Specificity and functional interaction of the polymerase complex proteins of human and avian metapneumoviruses

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INTRODUCTION

Human metapneumovirus (HMPV) is an enveloped, non-segmented, negative-strand RNA virus that causes respiratory tract illnesses, primarily in infants, young children, the frail elderly, and immunocompromised individuals (Crowe, 2004; Falsey et al., 2003; Kahn, 2006; Pelletier et al., 2002; van den Hoogen et al., 2001, 2003). HMPV is a member of the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus, and can be divided in two main genetic lineages (A and B) each consisting of two sublineages, A1 and A2, and B1 and B2 (van den Hoogen et al., 2004). The only other member of the genus Metapneumovirus is avian metapneumovirus (AMPV). AMPV has been found to infect domestic poultry worldwide, causing acute respiratory infections (Cook, 2000). AMPVs have been classified into four subgroups, A–D (Bayon-Auboyer et al., 1999; Eterradossi et al., 1995; Juhasz & Easton, 1994; Seal, 1998). AMPV-C was first detected in the USA and is more closely related to HMPV than the other AMPV subgroups (Govindarajan & Samal, 2004, 2005; Govindarajan et al., 2004; Toquin et al., 2003; van den Hoogen et al., 2002; Yunus et al., 2003). Human respiratory syncytial virus (HRSV) is the only other member of the subfamily Pneumovirinae that infects humans. In comparison with HRSV, metapneumoviruses lack the non-structural proteins NS1 and NS2, and the order of genes between the matrix (M) and large polymerase (L) is different: 3′-le-N-P-M-F-M2-SH-G-L-tr-5′ for HMPV and AMPV, and 3′-le-NS1-NS2-N-P-M-SH-G-F-M2-L-tr-5′ for HRSV.

The viral genome of all members of the subfamily Pneumovirinae is of antisense polarity and is assembled into a ribonucleoprotein complex (RNP). This RNP contains the viral genomic RNA (vRNA) encapsidated by the nucleocapsid protein (N), the phosphoprotein (P) and the large polymerase protein (L). By analogy with other paramyxoviruses, the L protein is responsible for the main catalytic activities of the viral polymerase complex
(Grdzelishvili et al., 2005; Hercyk et al., 1988; Ogino et al., 2005). The assembly and polymerase co-factor P and the L protein form the minimal complex needed for viral polymerase activity (Mazumder & Barik, 1994). HRSV RNA synthesis involves an additional viral protein, the M2.1 protein, a transcriptional elongation factor that enhances the synthesis of readthrough mRNAs (Collins et al., 1996; Fears & Collins, 1999; Hardy & Wertz, 1998). For HMPV, the function of M2.1 is not completely understood, as recombinant HMPV can be recovered in the absence of M2.1 and viruses from which the M2.1 gene is deleted grow efficiently in vitro but not in vivo (Buchholz et al., 2005; Herfst et al., 2004). The 3′ (leader) and 5′ (trailer) ends contain the viral promoters necessary for replication and transcription. Transcription of paramyxovirus is further directed by gene start (GS) and gene end (GE) sequences flanking each of the open reading frames (ORFs) in the viral genome. Transcription of the viral genome results in a gradient of transcripts, steadily decreasing towards the 5′ end of the genome. Thus, the gene order roughly reflects the relative amounts of gene products required for efficient virus replication (Lamb & Parks, 2007).

