Identification of a Novel Virulence Factor in Recombinant Pneumonia Virus of Mice

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Pneumonia virus of mice (PVM) is a murine relative of human respiratory syncytial virus (HRSV). Here we developed a reverse genetics system for PVM based on a consensus sequence for virulent strain 15. Recombinant PVM and a version engineered to express green fluorescent protein replicated as efficiently as the biological parent in vitro but were 4- and 12.5-fold attenuated in vivo, respectively. The G proteins of HRSV and PVM have been suggested to contribute to viral pathogenesis, but this had not been possible to study in a defined manner in a fully permissive host. As a first step, we evaluated recombinant mutants bearing a deletion of the entire G gene (ΔG) or expressing a G protein lacking its cytoplasmic tail (Gt). Both G mutants replicated as efficiently in vitro as their recombinant parent, but both were nonpathogenic in mice at doses that would otherwise be lethal. We could not detect replication of the ΔG mutant in mice, indicating that its attenuation is based on a severe reduction in the virus load. In contrast, the Gt mutant appeared to replicate as efficiently in mice as its recombinant parent. Thus, the reduction in virulence associated with the Gt mutant could not be accounted for by a reduction in viral replication. These results identified the cytoplasmic tail of G as a virulence factor whose effect is not mediated solely by the viral load. In addition to its intrinsic interest, a recombinant virus that replicates with wild-type-like efficiency but does not cause disease defines optimal properties for vaccine development.

Human and bovine respiratory syncytial viruses (HRSV and BRSV, respectively) are the most extensively characterized representatives of the genus Pneumovirus, subfamily Paramyxovirinae. HRSV and BRSV are important agents of severe lower respiratory tract infections in their respective hosts. Reverse genetics systems are available for HRSV and BRSV (9, 10) and have been useful for determining the functions of viral proteins and RNA signals on a molecular level. However, in vivo characterization of the contribution of these factors to viral pathogenesis has been hampered by the difficulty of performing defined experiments in the natural host. Mice, cotton rats, and monkeys are frequently used as experimental animal models of HRSV infection, but viral replication is only semipermissive and disease is either lacking or minimal (29). Chimpanzees approach humans with regard to the magnitude of HRSV replication and disease but are inconvenient, expensive, and scarce (45). BRSV has been used as a surrogate model of HRSV infection. The genome sequence is closely related to that of HRSV (9), and infection of calves can result in a disease resembling that caused by HRSV in humans (reviewed in reference 29). However, the virulence of BRSV in experimental infections can be variable, and there are limitations to the manageability of this large-animal model and the availability of immunological tools (38, 43).

Pneumonia virus of mice (PVM), a murine member of the genus Pneumovirus, is another potential surrogate for HRSV (15). PVM was first isolated by Horsfall and Hahn in the 1930s when mice were used during an attempt to isolate new human respiratory viruses (21, 22). Mice were inoculated with nasopharyngeal aspirates from patients with acute noninfluenza respiratory tract disease, followed by serial passage of mouse lung homogenates from animal to animal. Following several passages, the mice frequently developed morbidity and fatal pneumonia. PVM was isolated and identified as a potential murine pathogen rather than a human pathogen. Thus, the natural history of PVM is unclear, and reports of naturally occurring infection and disease are rare and limited to immunocompromised animals (36, 44). Infection of immunocompetent animals is thought to be inapparent or latent. However, PVM infections of other rodents have been described, and antibodies specific to PVM or a serologically related virus have been detected in many rodent species as well as in other mammals, including humans (16, 20, 28, 31, 33).

Two laboratory strains, strain 15 and strain J3666, have been described for PVM. An isolate of strain 15 has been used as a prototype in several experiments (13, 34), although this now appears to be a nonpathogenic mutant that was attenuated by passage in primate cell culture. However, we recently showed that the isolate of strain 15 that is available from the American Type Culture Collection (ATCC) is a virulent virus that thus resembles the original strain 15 isolate described by Horsfall and Hahn (21–23). The second laboratory strain of PVM, strain J3666, is virulent in mice and has been maintained entirely by passage in mice (12, 34). Presumably it was isolated at approximately the same time and place as strain 15 (42). How-
ever, there are no reports of the isolation in the literature, and thus, its origin is somewhat unclear. Strain J3666 has been used for in vivo studies of pathogenicity.

