORF junction among members of the genus Enterovirus. Our results revealed that the 5′ UTRs of selected representatives of HRV-Cc, HRV-Ca, HRV-B and HEV-A were all functional in the context of an HRV-A (HRV-A16) genome. This implies that the 5′ UTR including the IRES and CL cis-acting elements of each of these species are recognized efficiently by HRV-A16 proteins, resulting in productive translation and replication. This functional compatibility hints at an interspecies recombination potential between the 5′ UTR and the rest of the genome within members of the genus Enterovirus. This interspecies recombination opportunity may, however, be limited by several factors. First, two different HRVs and/or HEVs have to co-infect a target cell in the same time frame. This may seem particularly unlikely with regard to HEV/HRV recombination, as HEVs are known for their enteric tropism. However, several HEV strains exhibit a respiratory tropism, and the simultaneous presence of an HEV and an HRV genome in the same clinical specimens has been documented (Lu et al., 2008).

Regarding HRV/HRV co-infection, tropism is not a limiting factor, and we have observed such events in the past following the use of separate HRV-A and HRV-B real-time PCR assays to screen respiratory samples. Although these observations do not imply that the viruses co-detected in the same anatomical site are also present in the same cell, reports of intraspecies HRV recombinants suggest that such single-cell co-infection indeed occurs. Secondly, to outcompete the non-chimeric parental strains present in superior amounts in the co-infected host and to spread in the population, recombinant viruses need to be fitter than the non-chimeric viruses. In the setting of our competition experiments, the non-chimeric HRV-A16 virus was revealed to be fitter than the recombinants. This may be explained by suboptimal interactions between the 5′ UTR and other parts of the genome, as well as viral proteins. These interactions have probably been optimized over time by co-evolution thanks to both purifying and diversifying selective pressure (Kistler et al., 2007), and well-adapted strains may be difficult to outcompete. However, our competition assays were performed in HeLa Ohio cells, and it is not certain whether these considerations are valid for natural recombinants occurring in vivo.

Productive interspecies recombination within the polyprotein region may be even more complicated, as chimeric polyproteins may harbour protein cleavage sites incompatible with 2A and 3C proteases, and cre motifs, situated at different positions among different species, may be deleted or duplicated.

Surprisingly, we observed that the recombinant virus with the most divergent 5′ UTR sequence and the second most divergent 5′ UTR structure with regard to HRV-A16 (Supplementary Fig. S1), namely HEV-A71/A16 (64% sequence similarity to HRV-A16), was the second fittest virus, both in independent replication assays and in competition experiments, and displayed an efficient translation potential. In contrast, HRV-Ca/A16 and HRV-B37/A16 replicated with low efficiency. Interestingly, these two chimeras clustered in the structure-based RNA tree shown in Supplementary Fig. S1(b), suggesting that their secondary RNA structure may drive suboptimal replication and virus propagation in the HRV-A16 background. However, as mentioned earlier, structural predictions made with current programs should be interpreted carefully. We also demonstrated that there was no strict correlation between the translation and replication efficiencies of our chimeras. Indeed, HRV-Cc/A16 displayed a replication pattern almost equivalent to that of the parental HRV-A16, despite a notably lower translation ability, whereas HRV-Ca/A16 replicated markedly less, despite exhibiting a translation ability similar to that of HRV-A16.

Finally, we tested the viral culture phenotype of the HEV-A71/A16 chimera. Its optimal replication temperature and cellular tropism closely resembled those of the wild-type HRV-A16 and differed dramatically from those of HEV-A71, indicating that these parameters are not defined...
by the 5’ UTR part of the viral genome. Although the pathogenicity of such interspecies recombinants in vivo is hard to predict, our results suggest that most of the virus phenotype relies on the polyprotein rather than on the 5’ UTR sequence.

In summary, we have shown experimentally that the 5’ UTRs are functionally interchangeable between selected enterovirus species, and that propagation of such chimeras is limited by competition between parent and progeny in cell culture. Our data support phylogenetic analyses indicating ancient interspecies recombination among HRVs and HEVs, as well as between them. Similar recombination events may occur in the future, further contributing to the variability of these viruses.

**METHODS**

Construction of the chimeric HRV-A16 derivatives. The pR16.11 plasmid (kindly provided by W.-M. Lee, University of Wisconsin, USA) containing a full HRV-A16 genome was used to create four chimeras with different 5’ UTRs. An SfI site was introduced by site-directed mutagenesis (QuickChange II XL Site-Directed Mutagenesis kit; Stratagene) at the 5’ UTR–VP4 junction with primers RV50 and RV51 (Supplementary Table S1, available in IGV Online), so that the SfI site spanned codons 2 and 3 of the HRV-A16 ORF. The resulting plasmid, designated QCpHRV-A16, was then depleted of its 5’ UTR by SalI/SfI digestion to receive the 5’ UTR prepared from HRV-Ca, HRV-Cc and HRV-B and HEV-A71, as described below.