A tool frequently used for the analysis of cis- and trans-acting elements influencing viral RNA synthesis is minireplicon systems. In such systems, all components of the viral polymerase complex are transfected, and the replication and transcription of a synthetic vRNA-like molecule is measured using reporter genes. Exchanging proteins of the polymerase complex or synthetic vRNA-like molecules allows analysis of specificity and functional interactions. For the genera Respirovirus, Henipavirus and Pneumovirus of the family Paramyxoviridae, it has been shown that polymerase complexes provided by expression plasmids or co-infection can replicate vRNA-like molecules of other viruses belonging to the same genus (Halpin et al., 2004; Pelet et al., 1996; Yunus et al., 1999). vRNA-like molecules of the genus Morbillivirus are efficiently replicated by polymerase complex proteins of other morbilliviruses, but not or less efficiently by polymerase complexes consisting of proteins of two different morbilliviruses (Bailey et al., 2007; Brown et al., 2005). For pneumoviruses, it has been shown that vRNA-like molecules based on AMPV-A can be replicated by the polymerase complex proteins of HRSV (Marriott et al., 2001). For metapneumoviruses, it has been shown that polymerase complexes consisting of both human and avian metapneumovirus components are able to rescue virus from cDNA (Govindarajan et al., 2006).

Chimeric viruses in which polymerase genes are exchanged between two related viruses are frequently used to generate attenuated vaccine strains (Bailly et al., 2000; Govindarajan et al., 2006; Pham et al., 2005; Skiadopoulos et al., 2003). We hypothesized that minireplicon assays in which polymerase complex components of human and avian metapneumoviruses are exchanged could provide a rational basis for the design of live, attenuated metapneumovirus vaccine strains. To this end, an AMPV-C minireplicon system was generated and used in combination with minireplicon systems for HMPV-A1 and -B1. Each of these sets of metapneumovirus polymerase complex proteins was able to replicate synthetic vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C and HRSV, but not bovine parainfluenza virus 3 (BPIV-3). To test the functional interaction of polymerase complex proteins of HMPV-A1 and -B1 and AMPV-C, vRNA-like molecules were co-transfected with different combinations of N, P, L and M2.1 expression plasmids, revealing that chimeric polymerase complexes were functional but with different efficiencies. Subsequently, several chimeric viruses were created that contained polymerase complex genes of HMPV-A1 and -B1 or HMPV-B1 and AMPV-C. Most of these chimeric viruses replicated with similar efficiency to the wild-type viruses in vitro. A subset of these viruses was tested for attenuation in Syrian golden hamsters and was found to replicate to lower titres than the wild-type viruses. This study provides insight into the specificity and functional interaction of polymerase complex proteins of human and avian metapneumoviruses.

METHODS

Cells, media and viruses. Vero-118 cells were cultured in Iscove’s modified Dulbecco’s medium (BioWhittaker) supplemented with 10% fetal calf serum (FCS), 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM glutamine as described previously (Kuiken et al., 2004). Baby hamster kidney cells stably expressing T7 RNA polymerase (BSR-T7) (a kind gift of Dr K. Conzelmann; Buchholz et al., 1999) were grown in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) supplemented with 10% FCS, non-essential amino acids, 100 IU penicillin ml⁻¹, 10 μg streptomycin ml⁻¹, 2 mM glutamine and 0.5 mg G418 (Life Technologies) ml⁻¹. For HMPV rescue, Vero-118 cells and BSR-T7 cells were co-cultured in DMEM supplemented with 3% FCS, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 2 mM glutamine and 0.25 mg tetracycin ml⁻¹. For virus propagation and titration of HMPV-A1 and -B1, all chimeric viruses and AMPV-C (Colorado strain; Intervet), Vero-118 cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 4% BSA fraction V (Invitrogen), 100 IU penicillin ml⁻¹, 2 mM glutamine and 3.75 μg tetracycin ml⁻¹.

