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Chimeric Recombinant Human Metapneumoviruses with the Nucleoprotein or Phosphoprotein Open Reading Frame Replaced by That of Avian Metapneumovirus Exhibit Improved Growth In Vitro and Attenuation In Vivo

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Chimeric versions of recombinant human metapneumovirus (HMPV) were generated by replacing the nucleoprotein (N) or phosphoprotein (P) open reading frame with its counterpart from the closely related avian metapneumovirus (AMPV) subgroup C. In Vero cells, AMPV replicated to an approximately 100-fold-higher titer than HMPV. Surprisingly, the N and P chimeric viruses replicated to a peak titer that was 11- and 25-fold higher, respectively, than that of parental HMPV. The basis for this effect is not known but was not due to obvious changes in the efficiency of gene expression. AMPV and the N and P chimeras were evaluated for replication, immunogenicity, and protective efficacy in hamsters. AMPV was attenuated compared to HMPV in this mammalian host on day 5 postinfection, but not on day 3, and only in the nasal turbinates. In contrast, the N and P chimeras were reduced approximately 100-fold in both the upper and lower respiratory tract on day 3 postinfection, although there was little difference by day 5. The N and P chimeras induced a high level of neutralizing serum antibodies and protective efficacy against HMPV; AMPV was only weakly immunogenic and protective against HMPV challenge, reflecting antigenic differences. In African green monkeys immunized intranasally and intratracheally, the mean peak titer of the P chimera was reduced 100- and 1,000-fold in the upper and lower respiratory tracts, whereas the N chimera was reduced only 10-fold in the lower respiratory tract. Both chimeras were comparable to wild-type HMPV in immunogenicity and protective efficacy. Thus, the P chimera is a promising live HMPV vaccine candidate that paradoxically combines improved growth in vitro with attenuation in vivo.

Human metapneumovirus (HMPV) is an enveloped RNA virus of the genus *Metapneumovirus*, subfamily *Pneumovirinae*, family *Paramyxoviridae*, order *Mononegavirales*. HMPV was first described in 2001 (34) and is now regarded as an important viral respiratory pathogen of worldwide distribution (9, 20, 35). Its clinical impact warrants the development of a vaccine, particularly for the pediatric population. Avian metapneumovirus (AMPV) is a closely related virus that causes respiratory disease in turkeys, chickens, and other birds (24).

HMPV has a nonsegmented negative-strand RNA genome of approximately 13.3 kb that contains eight genes in the order 3′-N-P-M-F-M2-SH-G-L-5′ (5, 33). As is the case for other members of *Mononegavirales*, the nucleoprotein N, the phosphoprotein P, the RNA-dependent RNA polymerase L, and the viral genomic RNA form the ribonucleoprotein complex, which is the minimal replication/transcription unit. The other genes encode matrix protein M, fusion protein F, M2 mRNA, small hydrophobic glycoprotein SH of unknown function, and putative attachment glycoprotein G. The genomic RNA and its positive-sense replicative intermediate, the antigenomic RNA, are tightly encapsidated by the N protein. The N and L proteins are among the more highly conserved proteins between different *Paramyxoviridae* species, whereas P is somewhat more variable. These proteins often exhibit functional interchangeability, albeit with reduced efficiency, between closely related species (19, 26, 27, 36). HMPV also encodes M2-1 (product of the first open reading frame [ORF] in the M2 mRNA) and M2-2 (product of the second ORF in the M2 mRNA) proteins that appear to have roles in RNA synthesis (10).

An innovative method for creating live attenuated vaccines is to genetically modify the virus of interest such that its major neutralization and protective antigen genes are left undisturbed while replacing one or more of the other genes or open reading frames with the counterpart(s) from a closely related virus that has a different natural host. This is based on the premise that a given virus has evolved to operate optimally in its respective natural host. Thus, replacing one or more genes with the corresponding gene(s) from a related virus with a different natural host can result in a restriction of replication in vivo due to host incompatibility. Success with this strategy requires that the introduced gene(s) functions sufficiently well in the heterologous background for efficient growth in vitro, which is necessary for vaccine manufacture. There have been several reports of vaccine candidates made in this way. For example, chimeric vaccine candidates for human rotavirus were made using reassortment to place the human rotavirus VP7 gene into the genetic background of rhesus or bovine rotavirus (15). In

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the case of mononegaviruses, reverse genetics has been used to generate host-range-restricted vaccine candidates based on chimeras between rinderpest and peste-de-petits-ruminants viruses, between human and bovine parainfluenza type 3 viruses, and between human and bovine respiratory syncytial viruses (1, 12, 16, 28, 31).

HMPV was a candidate for this approach because it has a closely related animal counterpart, AMPV. One aspect that makes this approach attractive is that both viruses replicate reasonably well in Vero cells, which is a suitable substrate for preparing vaccines for human use. Thus, it was possible that substituting one or more genes or ORFs from AMPV into HMPV would yield recombinants that retain competence for replication in Vero cells but might be satisfactorily attenuated in vivo. Such recombinants would bear the major protective surface antigens of HMPV in an attenuated backbone and would be suitable for evaluation as a live vaccine.

In the present work, we have generated chimeras in which the N or P ORF of HMPV was replaced with that of AMPV subtype C, which is the subtype most closely related to HMPV. At the amino acid level, the AMPV N and P proteins share 89 and 68% identity with their HMPV counterparts, and the lengths of these proteins (394 amino acids for N and 294 amino acids for P) are identical between AMPV and HMPV. At the nucleotide level, the N and P genes are 75% and 68% identical between AMPV and HMPV (5, 33). For both viruses, the N and P mRNAs do not contain additional significant ORFs. Surprisingly, the resulting chimeras replicated more efficiently in vitro than their HMPV parent; nonetheless, they were attenuated in hamsters. Further evaluation in African green monkeys (AGMs) identified the P chimera as an attractive vaccine candidate suitable for clinical evaluation.

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were grown in OptiProSFM (Invitrogen) supplemented with 4 mM L-glutamine. BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that constitutively express T7 RNA polymerase (11). They were maintained in Glasgow minimal essential medium supplemented with glutamine, amino acids (Invitrogen), and 10% fetal bovine serum. The Canadian HMPV clinical isolate CAN97-83 (5) and its recombinant derivatives were propagated in Vero cells at 32°C in the absence of serum and the presence of 5 μg/ml of trypsin. Trypsin was replenished every second day as previously described (6). AMPV subtype C, strain Colorado (17), was a generous gift from Siba Samal, University of Maryland, and was propagated on Vero cells in the presence of 3% fetal bovine serum without trypsin.