Recently, complete genomic sequences were reported for the virulent strain 15, its nonpathogenic variant, and strain J3666 (24, 42). The prototypic virulent PVM strain 15 has a nonsegmented negative-sense RNA genome of 14,886 nucleotides (nt) (24). The genome contains 10 genes that encode 12 proteins. Eleven PVM proteins appear to correspond to the following HRSV proteins: the nucleocapsid protein N; the phosphoprotein P; the large polymerase protein L; the M2-1 and M2-2 proteins, which are encoded by overlapping open reading frames (ORFs) in the M2 mRNA and are involved in RNA synthesis (3, 11, 18); the matrix protein M; the nonstructural NS1 and NS2 proteins, involved in inhibiting the host interferon system (7, 27, 37, 39, 43); and three transmembrane surface glycoproteins, namely, the G protein, which is involved in attachment, the F protein, which is involved in membrane fusion, and the small hydrophobic protein, which might be an antipapotropic factor (46). The 12th PVM protein does not have a counterpart in HRSV or BRV; it is a 137-amino-acid protein of unknown function that is encoded by a second ORF in the P mRNA (2).

Studies of PVM pathogenesis to date have compared strain J3666 as the virulent strain with the nonpathogenic version of strain 15 as the nonpathogenic strain (see, e.g., references 13, 34, and 42). The recent sequence analysis mentioned above showed that these two viruses differ by 59 nucleotide substitutions involving 37 amino acid substitutions, complicating the identification of the relevant differences. In addition, the G gene of the nonpathogenic variant of PVM strain 15 appears to have sustained a single-nucleotide insertion in a run of four U residues at gene positions 169 to 172 (GenBank accession number AY729016), which immediately follows codon 29 in the G ORF. This would shift translation into a second frame that terminates after six additional codons. However, 5 nt upstream of this termination site, there is a second AUG in the G ORF (codon 34), and initiation there would yield a G protein that lacks the first 33 amino acids at its N terminus. The pneumovirus G protein is a type II glycoprotein: in the case of PVM, amino acids 1 to 34 are predicted to constitute its cytoplasmic tail, amino acids 35 to 59 would constitute its signal/anchor sequence, and the remainder of the molecule would be the ectodomain. Thus, initiation at Met-34 would eliminate essentially the entire cytoplasmic tail. This mutation was yet another candidate to be involved in the attenuation phenotype of the nonpathogenic version of strain 15, but it was necessary to evaluate this in a defined genetic background.

In the present study, we developed a reverse genetic system for the study of pneumovirus virulence factors in a susceptible in vivo model.

**MATERIALS AND METHODS**

**Viruses and cells.** PVM virulent strain 15 and its recombinant derivatives were propagated in BHK-21 (ATCC CCL-10) cells as described previously (24). BHK-21 cells and the BHK-derived BSR-T7/5 cells (9) were maintained in Glasgow minimal essential medium (GMEM) (Invitrogen Gibco), and Vero cells (ATCC CCL-81) were maintained in Eagle’s MEM (Biochrom), all supplemented with 10% fetal calf serum. Virus titers were determined by a plaque assay on Vero cells using 0.8% methylcellulose for 5 days at 32°C, followed by fixation with 80% methanol. Plates were visualized by incubation with a polyclonal rabbit serum raised against the PVM G protein, followed by incubation with biotinylated goat anti-rabbit immunoglobulin G (IgG) (Pierce) as a secondary antibody. Bound antibodies were detected by incubation with streptavidin coupled to horseradish peroxidase (HRP) (BD Pharmingen), followed by color development by incubation with 3,3’-diaminobenzidine tetrahydrochloride (DAB) substrate (Merek). Growth curves were performed in replicate wells. At the indicated time points, duplicate wells were harvested by scraping the cells into the overlying medium. The cells were pelleted by low-speed centrifugation, subjected to two cycles of freeze-thawing, resuspended in small volumes of medium, and centrifuged a second time. The supernatant was harvested, combined with that from the first centrifugation, flash frozen, and stored for titration. Each aliquot was titrated in duplicate, and the mean was calculated.

**RNA isolation, RT-PCR, and nucleotide sequencing.** Total cellular RNA or virion-associated RNA (vRNA) was isolated as described previously (24). Reverse transcription (RT) of the virion RNA was performed with 5 pmol of a specific primer. RNA was mixed with 5 pmol of a specific primer and 1 ml of vRNA. For construction of support plasmids expressing the nucleoprotein N, the phosphoprotein P, and the putative transcription factor M2-1, the respective ORFs were amplified from vRNA by RT-PCR using specific primers and cloned into the Neol restriction site of the T7 expression vector pTM1 such that the ATG of the Neol site became the translational start site of the ORF (17). The expression plasmid carrying the ORF of the RNA polymerase was assembled from two overlapping cDNA fragments derived by RT-PCR such that it could also be used as a shuttle vector for construction of the full-length plasmid (Fig. 1A). Fragment 1, of 2,895 nt (fragment 4 of the full-length construct), encompassed a naturally occurring BamHI site, which contained the last nucleotide of the translation initiation codon of the L ORF, and an XhoI site (nt 8475 to 8,480 and 11374 to 11,379, respectively, of PVM 15). This fragment was cloned into the BamHI-XhoI window of pT7DNA MV L, a plasmid encoding the L protein of human metapneumovirus (HMPV) (5), thereby replacing the HMPV sequence. Fragment 2, of 3,541 nt (fragment 5 of the full-length construct), encompassed the residual part of the L gene including the XhoI site and the L gene end (GE), as well as the complete PVM 15 trailer sequence and 35 nt of the sequence of the hepatitis D virus (HDV) ribozyme. This fragment was cloned into a separate pTM1 vector. After confirmation of the sequence, the second L fragment was transferred into the XhoI-Stul window of pT7 pmvl, thus creating pTM pmvL.