Plasmids. The minireplicon systems of HMPV-A1 and -B1 have been described previously (Herfst et al., 2004). The minireplicon system for AMPV-C was constructed using the same vectors, with primers designed on the basis of the published sequence of AMPV-C (GenBank accession no. AY579780). For construction of the AMPV-C vRNA-like molecule, the leader and the GS of N and the trailer and GE of L were amplified by PCR and ligated, separated by two BamBI sites. This fragment was ligated into a plasmid containing T7 RNA polymerase promoter (P₇) and terminator (T₇) sequences and a hepatitis delta ribozyme (pSP72-P₇-Tr-Le-Δ-T₇). The ORF of the chloramphenicol acetyltransferase (CAT) reporter gene was amplified by PCR and cloned in the BamBI sites between the GS of N and GE of L to yield pSP72-P₇-Tr-CAT-Le-Δ-T₇. For the construction of plasmids expressing the polymerase complex proteins, N, P and M2.1, ORFs of AMPV-C were amplified by PCR using primers spanning the start and stop codons and flanked by Nco and XhoI sites, respectively, and were cloned in the multiple cloning site of pCITE (Novagen) to yield plasmids pCITE-N, pCITE-P and pCITE-M2.1. Constructs encoding
the L gene of AMPV-C were assembled from overlapping PCR fragments using restriction sites in the L gene and were cloned in pCITE. The restriction sites used were NcoI (introduced at nt 6935 before the start codon of L), SceI (nt 8557), NdeI (nt 9770), and BclI (nt 11535) and XhoI (introduced at nt 13135 after the trailer). The minireplicon system of AMPV-A was a kind gift from Dr A. Easton (Naylor et al., 2004; Randhawa et al., 1997). The minireplicon systems of BPIV-3 and HRSV (Jin et al., 1998) were a kind gift from Dr R. Tang (MedImmune Vaccines, Inc., Mountain View, CA, USA).

The full-length HMPV cDNA plasmids for HMPV-A1 and -B1 have been described previously (Herfst et al., 2004). For the construction of the full-length chimeric HMPV-B1 cDNA plasmids containing the N, N and P, P, M2.1 or L of HMPV-A1 or the N, P or L of AMPV-C cDNA, fragments of HMPV-A1 were amplified by PCR and cloned into pCR4-TOPO (Invitrogen). All fragments were cloned such that the desired ORF and type II restriction sites flanking GS and GE were introduced. Using unique restriction sites, the fragments containing the desired ORF were swapped back into the full-length HMPV-B1 cDNA plasmids. All plasmid inserts were sequenced to ensure the absence of undesired mutations. All primer sequences are available upon request.

**Minigenome assays.** BSR-T7 cells grown to 80–95 % confluency in six-well plates were transfected with 1 µg of the vector expressing the vRNA-like molecule, 1 µg pCITE-N, 0.5 µg pCITE-P, 0.5 µg pCITE-L, 0.5 µg pCITE-M2.1 and 0.4 µg PT27 (a vector expressing β-galactosidase under the control of a cytomegalovirus immediate-early promoter; a kind gift from Dr. M. Malim, King’s College, London School of Medicine, UK). Cells were analysed 3 days after transfection by ELISA for CAT and β-galactosidase (Roche Diagnostics) according to the instructions of the manufacturer. All transfections were carried out in triplicate and CAT values were standardized to 10 ng β-galactosidase to control for transfection efficiency and sample processing.

**Recovery of recombinant virus.** Recovery of recombinant HMPV was performed as described previously (Herfst et al., 2004). Briefly, BSR-T7 cells were transfected for 5 h with 5 µg full-length HMPV cDNA plasmid, 2 µg pCITE-N, 2 µg pCITE-P, 1 µg pCITE-L and 1 µg pCITE-M2.1 using Lipofectamine 2000 (Invitrogen). The HMPV-B1 polymerase expression plasmid was used for the recovery of all chimeric HMPV-B1/HMPV-A1 and HMPV-B1/AMPV-C viruses. After transfection, the medium was replaced with fresh medium supplemented with trypsin. Three days after transfection, the BSR-T7 cells were scraped and co-cultured with Vero-118 cells for 8 days.

**Virus titrations.** Viruses were propagated in Vero-118 cells and virus titres were determined as described previously (Herfst et al., 2004). Confluent monolayers of Vero-118 cells in 96-well plates were spin-inoculated (15 min, 2000 g) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37 °C. After 2 h and again after 3–4 days, the inoculum was replaced with fresh infection medium. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV-specific polyclonal anti-serum raised in guinea pigs, as described previously (van den Hoogen et al., 2001). Titres expressed as TCID50 were calculated as described by Reed & Muench (1938).