Construction and recovery of recombinant HMPV containing the N or P ORF of AMPV. A reverse genetic system for HMPV based on HMPV CAN97-83 (GenBank accession number AY297749) was described previously (6). Compared to the clinical isolate, wild-type recombinant HMPV differs by only four nucleotide substitutions that create an NheI restriction site in the M-F intergenic region as a marker (Fig. 1C). In the present study, we used a wild-type derivative in which the naturally occurring SH gene was replaced with a modified version called "stabilized SH." The stabilized SH gene was created by modifying a number of naturally occurring A and T homo-oligomer tracts in the SH ORF so that selected A or T residues were replaced by C or G without changing amino acid coding (not shown). This was done because we recently realized that some virus preparations appear to contain subpopulations with one or more small insertions in these tracts within the SH gene, perhaps due to stuttering by the viral polymerase. This phenomenon is being studied and will be reported in detail in the future. Recombinant HMPV bearing the stabilized SH gene (rHMPV) replicated with the same efficiency as unmodified recombinant HMPV in vitro and in the respiratory tracts of hamsters and AGMs (not shown). Thus, recombinant HMPV with the stabilized SH gene has the phenotype of a wild-type virus, as might be expected. It was used as the parent to create the N and P chimeras in the present study (rHMPV-N_A and rHMPV-P_A).

Antigenome cDNAs encoding rHMPV-N_A and rHMPV-P_A were constructed as outlined in Fig. 1. PCR fragments were prepared containing either the HMPV or AMPV N ORF (1,182 nucleotides [nt] in length) and including the naturally occurring upstream MluI site (HMPV nt 12) preceding the N-gene start signal as well as a newly added BbsI or BsmBI site, respectively, in the downstream end of the fragment (Fig. 1A). To introduce the BbsI or BsmBI site, the sequence corresponding to positions 1242 to 1248 in the antigenome, 5′-AAAAAAG-3′, was changed to contain a BbsI site (5′-AGTCTTC-3′ [changes underlined]) or a BsmBI site (5′-GAGACGG [changes underlined]), using PCR primers bearing these changes.

PCR fragments containing either the HMPV or AMPV P ORF (882 nt) were designed so that the upstream end contained a BfuAI site (the sequence corresponding to positions 1227 to 1232 in the antigenome, 5′-TGATTA-3′, was changed to 5′-ACCTGC [substitutions underlined]), HMPV N-gene end signal, N/P intergenic region, and P-gene start signal, while the downstream end was modified to contain another BfuAI site (the sequence corresponding to positions 2152 to 2157 in the antigenome, 5′-ATAAAA, was changed to 5′-GCAGGT [substitutions underlined]; Fig. 1B). A PCR fragment containing the HMPV M ORF was designed so that the upstream end contained a BbsI site (the sequence corresponding to positions 2136 to 2141 in the antigenome, 5′-TTAATT, was changed to 5′-GAAGAC [substitutions underlined]), P-gene end, intergenic region, and M-gene start signal, while the downstream end contained the already-existing NheI site (Fig. 1C).

Assembly of the chimeric viruses is shown in Fig. 1D. Cleavage of the BbsI or BsmBI site at the downstream ends of the N fragments would remove the recognition sequences and yield 5′-TAAT overhangs, which were designed to be compatible with the 3′-ATTA overhangs left by cleavage with BfuAI at the upstream end of the P fragment (Fig. 1D). The BfuAI site at the downstream end of the P fragments was designed to leave a 5′-GTAG overhang, which could be compatible with the 3′-CATC overhang left by BbsI cleavage at the upstream end of the M ORF (Fig. 1D). Note that the cleavage sites for BbsI, BsmBI, and BfuAI are located apart from their respective recognition sites and are not sequence specific, permitting the overhangs to be designed as needed. Also, these restriction enzyme recognition sites, with all of the substitutions, are removed from the DNA fragments by digestion with the restriction enzymes, leaving the sequence and nucleotide length of the genes unaffected. The N, P, and M PCR fragments were individually cloned in pBluescript, sequenced, and then excised by digestion with the restriction enzymes noted above. To assemble each full-length antigenome plasmid, the 3-kb MluI-NheI fragment containing the N, P, and M genes was excised from the complete rHMPV-SHs antigenomic cDNA, and this MluI-NheI window was used to accept the N, P, and M fragments in a four-fragment ligation (Fig. 1D). The antigenome cDNAs were cloned, and correct structures were confirmed by restriction enzyme digestion and sequencing.

The HMPV/AMPV chimeric viruses were recovered from transfected BSR T7/5 cells. Confluent BSR T7/5 cells in six-well dishes were transfected with 5 μg of an individual antigenome plasmid, 2 μg each of the support plasmids pT7-N and pT7-P, and 1 μg each of pT7-M2-1 and pT7-L per well. Transfections were done with Lipofectamine 2000 (Invitrogen) in OptiMEM without trypsin or serum and maintained overnight at 32°C. The transfection medium was removed 1 day later and replaced with Glasgow minimal essential medium without trypsin or serum. Trypsin was added on day 3 to a final concentration of 5 μg/ml, and cell-medium mixtures were passaged onto fresh Vero cells on day 6.

The genome of each recovered virus was analyzed by reverse transcriptase PCR (RT-PCR) performed on total RNA from infected Vero cells. Two regions were amplified: nt 1 to 2287, containing the leader, N, P, and upstream end of M; and nt 4656 to 7499, containing the M2, SH, G, and upstream end of L. Parallel reaction mixtures that lacked RT enzyme did not yield detectable product, indicating that the template was RNA and not contaminating DNA. Each amplified product was sequenced in its entirety from uncloned material, showing that each virus contained the designed sequence free of adventitious mutations.