For construction of the plasmid encoding the complete antigenome of PVM 15 (Fig. 1), plasmid pKS II 3/7 HDV-T7ter, containing the HDV ribozyme followed by a terminator for the T7 RNA polymerase (14), was modified to contain a polylinker with NheI, AgeI, and BstBI sites, as well as the first 17 nt of the PVM L gene, including the gene start (GS) signal and the naturally occurring BamHI site. This polylinker was cloned into the XmnI-XhoI window of pT7DNA MV L, a plasmid encoding the L protein of human metapneumovirus (HMPV) (5), thereby replacing the HMPV sequence. Fragment 2, of 3,541 nt (fragment 5 of the full-length construct), encompassed the residual part of the L gene including the XhoI site and the L gene end (GE), as well as the complete PVM 15 trailer sequence and 35 nt of the sequence of the hepatitis D virus (HDV) ribozyme. This fragment was cloned into a separate pTM1 vector. After confirmation of the sequence, the second L fragment was transferred into the XhoI-Stul window of pT7 pmvL, thus creating pTM pmvL.

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plasmid was added on the downstream site of fragment 2 (Fig. 1B). Fragment 3 encompassed the complete G, F, and M2 genes. On the upstream end, the fragment overlapped with the added AgeI site, whereas a BstBI restriction site that would be located in the M2–L intergenic region was added to the downstream end. The subgenomic fragments were cloned and the sequences confirmed. Confirmed fragments were added to the corresponding restriction sites of the vector described above containing the PVM L gene, thus creating pPVM. The sequence of the complete assembled antigenome was confirmed in its entirety.

To construct a variant encoding green fluorescent protein (GFP), pPVM-GFP, the pPVM plasmid was modified by addition of a transcription cassette containing the ORF for enhanced GFP (Clontech, Inc.) to the AgeI restriction site in the SH–G intergenic region (Fig. 1C). To create the GFP insertion cassette, the GFP ORF was amplified by PCR to add an AgeI site, followed by the GS sequence of the PVM N gene (which is identical to that of the P gene), and then the Kozak sequence, on the upstream side and the GE sequence of the PVM F gene, followed by an AgeI site, on the downstream end (Fig. 1C). The structure of the GFP transcription cassette was confirmed by sequencing.

To construct a mutant in which the cytoplasmic tail of G was deleted (pPVM-GFP-Gt), the pPVM-GFP7 cDNA was subjected to PCR using a forward primer that contained the AgeI restriction site and G GS sequence, followed by 22 nt of the G ORF starting at nt 178 of the G gene. PCR amplification using this forward primer and the same reverse primer as for the generation of fragment 3 resulted in a cDNA containing a truncated G gene with the GS sequence fused to nt 178 of the G gene (thereby deleting the intervening167 nt), the complete naturally occurring G gene downstream of nt 178, and the G–F intergenic region, followed by the complete downstream part of fragment 3 of pPVM1. The insert was then digested with AgeI and BstBI and cloned into the corresponding window of pPVM, resulting in pPVM-Gt. The antigenomic cDNAs of pPVM-ΔG and pPVM-GFP6 were digested with AgeI and BstBI to confirm the deletion and inclusion of the GFP ORF, respectively.
RESULTS

Reverse genetic system for virulent PVM strain 15. A plasmid encoding the complete antigenic RNA of virulent PVM strain 15 (pPVM) was constructed from five overlapping subgenomic RT-PCR fragments as illustrated in Fig. 1A. As markers, a unique AgeI site was added to the SH–G intergenic region by the substitution of 1 and addition of 3 nt, increasing its length from 2 nt to 5 nt, and a unique BstBI site was added to the M2–L intergenic region by the addition of 2 and deletion of 1 nt, increasing its length from 9 nt to 10 nt. The encoded rPVM antigenome would be 14,890 nt long, compared to 14,886 nt for its biological parent. Apart from these markers, the sequence of the rPVM cDNA was identical to the consensus sequence of its biological parent (GenBank accession number AY729016) (24).