**RESULTS**

**Replication of paramyxovirus vRNA-like molecules by heterologous polymerase complexes**

To determine whether the polymerase complexes of different members of the family Paramyxoviridae recognize heterologous templates, vRNA-like molecules containing a CAT ORF in antisense orientation flanked by the genomic termini of HMPV-A1 and -B1, AMPV-C or one of the chimeric virus strains at an m.o.i. of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed twice with medium before the addition of 7 ml fresh medium and incubation at 37 °C. Every day, 0.5 ml supernatant was collected and replaced with fresh medium. Plaque assays were performed to determine viral titres.

**Plaque assays.** Plaque assays were performed as described previously (Herfst et al., 2004), with minor adjustments. Twenty-four-well plates containing 95 % confluent monolayers of Vero-118 cells were inoculated with 10-fold serial virus dilutions for 1 h at 37 °C, after which the medium was replaced by 0.5 ml fresh medium and 0.5 ml 2 % methylcellulose (MSD) and cells were incubated at 37 °C for 4 days. Methylcellulose overlays were removed and cells were fixed with 80 % acetone. Cells were incubated with HMPV-specific polyclonal antiserum for 1 h at 37 °C, followed by incubation with horseradish peroxidase-labelled rabbit anti-guinea pig antibodies (DakoCytomation). Plaques were quantified after incubation with a freshly prepared solution of 3-amino-9-ethylcarbazole substrate chromogen (Sigma-Aldrich) to determine viral titres.

**Animal experiments.** Six-week-old, female Syrian golden hamsters (Mesocricetus auratus) (Harlan Sprague–Dawley) were inoculated intranasally with 106 TCID50 of virus in 100 µl, diluted in PBS. Four days after inoculation, lungs and nasal turbinates (NTs) were collected, snap-frozen immediately and stored at –80 °C until further processing. All intranasal inoculations and euthanasia were performed under anaesthesia with inhaled isoflurane. All animal studies were approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines. Tissues from the inoculated hamsters were homogenized using a Polytron homogenizer (Kinematica AG) in infection medium. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titres were calculated as TCID50 (g tissue)−1, with a detection limit of 101.6 and 101.2 TCID50 (g tissue)−1 for NT and lung samples, respectively.
homologous vRNA-like molecules, the reporter gene CAT was expressed efficiently (Fig. 1). Polymerase complex proteins of HMPV-A1 and -B1 and AMPV-C could replicate the vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C, and HRSV, but not BPIV-3. Conversely, the BPIV-3 polymerase complex only replicated the homologous vRNA-like molecule. The metapneumovirus polymerase complexes revealed little substrate specificity, as they replicated heterologous metapneumovirus vRNA-like molecules with similar efficiency to homologous molecules. vRNA-like molecules based on the HRSV genome were replicated less efficiently than the metapneumovirus vRNA-like molecules by the HMPV polymerase complexes.

Replication of metapneumovirus vRNA-like molecules by chimeric polymerase complexes