Evaluation in hamsters. Groups of 6-week-old Golden Syrian hamsters (18 animals per group) were infected intranasally, under light methoxyflurane anesthesia, with 0.1 ml of L15 medium containing $10^{5.7}$ PFU of rHMPV, rHMPV-N_A, rHMPV-P_A, or AMPV. On days 3 and 5 postinfection, six animals from each group were sacrificed, and the lungs and nasal turbinates were harvested. Virus titers were determined by plaque assay of tissue homogenates on Vero cells under methylcellulose overlay containing 5 μg/ml trypsin. The cultures were incubated at 32°C for 6 days, and plaques were visualized by immunostaining with a rabbit antiserum raised against gradient-purified CAN97-83 (6). The remaining six animals in each group were used to measure immunogenicity and protective efficacy. Sera were collected 2 days prior to infection and 27 days postinfection. The titers of HMPV-neutralizing antibodies were determined by an endpoint dilution neutralization assay on Vero cells as described previously

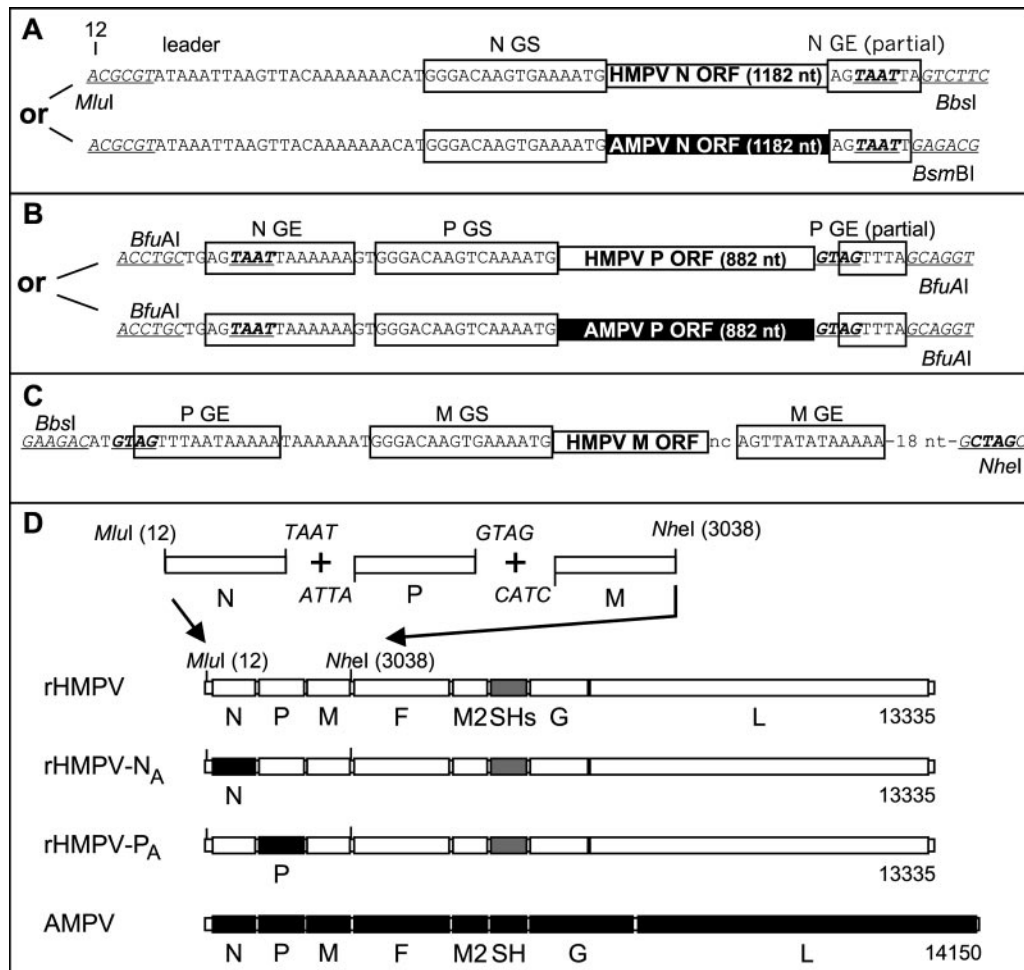

FIG. 1. Structures of the engineered N (A), P (B), and M (C) genes and flanking sequences and strategy for constructing HMPV/AMPV chimeras (D). The N and P genes and flanking sequences in panels A and B are shown with the HMPV construct on top and the HMPV/AMPV chimeric construct underneath. The BbsI, BsmBI, and BfuAI sites in panels A to C were introduced by PCR adapters to facilitate assembly; the MluI site (panel A) occurred naturally and the NheI site (panel C) had been introduced previously. Restriction sites are in italic type and underlined; the 4-nt compatible cohesive ends generated by cleavage with the respective enzymes are shown in bold italic type and underlined. The sequences of gene start (GS) and gene end (GE) signals are boxed. Panel D illustrates assembly of the N, P, and M fragments and shows the genomes of the resulting recombinants. HMPV genes are shown in white, and AMPV genes are shown in black. The synthetic SH gene (SHs) is gray.

(8). On day 28 postinfection, hamsters were challenged by the intranasal administration of $10^{5.7}$ PFU of HMPV CAN97-83 in a 0.1-ml inoculum under light methoxyflurane anesthesia. Nasal turbinates and lungs were harvested 3 days later, and the virus titer in each tissue homogenate was determined by plaque assay as described above.

Evaluation in AGMs. Young adult AGMs (*Chlorocebus aethiops*, imported from St. Kitts in the Caribbean; average weight, 4.4 ± 0.6 kg) were determined to be negative for serum-neutralizing antibodies to HMPV. The animals were inoculated simultaneously by the intranasal and intratracheal routes with a 1-ml inoculum per site containing $10^{6.0}$ PFU of rHMPV, rHMPV-N_A, or rHMPV-P_A in L15 medium (Invitrogen). A mock-infected control group received L15 medium alone. Each group included four animals except for the mock-infected group, which contained two animals. Clinical observations were made on days 0 to 12 following inoculation. Nasopharyngeal (NP) swabs were collected daily for 10 days following inoculation and on day 12. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, 10, and 12 postinfection. The amount of virus present in NP and TL samples was quantified by plaque assay as described above. On day 28 each monkey was challenged by the intranasal and intratracheal routes with $10^{6.0}$ PFU of CAN97-83 in a 1.0-ml volume per site. Virus shedding was examined in NP and TL samples collected on days 2, 4, 6, and 8 postchallenge. Serum samples were collected on days 0, 28, and 56, and the titers of HMPV-neutralizing antibodies were determined by the endpoint dilution assay noted above (8).

RESULTS

Recovery of recombinant HMPV bearing the AMPV N or P ORF. Reverse genetics was used to design HMPV mutants in which the N or P ORF was replaced by its counterpart from AMPV subtype C, the subtype that is most closely related to HMPV. The cloning strategy was designed to place each AMPV ORF under the control of the existing set of HMPV gene start and gene end signals. The PCR adapters used for cloning of the N and P ORFs contained nonpalindromic BbsI, BsmBI, and BfuAI restriction sites that yielded compatible overhangs, while excising the recognition sites; this facilitated the construction and did not introduce any mutations into the final antigenomic cDNAs (Materials and Methods and Fig. 1).