For the HRV-Ca/A16 construct, a pWR3.26 plasmid containing a complete HRV-C11 genome (GenBank accession no. EU840952) generated in our laboratory was digested with SalI and SfI. The HRV-Ca 5’ UTR was gel purified and cloned into the purified SalI/SfI-digested QCpHRV-A16 vector.

For all other constructs, PCR was used to amplify 5’ UTR products (using Platinum Taq DNA Polymerase High Fidelity; Invitrogen) directly from a clinical specimen (HRV-Cc, GenBank accession no. JN087518), an ATCC strain (HRV-B37, GenBank accession no. EF173423) or an infectious clone (plasmid pEV71; BrCr-TR, GenBank accession no. AB204852; kindly provided by A. Minetaro, National Institute of Infectious Diseases, Japan), using a SalI–T7 promoter-tagged forward primer and an SfI-tagged reverse primer (primers RV9–RV96; Supplementary Table S1). The PCR products were subcloned into a pCR2.1-TOPO vector (Invitrogen) before being digested by SalI and SfI and cloned into the purified SalI/SfI-digested QCpHRV-A16 vector.

The *in vitro*-transcribed parental QCpHRV-A16 and pEV71 (BrCr-TR) RNA (HEV-A71 in the text) were used as controls in various assays.

Cell culture and infection. HeLa Ohio cells (kindly provided by F. H. Hayden, University of Virginia, USA) were grown in Eagle’s minimum essential medium (Lonza) supplemented with 2 mM l-glutamine, 1 μg amphotericin ml⁻¹, 100 μg gentamicin ml⁻¹, 20 μg vancomycin ml⁻¹ and 10 % FCS at 37 °C in a 5 % CO₂ atmosphere. Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 2 mM l-glutamine, 100 μg penicillin/streptomycin ml⁻¹, 10 % FCS and 0.2 % NaHCO₃ at 37 °C in a 5 % CO₂ atmosphere. The infection media were used or 0.04 μg for the tenfold-diluted HRV-A16 RNA.

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Viral stocks were prepared as follows: RNA derived from HRV-Ca/A16, HRV-Cc/A16, HRV-B37/A16, HEV-A71/A16 and HRV-A16 constructs was transfected into HeLa Ohio cells (see below). Cells and supernatants were collected at 72 h post-transfection and submitted to three freeze (−80 °C)/thaw cycles. The resulting mixture was clarified and distributed into aliquots. The CCID₅₀ ml⁻¹ was determined for each virus stock using the method of Karber (1931).

Virus stocks were diluted in infection medium to 10⁵ CCID₅₀ ml⁻¹ to infect HeLa Ohio cells, as described elsewhere (Cordey et al., 2010). For virus passaging, 10 or 20 μl infected cell supernatant was added directly to the infection medium overlying fresh HeLa Ohio cells seeded in 12- or six-well plates, respectively. Virus was passaged every 48 h.

**In vitro transcription and transfection.** For each construct, 5–10 μg plasmid was linearized at a unique SacI restriction site downstream of the viral 3’ poly(A) tail. RNA transcripts were synthesized from their linear templates with a MEGAscript T7 kit (Ambion) for 3 h at 37 °C and purified with an RNeasy Mini kit (Qiagen). *In vitro*-transcribed RNA was quantified and checked by 0.1 % SDS/1 % agarose gel analysis. For single transfections in six- or 12-well plates, 2 or 1 μg in *in vitro*-transcribed RNA was transfected per well in HeLa Ohio or Vero cells using a TransMessenger Transfection Reagent kit (Qiagen), as described previously (Cordey et al., 2008). For co-transfection experiments, 0.4 μg RNA of each construct was used or 0.04 μg for the tenfold-diluted HRV-A16 RNA.

RNA extraction, reverse transcription, PCR, subcloning and sequencing. Transfected or infected HeLa Ohio cells were checked daily for cytopathic effect. At selected time points, 200 μl supernatant was used for RNA extraction with a NucleiSens easyMAG magnetic beads system (bioMérieux) according to the manufacturer’s instructions. Reverse transcription was performed with Superscript II (Invitrogen) and random hexamer primers (Roche). Primers 7 and P1.204 were used to identity the viruses resulting from the competition experiments (Supplementary Table S1).

PCR products were purified with Microcon columns (Millipore) before sequencing.

Gel purification was performed using a QIAEX II Gel Extraction kit (Qiagen), according to the manufacturer’s instructions.

PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen) before being ligated with SalI and SfI and cloned into the purified SalI/SfI-digested QCpHRV-A16 vector.

The 

**Quantitative real-time RT-PCR.** Reverse transcription was performed on RNA extracted from total cell lysates using oligo(dT) primers (Roche) to specifically quantify the plus-strand RNA. cDNA was analysed by reverse transcription using a reverse transcriptase enzyme (Promega) and random hexamer primers (Roche). Primers 7 and P1.204 were used to identify the viruses resulting from the competition experiments (Supplementary Table S1).

PCR products were purified with Microcon columns (Millipore) before sequencing.

Gel purification was performed using a QIAEX II Gel Extraction kit (Qiagen), according to the manufacturer’s instructions.

PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen), according to the manufacturer’s instructions. Minipreps were performed with a NucleoSpin Plasmid Miniprep kit (Macherey-Nagel) and sequenced with M13 forward and reverse primers.