For morbilliviruses, it has been found that vRNA-like molecules can be replicated by heterologous polymerase complexes, but not or less efficiently by chimeric polymerase complexes (Bailey et al., 2007; Brown et al., 2005). To investigate the functional interaction between polymerase complex proteins of human and avian metapneumoviruses, the N, P, L and M2.1 expression plasmids were exchanged individually between the HMPV-A1 and -B1 and AMPV-C minireplicon systems (Fig. 2). All chimeric HMPV-A1/HMPV-B1 polymerase complexes were functional and replicated vRNA-like molecules with similar efficiency to the homologous complex protein sets (Fig. 2a and c). Chimeric polymerase complexes consisting of HMPV-A1 and AMPV-C or HMPV-B1 and AMPV-C components were functional but differed in their replication efficiency (Fig. 2b, d–f). Furthermore, HMPV-A1 and HMPV-B1 polymerase complex proteins appeared to be highly conserved, as they generally caused similar increases and decreases in replication efficiency when exchanged with those of AMPV-C (compare Fig. 2b and d or Fig. 2e and f). Chimeric HMPV-A1 (Fig. 2b) and HMPV-B1 (Fig. 2d) polymerase complexes in which the P protein was substituted with the P protein of AMPV-C were less efficient in the replication of vRNA-like molecules than the wild-type HMPV polymerase complexes. Smaller differences were observed when the N or M2.1 proteins were substituted. Chimeric polymerase complexes in which the HMPV-A1 or -B1 L protein was substituted with the L...
protein of AMPV-C replicated HMPV-A1 or -B1 vRNA-like molecules with higher efficiency compared with polymerase complexes consisting of HMPV-A1 or -B1 or AMPV-C proteins only (Fig. 2b, d). In agreement with this observation, chimeric polymerase complexes in which the AMPV-C L protein was substituted with the L protein of HMPV-A1 or -B1 replicated AMPV-C vRNA-like molecules with lower efficiency compared with polymerase complexes consisting of HMPV-A1 or -B1 or AMPV-C proteins only (Fig. 2e, f). Substitution of the P protein of AMPV-C with P of HMPV-A1 resulted in similar CAT expression, whereas substitution with P of HMPV-B1 resulted in lower CAT expression. Substitution of the N or M2.1 proteins had less of an impact on replication efficiency. It should be noted that the M2.1 expression plasmid of pneumovirus and metapneumovirus minireplicon systems can be omitted without significant effects on the levels of CAT (Collins et al., 1995, 1996; Herfst et al., 2004; Naylor et al., 2004).

Rescue of HMPV-B1 by chimeric polymerase complexes

As chimeric polymerase complexes consisting of human and avian metapneumovirus proteins revealed differences in minireplicon assays, we next tested whether it was possible to rescue recombinant HMPV using these chimeric polymerase complexes. The full-length HMPV-B1 cDNA plasmid was co-transfected into BSR-T7 cells with the N, P, L and M2.1 expression plasmids of HMPV-B1 or AMPV-C or sets in which the HMPV-B1 N, P, L and M2.1 expression plasmids were individually exchanged with those of AMPV-C. It was possible to rescue HMPV-B1 using the HMPV-B1, AMPV-C and all chimeric HMPV-B1/AMPV-C polymerase complexes (data not shown).

Replication characteristics of chimeric HMPV-B1/AMPV-C viruses in tissue culture

Minireplicon systems only include the components of the viral polymerase complex necessary for replication and transcription of the viral genome. To investigate the functionality of chimeric polymerase complexes in the context of a complete virus, a panel of chimeric viruses was made. The N, P and L genes of HMPV-B1 were replaced with those of AMPV-C, resulting in HMPV-B1/AMPV-C viruses. The level of replication of the chimeric viruses with those of the parental viruses HMPV-B1 and AMPV-C (Fig. 4). This revealed that AMPV-C replicated faster than its human counterpart HMPV-B1. Furthermore, HMPV-B1/LAMPV-C and HMPV-B1/NAMPV-C grew to similar titres as the backbone virus HMPV-B1. In contrast, HMPV-B1/PAMPV-C grew to higher titres than HMPV-B1.

Characterization of chimeric HMPV-B1/AMPV-C viruses in a hamster model

The level of replication of the chimeric HMPV-B1/AMPV-C viruses in the upper and lower respiratory tract was evaluated in Syrian golden hamsters, which represent a permissive small-animal model for HMPV (MacPhail et al., 2004). Five groups (n=6) of hamsters were intranasally inoculated with 10^6 TCID50 of the parental and chimeric viruses; the lungs and NT were harvested on day 4 post-infection, and the virus titres present in tissue homogenates were determined (Fig. 5). AMPV-C replicated to 100-fold higher titres in the lungs, but 10-fold lower titres in the NT
compared with HMPV-B1. The HMPV-B1/NAMPV-C and HMPV-B1/LAMPV-C chimeric viruses did not replicate in the lungs and replicated slightly less efficiently in the NT compared with HMPV-B1. HMPV-B1/P AMPV-C did not replicate in the lungs and resulted in 10 000-fold lower titres in the NT compared with HMPV-B1.