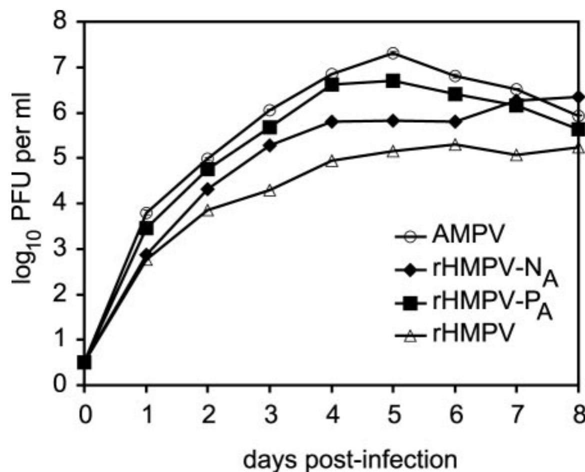

FIG. 2. Comparison of the multistep growth kinetics of rHMPV, rHMPV-N, rHMPV-P_A, and AMPV. Vero cells were infected at a MOI of 0.01 PFU per cell. At 24-h intervals, medium samples (0.5 ml of the 2-ml overlay) were taken and replaced by an equivalent volume of fresh medium containing 5 μg/ml of trypsin; in this case, the AMPV cultures also contained trypsin. The samples were analyzed by plaque assay on Vero cells. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown. The detection limit was 5 PFU per ml.

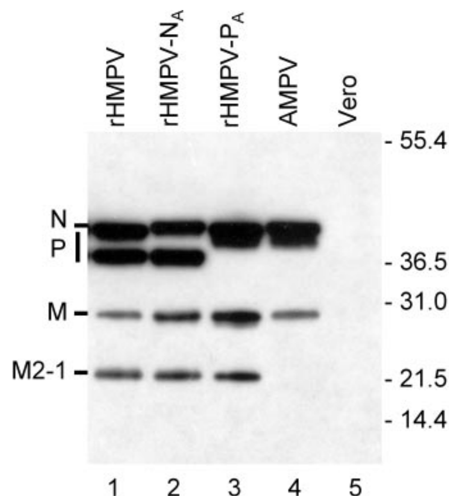

FIG. 3. Western blot analysis of viral proteins expressed in Vero cells infected at an input MOI of 1 PFU per cell with rHMPV (lane 1), rHMPV-N_A (lane 2), rHMPV-P_A (lane 3), or AMPV (lane 4) or mock infected (lane 5). The cells were harvested 72 h after infection, and lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and denaturing conditions using a 10% gel (NuPAGE; Invitrogen). After Western blot transfer to nitrocellulose, the membrane was incubated with a rabbit hyperimmune serum against purified HMPV followed by goat anti-rabbit antibodies conjugated with horseradish peroxidase, and the bound antibodies were visualized by chemiluminescence. The positions of the N, P, M, and M2-1 proteins are shown to the left of the gel. The positions of molecular mass markers (in kDa) are indicated to the right of the gel.

Thus, each ORF was swapped without introducing any changes to the gene start and gene end signals, noncoding regions, or the flanking intergenic regions (Fig. 1).

To recover the recombinant viruses, the individual antigenomic plasmids were transfected, together with a set of four support plasmids encoding the HMPV N, P, M2-1, and L proteins into BHK21 cells that constitutively expresses T7 RNA polymerase (11). The recovered viruses were amplified on Vero cells. Correct structures for the recovered viral genomes were verified by direct sequencing of RT-PCR products covering the leader region, N, P, and upstream end of the M gene (nt 1 to 2287) and M2, SH, G, and the upstream end of the L gene (nt 4656 to 7499). This confirmed that these genome regions were as designed and lacked adventitious mutations.

Multicycle growth of the N and P chimeric viruses in vitro. To evaluate the ability of the chimeric viruses to grow in vitro, replicate cultures of Vero cells were infected with the N and P chimeras in parallel with their rHMPV and AMPV parents at a multiplicity of infection (MOI) of 0.01 PFU/cell. At 24-h intervals, medium supernatant samples were harvested, frozen, and analyzed later in parallel by plaque titration (Fig. 2). AMPV grew to a peak titer of $10^{7.3}$ PFU per ml on day 5 postinfection, which was approximately 100-fold higher than the peak titer for rHMPV. The N and P chimeric viruses grew more efficiently than rHMPV: the replication of rHMPV-P_A was 25-fold higher than that of rHMPV and was almost as high as that of AMPV, whereas the peak titer of rHMPV-N_A was 11-fold higher than that of rHMPV.

Viral protein synthesis in infected cells. The viral proteins produced in virus-infected cells were analyzed in order to confirm the expression of the AMPV N and P proteins by the N and P chimeras, as well as to evaluate the overall level of gene expression. Vero cells were infected at a MOI of 1 PFU per cell with the N and P chimeric viruses or their rHMPV and AMPV parents. After 72 h of incubation, cell lysates were prepared and subjected to Western blot analysis using a polyclonal serum raised against gradient-purified HMPV (6).

In the rHMPV sample (Fig. 3, lane 1), the HMPV-specific antiserum detected bands that we previously identified as N, P, and M2-1 on the basis of analysis of proteins expressed from transfected cDNAs and virus with the M_2 gene deleted; in addition, another band was detected of the appropriate size to be the M protein (10). The HMPV glycoproteins were not detected in this Western blot, which probably is a consequence of the highly denaturing conditions as previously noted (10). The HMPV-specific serum was also cross-reactive with AMPV (Fig. 3, lane 4), detecting bands of approximately 42, 41, and 30 kDa, which are of the appropriate sizes to be the N, P, and M proteins, respectively. The AMPV N and P proteins migrated more slowly than their HMPV counterparts despite their nearly identical calculated molecular weights: the difference was slight in the case of N and was more considerable in the case of P. In the samples from cells infected with the N and P chimeric viruses (Fig. 3, lanes 2 and 3), bands corresponding to the AMPV N and P proteins could be distinguished in the appropriate lanes by this difference in electrophoretic mobility, confirming expression of the correct AMPV proteins by the appropriate viruses.