Chromatograms produced with an ABI Prism 3130XL DNA Sequencer (Applied Biosystems) were imported directly for reading using Geneious Pro 5.0.3 software (Biomatters Ltd).

**Experimental HRV and HEV recombination**

http://vir.sgmjournals.org
thermocycler. Results were analysed using the sds version 1.4 program (Applied Biosystems). Viral amplicon C values were normalized to those of the housekeeping gene. Relative quantification was calculated using the 2−ΔΔCt method (Livak & Schmittgen, 2001). The quantitative HRV-A16 3D assay was run using a tenfold dilution series of the pR16.11 plasmid, which was used as a quantitative reference curve for each run.

**SDS-PAGE analyses and Western blotting.** Total cellular extracts, lysed in NP-40 lysis buffer [10 mM NaCl, 50 mM Tris/HCl (pH 8), 0.6% NP-40] supplemented with the protease inhibitors 2 mM 4-(2-aminoethyl)-benzenesulfonyl (Sigma-Aldrich) and aprotinin (diluted 1:1000; Sigma-Aldrich), were analysed by SDS-PAGE (15% acrylamide). After electrophoresis, the proteins were transferred using a semi-dry system onto PVDF membranes (Millipore). Blots were then incubated with a mouse anti-HRV-A16 mAb (kindly provided by W.-M. Lee) and a mouse anti-actin mAb (Millipore), followed by a goat anti-mouse HRP-conjugated secondary antibody (Bio-Rad). Protein detection was performed using an enhanced chemiluminescence system (Amersham Biosciences).

**Immunofluorescence.** At 48 h post-transfection, cells were washed twice with PBS lacking Ca2+ and Mg2+ (PBS−/−) and fixed for 20 min in methanol:acetone (1:1) at −20 °C. Cells were air dried for a few minutes at room temperature before incubation with the primary antibody, a mouse anti-HRV-16 mAb diluted 1:3000 in PBS−/−/1% BSA, for 45 min at 37 °C. After intensive washing with PBS−, FITC-conjugated anti-mouse IgG antibody (Light Diagnostics) was added to the cells for 45 min at 37 °C in the dark. Cells were then washed three times with PBS−− and stained with DAPI for 5 min at room temperature. After a final rinse with PBS−−, the coverslips were mounted in Fluoroprep mounting medium (bioMérieux).

**Quantification of immunofluorescence-positive cells.** Images were acquired with a Mirax microscope (Carl Zeiss) using a ×20 objective. Image analysis was performed with Metamorph/MetaXpress software ( Molecular Devices). The blue channel recorded DAPI-stained nuclei, whereas the green channel recorded cells marked with the FITC-coupled antibody. The first step of processing was separation of the two channels. Respective channels were converted from 8 to 16 bits by multiplication. The ‘CellScoring’ tool of the Metamorph software was applied to 16-bit versions of the blue and green channels. The parameters used to analyse the images were a cell minimal width of 13 μm and a maximal width of 40 μm, and an intensity above the local threshold of 20. Positive staining was determined in the cytoplasm (parameter ‘Stained area’). The reported data included total cell number, positive-cell number and their relative percentages.

**Phylogenetic trees based on RNA sequences.** RNA sequences for the 5′ UTR, CL and IRES were aligned using the MUSCLE program (Edgar, 2004). Well-aligned regions were extracted using Gblocks (Castresana, 2000) and maximum-likelihood trees were estimated using PhyML (Guindon et al., 2010). One hundred bootstrap replicates were performed using the general time reversible (GTR) model for correcting nucleotide substitution rates. All trees were rooted on the simian virus 2 (SV2) outgroup strain.

**Phylogenetic trees based on RNA structure distances.** Neighbour-joining trees were computed for RNA structures in the full 5′ UTR, CL and IRES. The respective sequences were extracted for each strain used and folded into their minimum free-folding energy structure using RNAfold (Hofacker et al., 1994). Secondary RNA structures were converted to a tree representation of the structure, and a tree-edit distance for all pairwise combinations of strains was computed using RNA distance from the Vienna RNA package (Hofacker, 2009). Distance matrices were used with the program NEIGHBOUR from the PHYLIP package (Felsenstein, 2005) to compute the final neighbour-joining trees on RNA structure distances. Strains that group closely together in the trees show more similarity regarding the underlying RNA secondary structures, which can be interconverted with fewer edit operations than distantly placed strains.

**ACKNOWLEDGEMENTS**

We would like to thank Chantal Gaille, Carole Bampi, Manel Essaidi and Geneviève Mottet-Osman for technical assistance. We also thank Dominique Garcin and Laurent Roux for useful suggestions. This study was supported by the Swiss National Science Foundation (grant 310030-127159 to C.T.; grant 32003B_127160 to L.K.) and partly supported by the Research Fund of the Department of Internal Medicine of the University Hospital and the Faculty of Medicine of Geneva; this Fund receives an unrestricted grant from AstraZeneca Switzerland, GlaxoSmithKline and Merck Sharp & Dohme.

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