**DISCUSSION**

Here, a newly developed minireplicon system for AMPV-C has been described and used to study the specificity and functional interactions of the polymerase complex proteins of human and avian metapneumoviruses. As expected, replication and transcription of metapneumovirus vRNA-like molecules was efficient when the homologous virus polymerase complex proteins were present. Replication and transcription of vRNA-like molecules of HMPV-A1 and -B1, AMPV-A and -C and HRSV also occurred when heterologous polymerase complex proteins derived from HMPV-A1 or -B1 or AMPV-C were provided. However, these polymerase complex proteins were not able to replicate the vRNA-like molecules representing BPIV-3. Thus, the cis-acting elements in the genomic termini of HMPV-A1 and -B1, AMPV-A and -C and HRSV are conserved and functionally interchangeable. This is in agreement with the fact that the leader and trailer regions within the subfamily Pneumovirinae display a high degree of sequence conservation, but less so between pneumoviruses and BPIV-3 (Fig. 6). The N, P, L and M2.1 expression plasmids of AMPV-A and HRSV also gave rise to replication and transcription of vRNA-like molecules derived from viruses of the same genus but not other genera (data not shown). Because the polymerase protein expression vectors for AMPV-A and HRSV were far less efficient than for HMPV-A1 and -B1, AMPV-C and BPIV-3 under our experimental conditions, solid conclusions about specificity and functional interactions could not be made. However, it has been shown previously that HRSV
Chimeric metapneumovirus polymerase complexes

Chimeric polymerase complexes of members of the family Paramyxoviridae vary in their ability to replicate vRNA-like molecules or rescue recombinant virus (Bailey et al., 2007; Brown et al., 2005; Govindarajan et al., 2006). Exchanging polymerase genes between two related viruses with a different host range is a method frequently used for the design of live, attenuated metapneumovirus vaccine strains. To this end, HMPV-A1 and -B1 and AMPV-C vRNA-like molecules were co-transfected with different combinations of N, P, L and M2.1 expression plasmids to investigate the functional interaction between the polymerase complex proteins. All chimeric polymerase complexes based on HMPV-A1 and -B1 were functional and displayed similar replication and transcription efficiency to the wild-type polymerase complexes. In contrast, when polymerase complex proteins were exchanged between human and avian metapneumoviruses, CAT expression levels varied significantly. The most striking differences in CAT expression were observed when the L or P expression plasmid was exchanged. When the P proteins of AMPVs were replaced with the P protein of AMPV-C, CAT expression was lower than when the homologous P protein was used. When the L proteins of AMPVs were replaced with the L protein of AMPV-C, CAT expression increased compared with the wild-type polymerase complex, and, conversely, when the L protein of AMPV-C was replaced with L of HMPVs, CAT expression decreased. Exchange of the N or M2.1 proteins between the HMPVs and AMPV-C in general had a modest effect. These data suggested that chimeric viruses consisting of HMPV-A1 and -B1 genes would replicate similarly to wild-type virus, whilst chimeras based on HMPV and AMPV-C, in particular those where L or P are exchanged, could display differences in virus replication.

Indeed, growth curves of chimeric HMPV-B1 viruses in which the N, P, L or M2.1 gene was replaced with that of HMPV-A1 revealed similar replication kinetics to the wild-type HMPV-B1. However, replication kinetics of HMPV-B1 with the N, P or L gene replaced with that of AMPV-C only showed some differences, and these differences were unexpected: exchange of P resulted in low activity in minigenome assays but high virus production, whereas exchange of L resulted in high activity in minigenome assays but had no effect on virus replication. Thus, there was poor agreement between minigenome assays and virus replication.