The Western blot analysis in Fig. 3 also provided a rough comparison of the level of gene expression by the chimeric viruses compared to rHMPV. This was of particular interest given the more efficient in vitro growth of the chimeric viruses,

TABLE 1. Level of replication of the HMPV/AMPV chimeras in the upper and lower respiratory tracts of hamsters

Virus[a]	Day of harvest	Nasal turbinate			Lung		
		% with detectable virus	Mean titer ($\log_{10}$ PFU/g of tissue ± ME)[b]	Reduction of mean titer[c] ($\log_{10}$ PFU/g of tissue)	% with detectable virus	Mean titer ($\log_{10}$ PFU/g of tissue ± ME)	Reduction of mean titer ($\log_{10}$ PFU/g of tissue)
rHMPV	3	100	5.4 ± 0.2		100	3.7 ± 0.3	
rHMPV-N_A	3	83	3.1 ± 0.3[d]	2.3	66	1.7 ± 0.0[d]	2.0
rHMPV-P_A	3	100	3.6 ± 0.3	1.8	33	1.8 ± 0.1	1.9
AMPV	3	100	5.3 ± 0.1	0.1	83	4.3 ± 0.4	
rHMPV	5	100	6.5 ± 0.1		17	1.7 ± 0.0	
rHMPV-N_A	5	100	5.8 ± 0.2	0.7	50	1.7 ± 0.0	
rHMPV-P_A	5	100	5.1 ± 0.3	1.4	50	2.1 ± 0.2	
AMPV	5	83	3.4 ± 0.3	3.1	67	1.9 ± 0.1	

[a] Hamsters in groups of 12 were infected intranasally with $10^{5.7}$ PFU of the indicated virus on day 0.
[b] Six animals from each group were sacrificed on days 3 and 5. Nasal turbinates and lungs were harvested, and virus titers were determined by plaque assay. ME, mean error.
[c] Reduction of mean $\log_{10}$ titer compared to that of rHMPV.
[d] The value of 50 PFU per g of tissue (the lower limit of virus detection) was assigned to animals lacking detectable virus in order to calculate the mean titer.

in particular rHMPV-P_A. Because the serum used in the blot was HMPV specific, it presumably would react less efficiently with AMPV proteins due to antigenic differences. Nonetheless, comparison of the level of expression of a given protein between different viruses would be valid as long as the protein in each case had the same species origin, i.e., HMPV or AMPV. For example, comparison could be made between rHMPV and rHMPV-N_A with regard to the P, M, and M2-1 proteins, since these proteins are HMPV specific in each virus. Similarly, comparison could be made between rHMPV and rHMPV-P_A for the HMPV-specific N, M, and M2-1 proteins. Also, the AMPV N and P proteins expressed by rHMPV-N_A and rHMPV-P_A, respectively, could be compared to those expressed by AMPV, since these are all AMPV specific. In general, the levels of accumulation of the various viral proteins were similar for the various viruses. This suggests that the increased efficiency of replication by the N and P chimeras in vitro was not due to substantial increases in intracellular viral gene expression as assayed by intracellular protein accumulation.

Replication, immunogenicity, and protective efficacy of the N and P chimeric viruses in hamsters. To assay the ability of the chimeras to replicate in vivo, hamsters in groups of 18 were infected intranasally with the N and P chimeric viruses in parallel with rHMPV and AMPV. Six animals from each group were sacrificed on days 3 and 5 following inoculation, and the lungs and nasal turbinates were harvested and analyzed for infectious virus. rHMPV replicated to mean titers of $10^{5.4}$ and $10^{6.5}$ PFU per g of tissue in the upper respiratory tract on days 3 and 5 and to $10^{3.7}$ and $10^{1.7}$ PFU in the lower respiratory tract on days 3 and 5, respectively (Table 1). The level of replication of AMPV in the nasal turbinates and lungs on day 3 was essentially the same as for rHMPV, and on day 5 its titer in the lungs was also comparable to that of rHMPV, whereas in the nasal turbinates it was reduced by 3.1 $\log_{10}$. Thus, there was evidence of host range restriction, but on only one of the two days sampled and only in the nasal turbinates. The titers of the N and P chimeric viruses on day 3 were reduced approximately 100-fold in both the upper and lower respiratory tract compared to rHMPV, with rHMPV-N_A being slightly more reduced than the P chimera. On day 5, only the P chimera was substantially lower (1.4 $\log_{10}$) than rHMPV and only in the nasal turbinates.

For the six animals remaining in each of the rHMPV-N_A, rHMPV-P_A, rHMPV, and AMPV groups, serum samples were

TABLE 2. Immunogenicity and protective efficiency of the HMPV/AMPV chimeras in hamsters

Immunizing virus[a]	Mean HMPV-neutralizing serum antibody titer prior to challenge (reciprocal $\log_2$ ± SE)[b]		HMPV replication 3 days following challenge on day 28			
			Nasal turbinate		Lung	
	Preimmunization	27 days postimmunization	% with detectable virus	Mean titer ($\log_{10}$ PFU/g of tissue ± SE)[c]	% with detectable virus	Mean titer ($\log_{10}$ PFU/g of tissue ± SE)
None (mock)	≤1.5 ± 0	≤1.5 ± 0	100	6.2 ± 0.1	83	2.8 ± 0.3
rHMPV	≤1.5 ± 0	5.0 ± 0.3	0	≤1.5 ± 0.0[d]	0	≤1.5 ± 0.0
rHMPV-N_A	≤1.5 ± 0	5.6 ± 0.6	0	≤1.5 ± 0.0	0	≤1.5 ± 0.0
rHMPV-P_A	≤1.5 ± 0	4.9 ± 0.6	0	≤1.5 ± 0.0	0	≤1.5 ± 0.0
AMPV	≤1.5 ± 0	3.1 ± 0.3	100	4.6 ± 0.2	17	1.6 ± 0.1

[a] Hamsters in groups of six were immunized by intranasal infection with $10^{5.7}$ PFU of the indicated virus or mock infected with L15 medium.
[b] Sera were collected 2 days before and 27 days following immunization, and the neutralizing antibody titer against HMPV was determined.
[c] On day 28, hamsters from each group were challenged intranasally with $10^{5.7}$ PFU of HMPV CAN97-83. Nasal turbinates and lungs were harvested 3 days later, and the virus titers present in tissue homogenates were determined by plaque assay.
[d] The value of 50 PFU per g of tissue (the lower limit of virus detection) was assigned to animals lacking detectable virus in order to calculate the mean titer.

TABLE 3. Level of replication of the rHMPV/AMPV chimeras in the upper and lower respiratory tracts of AGMs

Virus[a]	Nasopharyngeal swab			Tracheal lavage specimen		
	Mean peak titer ($\log_{10}$ PFU/ml ± SE)[b] and statistical grouping[c]	Reduction of mean peak titer[d] ($\log_{10}$ PFU/ml)	Duration of shedding[e] (day ± SE)	Mean peak titer ($\log_{10}$ PFU/ml ± SE) and statistical grouping	Reduction of mean peak titer ($\log_{10}$ PFU/ml)	Duration of shedding (day ± SE)
rHMPV	3.7 ± 0.0 A		11.0 ± 0.0	5.4 ± 0.2 A		8.5 ± 1.0
rHMPV-N_A	3.3 ± 0.2 A	0.4	11.0 ± 0.0	4.5 ± 0.2 B	0.9	8.5 ± 0.5
rHMPV-P_A	2.0 ± 0.1 B	1.7	10.0 ± 0.0	2.7 ± 0.0 C	2.7	5.0 ± 0.8

[a] HMPV-seronegative AGMs in groups of four were inoculated on day 0 by the combined intranasal and intratracheal routes with $10^{6.0}$ PFU of the indicated virus in 1 ml per site. Nasopharyngeal swabs were collected daily on days 1 to 10 and 12, and tracheal lavage specimens were collected on days 2, 4, 6, 8, 10, and 12.

[b] Virus titers were determined by plaque assay on Vero cells (6). The level of virus replication is expressed as the geometric mean of the peak virus titers ($\log_{10}$ PFU/ml ± standard error) for the animals in each group irrespective of sampling day. The lower limit of detection was 0.7 $\log_{10}$ PFU/ml. A value of 0.7 $\log_{10}$ PFU/ml was assigned to samples with no detectable virus in order to calculate means.

[c] Mean peak virus titers were assigned to statistically similar groups by the Tukey-Kramer post hoc test. Values within a column that share a common letter are not significantly different, whereas those that do not are significantly different ($P < 0.05$).

[d] Reduction of mean peak titer compared to that of rHMPV.

[e] The period of days from the first to the last day on which virus was recovered, including negative days (if any) in between.

taken on day −2 (i.e., 2 days before the immunizing infection) and day 27 and assayed for the ability to neutralize HMPV in vitro (Table 2). The titer of HMPV-neutralizing serum antibodies induced by the N and P chimeric viruses was essentially indistinguishable from that induced by rHMPV. In contrast, the titer of HMPV-neutralizing serum antibodies induced by AMPV was substantially less than that induced by rHMPV and the N and P chimeras even though AMPV had replicated relatively efficiently: this presumably reflects antigenic differences between HMPV and AMPV.

The animals were challenged intranasally with HMPV CAN97-83 on day 28 postimmunization and were sacrificed 3 days later, and the lungs and nasal turbinates were harvested and assayed for infectious virus. All animals previously immunized with rHMPV or with the N or P chimera were completely protected against HMPV challenge virus replication in both the upper and lower respiratory tract, whereas the mock-infected control group had mean peak challenge virus titers of $10^{6.2}$ PFU per g of tissue in the nasal turbinates and $10^{2.8}$ PFU per g of tissue in the lungs (Table 2). The mean titers of challenge virus replication in the animals previously immunized with AMPV were reduced approximately 50-fold in the upper respiratory tract and approximately 10-fold in the lower respiratory tract compared to the mock-immunized group. This indicated that infection with AMPV provided a modest degree of cross-protection against HMPV, which is in accordance with the moderate titer of HMPV-neutralizing serum antibody titers induced by AMPV as noted above.

Replication of the N and P chimeric viruses in the respiratory tracts of AGMs. Since the chimeric viruses were replication competent and protective in the rodent model, they were chosen for further preclinical testing in AGMs, a primate host in which HMPV replicates efficiently (30). AGMs that were identified as being negative for HMPV-neutralizing serum antibodies (≤1.5 $\log_2$) (see Table 4) were inoculated intranasally and intratracheally with $10^{6.0}$ PFU per site of rHMPV, rHMPV-N_A, and rHMPV-P_A (Table 3). To monitor virus replication in the upper and lower respiratory tracts, respectively, nasopharyngeal swabs and tracheal lavage samples were collected at intervals over 12 days postinfection and were subsequently assayed for virus titer (Table 3). The AGMs were monitored daily for clinical symptoms, of which there were none.

rHMPV replicated over several consecutive days (Fig. 4) to mean peak titers of 3.7 ± 0.0 $\log_{10}$ PFU per ml in the upper respiratory tract and 5.4 ± 0.2 $\log_{10}$ PFU per ml in the lower respiratory tract (Table 3). The chimeras replicated to mean peak titers that were slightly (rHMPV-N_A) or significantly (60-fold for rHMPV-P_A) reduced in the upper respiratory tract compared to rHMPV. In the lower respiratory tract, replication of both chimeras was significantly reduced: 15-fold in the case of rHMPV-N_A and 1,000-fold in the case of rHMPV-P_A. Also, the duration of shedding in the lower respiratory tract was reduced for the latter virus (Table 3 and Fig. 4).

Immunogenicity and protective efficacy of the N and P chimeric viruses in AGMs. Serum samples were taken from the AGMs described above on day 0 prior to the initial immunizing infection and on day 28 postimmunization. Each immunized animal developed a high titer of HMPV-neutralizing serum antibodies, ranging from 6.0 ± 0.7 $\log_2$ for rHMPV to 5.0 ± 0.5 $\log_2$ for rHMPV-P_A (Table 4). There was a trend towards lower antibody titers for the more attenuated rHMPV-P_A virus, but the differences compared to rHMPV and rHMPV-N_A were not statistically significant (Table 4).

To evaluate protective efficacy, the AGMs were challenged on day 28 postimmunization by the combined intranasal and intratracheal routes with $10^{6.0}$ PFU of CAN97-83 per site. Challenge virus replication was monitored by collecting nasopharyngeal swabs and tracheal lavage samples on days 2, 4, 6, and 8 postchallenge (Table 4). As expected, challenge virus replication was highly restricted in the upper and lower respiratory tracts of AGMs previously immunized with rHMPV, with only trace amounts of shedding, just above the detection limit, in the upper respiratory tracts of two of four rHMPV-immunized animals. In the animals that had been infected with the N and P chimeric viruses, shedding was not detected in the upper or lower respiratory tract, showing that immunization with an HMPV/AMPV chimeric virus was highly protective.