In an attempt to explain this discrepancy, chimeric HMPV-B1/HMPV-A1 and HMPV-B1/AMPV-C viruses were rescued with reverse genetics, and wild-type HMPV-B1 virus was rescued using chimeric polymerase complexes. However, because the resolution of the latter type of assay to read out polymerase activity is low, no differences in virus rescue efficiency were observed (data not shown). It was also tested whether differences in results obtained with minigenome and virus replication assays were due to the choice of cell line of BSR-T7 and Vero-118, respectively. Minigenome assays performed with 293T and Vero-118 cells revealed similar trends in activity of HMPV-B1/AMPV-C polymerase complexes to those in Fig. 2(d), with lower activity of N and P chimeric complexes, and relatively high CAT activity of the L chimeric complex. Moreover, when virus replication assays were performed in BSR-T7 cells, the same cell line used for minigenome assays, there was still no agreement between the minigenome assays and virus replication data (data not shown).

The functionality of chimeric polymerase complexes was next tested in a hamster model, using chimeric viruses that displayed the largest differences in minireplicon assays and virus replication curves: those based on HMPV-B1 and AMPV-C. Each of the three chimeric viruses, HMPV-B1/N<sub>AMPV</sub>-C, HMPV-B1/P<sub>AMPV</sub>-C and HMPV-B1/M2<sub>AMPV</sub>-C, displayed some level of attenuation in the respiratory tract of inoculated hamsters. The virus that replicated to the highest titres in vitro, HMPV-B1/P<sub>AMPV</sub>-C, was the most attenuated in hamsters with more than 10 000-fold reduced viral titres in the NT and undetectable virus titres in the lungs. Whilst this could suggest that minireplicon assays are more predictive for attenuation than in vitro replication assays, the opposite was true for the L chimera, which was

![Fig. 6. Alignment of HMPV-A1, HMPV-B1, AMPV-C, AMPV-A, HRSV and BPIV-3 leader and trailer sequences. Differences in sequence compared with HMPV are underlined.](http://vir.sgmjournals.org)
attenuated in hamster but revealed increased levels of CAT expression in minigenome assays.

Exchanging genes between two related paramyxoviruses with different host range or replication properties has been shown to be useful for the rational design of live attenuated vaccine strains (Baill, et al., 2000; Govindarajan et al., 2006; Pham et al., 2005; Skiadopoulos et al., 2003). Intuitively, the level of attenuation of such chimeric viruses would increase when the relatedness of the two paramyxoviruses decreases. Our in vitro results suggest that this is indeed the case. Gene exchange between two HMPVs had no discernible effect on polymerase complex activity in vitro. Gene exchange between HMPV and AMPV-C resulted in modest differences in polymerase complex activity, both in vitro and in vivo. It is likely that gene exchange between HMPV and AMPV-A, HRSV and other paramyxoviruses such as BPIV-3 would result in higher levels of attenuation with decreasing relatedness of the viruses.

For efficacious live, attenuated vaccine candidates, a proper balance between the level of attenuation and immunogenicity is crucial. In this context, evaluation of vaccine candidates in more than one animal model is needed. Recently, Pham et al. (2005) evaluated chimeric HMPV/AMPV-C viruses in hamsters and African green monkeys. Recombinant HMPV (lineage A2) in which the N or P gene was replaced by that of AMPV-C was found to grow to high titres in cell culture but was attenuated in hamsters and African green monkeys, indicating that such strains are promising HMPV vaccine candidates. Further studies in animal models and humans are required to study the level of attenuation, immunogenicity and stability of such live, attenuated vaccines; exchange of multiple genes rather than just one and/or exchange of genes from more distantly related viruses may be required to design the ideal live, attenuated vaccine.

From these studies, we conclude that neither minireplicon assays nor in vitro replication kinetics can be used as predictive models for attenuation of metapneumoviruses. Whilst minireplicon assays and in vitro replication assays remain crucial tools to study fundamental aspects of virus transcription and replication, animal model systems remain indispensable to evaluate the level of attenuation of, for example, live, attenuated vaccine candidates.

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