Serum samples were collected 28 days following the challenge, and the titers of HMPV-neutralizing antibodies were determined (Table 4). In the mock-infected control group, the mean postchallenge titer of HMPV-neutralizing serum anti-

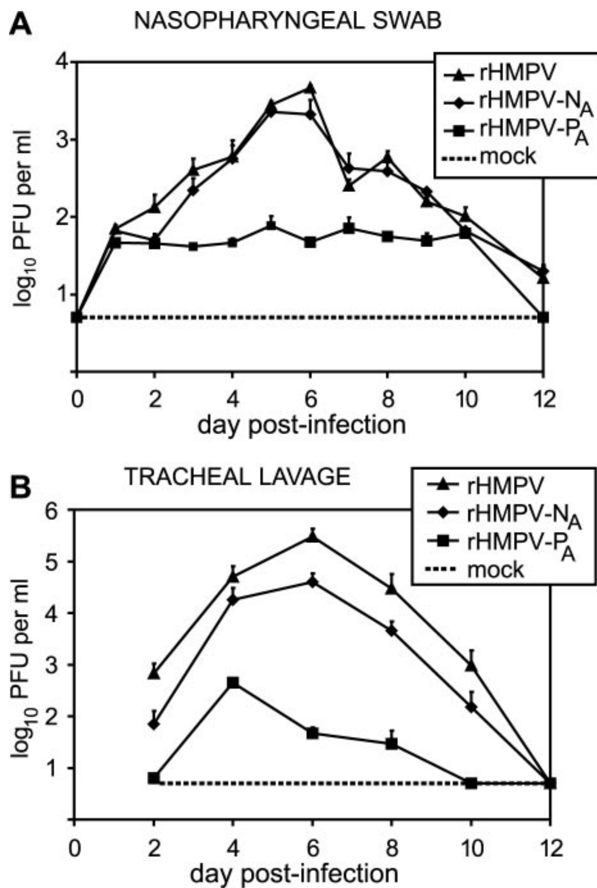

FIG. 4. Kinetics of replication of rHMPV and AMPV chimera mutants in the upper and lower respiratory tracts of AGMs. Four animals per group (except for the mock-infected control group, which was composed of two animals) were inoculated by combined intranasal and intratracheal routes by using a 1-ml inoculum per site containing $10^{6.0}$ PFU of the indicated virus on day 0. The nasopharyngeal swab (A) and tracheal lavage (B) specimens were taken on the indicated days, and the titer of virus shed was quantified by plaque assay. The detection limit was 0.7 $\log_{10}$ PFU/ml.

bodies was comparable to that induced by immunization with rHMPV during the initial immunization (6.8 ± 0.3 $\log_2$ for the mock group on day 28 postchallenge versus 6.0 ± 0.7 $\log_2$ for rHMPV on day 28 postimmunization), as would be expected. For animals that had been immunized with any of the viruses on day 0, including the highly attenuated rHMPV-P_A virus, there was no significant increase in the titers of HMPV-neutralizing serum antibodies following the challenge. This probably indicates that the restriction of challenge virus replication was so complete that there was insufficient generation of antigen to induce a secondary response. Alternatively, the immune response from the initial infection might have hit a maximal level beyond which boosting was insignificant.

DISCUSSION

HMPV is a recently discovered human respiratory pathogen for which a vaccine is needed. In the present study, we investigated the strategy of creating chimeric viruses between HMPV and AMPV subtype C, a closely related virus of birds, as potential live intranasal vaccine candidates. This would follow the general strategy of using an animal virus as a live vaccine against a closely related human pathogen virus, pioneered by Jenner's use of cowpox as a vaccine against smallpox, but with the use of reverse genetics to make swaps of individual genes. We generated chimeric recombinant HMPV in which the N or P ORF was derived from AMPV. Evaluation of these in hamsters and nonhuman primates showed that the P chimeric virus in particular is highly attenuated and immunogenic and is a promising HMPV vaccine candidate.

Since a primary goal was to develop vaccine viruses against HMPV, it was preferable that each chimeric virus retain the HMPV surface glycoprotein antigens and, in particular, the F protein, since it appears to be the predominant HMPV neutralization and protective antigen (25, 30). Among the remaining HMPV genes, we chose N and P as the first genes to evaluate for substitution, since a similar approach replacing the N or P ORF of human parainfluenza type 3 virus with their bovine parainfluenza virus counterparts resulted in generation of a good vaccine candidate (1, 31). The chimeric rHMPV-N_A and rHMPV-P_A viruses were readily recovered. Surprisingly, both viruses, and especially rHMPV-P_A, replicated substantially more efficiently (11- to 25-fold better with regard to peak titers) in Vero cells than did HMPV. Since wild-type AMPV replicated 100-fold more efficiently than HMPV under these conditions, the N and P chimeric viruses appeared to have partially acquired this replicative capacity. We had assumed that AMPV grows more efficiently than HMPV in vitro because it does not require added trypsin for cleavage of its F protein and activation of infectivity; however, the improved growth of the N and P chimeric viruses suggests that the difference lies with internal proteins. In any event, efficient growth in Vero cells is an important characteristic for a live vaccine virus, since Vero cells are one of the few acceptable cell substrates for the production of vaccines for human use.

It was surprising that the replacement of a viral protein with a heterologous counterpart would result in improved growth, exemplified here with two independent examples. Furthermore, the greater effect was observed for the P protein, which is the more divergent of the two swapped proteins (68% amino acid sequence identity between AMPV and HMPV compared to 89% for N). It is possible that, for some unknown reason, the AMPV N and P proteins function more efficiently in the context of the other HMPV proteins than do HMPV N and P. Alternatively, and perhaps more likely, the AMPV N and P proteins somehow function more efficiently in the Vero cell environment, perhaps involving interaction with cellular components that remain to be identified. It is well-known that cellular factors interact with the ribonucleoproteins of various members of *Mononegavirales*; for example, RNA synthesis in a test tube directed by human respiratory syncytial virus (HRSV) nucleocapsids isolated from infected cells was highly dependent on added cytoplasmic extract (2, 21). The improved growth of the chimeras in the present study did not seem to be due to a substantial increase in gene expression compared to HMPV, as least as measured by Western blot analysis of the total accumulation of viral proteins in cells infected with the chimeric viruses. However, the effect might be subtle. Also, it is possible that the effect operates at some other level, such as virion morphogenesis, rather than genome encapsidation,

TABLE 4. Immunogenicity and protective efficiency of rHMPV/AMPV chimeras in AGMs

Immunizing virus[a]	Mean serum neutralizing antibody titer (reciprocal $\log_2 \pm$ SE)[b]			HMPV replication following challenge on day 28					
				Nasopharyngeal swab			Tracheal lavage specimen		
	Preimmunization	28 days postimmunization and statistical grouping[c]	28 days postchallenge	Mean peak titer ($\log_{10}$ PFU/ml $\pm$ SE)[d]	Duration of shedding[e] (day $\pm$ SE)	No. of animals shedding virus[f]	Mean peak titer ($\log_{10}$ PFU/ml $\pm$ SE)	Duration of shedding (day $\pm$ SE)	No. of animals shedding virus
None (mock)	≤1.5	≤1.5	6.8 ± 0.3	2.9 ± 0.6	6.0 ± 1.0	2/2	3.6 ± 1.2	5.0 ± 0.0	2/2
rHMPV	≤1.5	6.0 ± 0.7 A	6.0 ± 0.7	0.8	0.5 ± 0.3	2/4	<0.7	0.0 ± 0.0	0/4
rHMPV-N_A	≤1.5	5.4 ± 0.4 A	5.8 ± 0.3	<0.7	0.0 ± 0.0	0/4	<0.7	0.0 ± 0.0	0/4
rHMPV-P_A	≤1.5	5.0 ± 0.5 A	6.7 ± 0.4	<0.7	0.0 ± 0.0	0/4	<0.7	0.0 ± 0.0	0/4

[a] The groups of AGMs are identified by the virus used in the initial immunizing infection as described in Table 3, footnote *a*. The mock-infected group is an additional group of two animals shown in Fig. 4 that had been mock infected in parallel with the other groups.

[b] Sera were collected on days 0 and 28 following the first infection and 28 days following the challenge, and the neutralizing antibody titer against HMPV was determined using an endpoint dilution assay (8).

[c] Mean antibody titers were assigned to statistically similar groups by the Tukey-Kramer post hoc test. Values within a column that share a common letter are not significantly different ($P < 0.05$).

[d] On day 28, AGMs from each group were challenged intranasally and intratracheally with 10^6 PFU of CAN97-83 in 1 ml per site. Nasopharyngeal swabs and tracheal lavage specimens were collected on days 2, 4, 6, and 8 postchallenge, and virus titers were determined by plaque assay. The level of virus replication is expressed as the geometric mean of the peak virus titers ($\log_{10}$ PFU/ml $\pm$ standard error) for the animals in each group irrespective of sampling day. The lower limit of detection is 0.7 $\log_{10}$ PFU/ml. A value of 0.7 $\log_{10}$ PFU/ml was assigned to samples without detectable virus.

[e] The period of days from the first to the last day on which virus was recovered, including negative days (if any) in between.

[f] Number of animals from which virus was detected out of the total number of animals in that group.

RNA replication, or transcription. We are creating chimeras involving additional "internal" AMPV genes, and we will examine the Vero cell phenotypes of AMPV and the AMPV/HMPV chimeras in greater detail when these additional viruses are available. Also, the effects of mixtures of AMPV and HMPV proteins on RNA synthesis and production of virus-like particles will be analyzed in a metapneumovirus minireplicon system.

While the N and P chimeric viruses exhibited increased growth in Vero cells, they were attenuated in hamsters and, especially, AGMs, a permissive primate host. The observation that the N and P chimeric viruses replicated more efficiently than HMPV in Vero cells—which are of AGM origin—but replicated less efficiently than HMPV in AGMs themselves illustrates the caution that must be taken in using a monolayer cell line of a given tissue (in this case kidney) of a given species to predict replication and host range effects in the corresponding whole animal.

AMPV is a causative agent of respiratory disease in poultry—especially turkeys—that was first observed in 1978 in South Africa (13, 14) and since has been reported throughout much of the world. Subtype C, represented by the Colorado strain used in the present study, is concentrated in the New World, where it was first observed in 1996 (29). There are no reports of humans being infected with AMPV, although there certainly would be exposure of humans to AMPV through infected poultry. This suggests that AMPV has a limited host range. This would be consistent with the experience with HRSV and bovine RSV and human and bovine parainfluenza type 3 virus, which are human/animal counterparts with roughly comparable levels of sequence relatedness and for which each animal counterpart exhibits a strong host range restriction in primates (12, 23, 32). Conversely, the ability of HMPV to infect birds was tested previously by experimental inoculation of turkeys: during the subsequent 3-week period there was no detectable virus shedding as assayed by RT-PCR of nasal swabs (34). This is consistent with the expectation that AMPV and HMPV have limited host ranges commensurate with the observed level of sequence divergence. We did not directly evaluate the ability of AMPV to replicate in nonhuman primates in the present study, although this will be done in the future; there is a limitation to the number of viruses that can be studied at any one time in nonhuman primates and a general difficulty in identifying AGMs that are seronegative for HMPV as are necessary for these studies.

The results of the AGM study indicated that the N and P chimeric viruses are attenuated and immunogenic and that the P chimeric virus in particular is a very promising vaccine candidate. The greater attenuation of HMPV-P_A compared to HMPV-N_A is consistent with the greater divergence of P (32% difference, involving a total of 95 residues) compared to N (11% difference, total of 44 residues) between AMPV and HMPV. This considerable level of sequence difference might confer stability to the attenuation phenotype, although this remains to be demonstrated.

Clinical experience with live intranasal vaccines for influenza virus, human parainfluenza type 3 virus, and HRSV indicates that a reduction in virus replication in the lower respiratory tracts of primates of approximately 1,000-fold is a desirable initial target (3, 18, 22). This is exactly what was achieved for the P chimeric virus in AGMs. However, wild-type and attenuated HMPV strains have not yet been administered experimentally to humans, and the levels of virus dose and virus replication that are associated with disease and protective immunity remain to be determined. We presently have three promising attenuated versions of HMPV as vaccine candidates, namely, HMPV with deletion of the G gene (4, 7), HMPV with deletion of the M2-2 ORF (4, 10), and the present rHMPV-P_A virus. The next step will be to evaluate these candidates in clinical studies in parallel with their wild-type parent to establish benchmarks for infectivity, replication, safety, immunogenicity, and protective efficacy of HMPV vaccines.

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