To facilitate virus detection, we also made a version of pPVM containing a gene encoding GFP. PCR was used to construct a transcription cassette containing the ORF of enhanced GFP under the control of the GS signal of N (which also is identical to that of the P gene) and the GE signal of F, flanked by AgeI sites (Fig. 1C). This cassette was inserted in the AgeI site of pPVM. Thus, the resulting plasmid, pPVM-GFP7, contained 11 genes, with the added GFP gene located at position 7 between the SH and G genes. The inset increased the genome length to 15,642 nt.

Recovery of recombinant PVM. Plasmid pPVM or pPVM-GFP7 was transfected, together with T7 expression plasmids encoding the N, P, M2-1, and L support proteins, into BSR-T7/5 cells that constitutively express T7 RNA polymerase (5, 9). In cultures transfected with pPVM-GFP7, individual GFP-expressing cells were detected 2 days after transfection. During the following days, foci of infected cells formed, followed by spreading over the residual monolayer. Final titers of both recombinant viruses reached more than 10^7 PFU/ml after the second passage. To confirm the recombinant origin of the recovered viruses, total intracellular RNA was isolated from infected BHK-21 cells and RT-PCR was used to amplify a fragment spanning nt 3312 (in the N gene) to 5981 (in the F gene) (positions relative to PVM15), which included the AgeI marker restriction site and the added GFP gene. Detection of the predicted PCR products depended on the presence of reverse transcriptase, indicating that the products represented RNA (data not shown). Restriction digestion confirmed the presence of the AgeI site in rPVM and the presence of the GFP insert in rPVM-GFP7, whereas neither was present in the biologically derived parent (Fig. 2A).

The in vitro replication efficiencies of rPVM and rPVM-GFP7 were compared to that of biologically derived PVM strain 15 (PVM15) in a multicyle time course in BHK-21 cells. Cell monolayers were infected in duplicate at a multiplicity of infection (MOI) of 0.01 PFU/cell, and cells and medium supernatants were harvested at 24-h intervals and stored for subsequent viral titration by a plaque assay as described in Materials and Methods. As shown in Fig. 2B, the kinetics and viral yields of PVM15, rPVM, and rPVM-GFP7 were essentially the same, reaching titers of more than 10^7 PFU/ml by day 7. Monitoring of the expression of GFP by fluorescent microscopy revealed a continuous spread of rPVM-GFP7 up to day 4, at which time almost 100% of the cells were expressing GFP (Fig. 2C). Interestingly, the monolayer stayed viable up to the end of the experiment at day 7, continuing to produce infectious particles and with no sign of syncytium formation. This was also observed for BHK-21 monolayers infected with PVM15 and rPVM and for Vero cell monolayers infected with each of the three viruses (data not shown).

Replication and virulence of rPVM and rPVM-GFP7 in mice. To investigate the virulence of the recombinant viruses, BALB/c mice were infected intranasally with graded doses of biologically derived PVM15 (500 PFU and 50 PFU), rPVM (5,000 PFU, 500 PFU, and 50 PFU), or rPVM-GFP7 (5,000 PFU and 500 PFU). The mice were observed closely and weighed daily.

All of the mice that received 500 PFU of PVM15 or rPVM...
died or were sacrificed in extremis by day 8 and day 10, respectively (Fig. 3A). However, the onset of symptomatic disease (day 5 versus day 6) (Fig. 3B) and occurrence of first fatalities (day 7 versus day 8) (Fig. 3A) were delayed 1 to 2 days for rPVM, indicating a modest degree of attenuation of rPVM compared to its biological parent. This attenuation was more pronounced in infections involving 50 PFU. In this case, all of the animals infected with rPVM survived (Fig. 3A), although substantial disease was evident, as indicated by weight loss (Fig. 3B). In contrast, following infection with 50 PFU of the biological wild-type virus, most of the mice had to be sacrificed in extremis, and just one mouse survived. The median lethal dose was calculated to be 40 PFU for PVM15 and 160 PFU for rPVM, indicating a fourfold attenuation for rPVM (35).

Although the insertion of GFP into the genome of rPVM did not have a detectable effect on replication in vitro, it had a modest attenuating effect in vivo. Fifty percent of the mice infected with 500 PFU of rPVM-GFP7 survived (Fig. 3A), although they experienced substantial weight loss (Fig. 3B). In this comparison, the onset of symptomatic disease, as indicated by ruffled fur and weight loss, was delayed 1 day for rPVM-GFP7 compared to rPVM (Fig. 3B and data not shown). Based on this 50% lethal dose (LD$_{50}$) of 500 PFU, rPVM-GFP7 was 3-fold and 12.5-fold attenuated compared to its recombinant and biological parents, respectively (Fig. 3B). At a dose of 5,000 PFU of rPVM-GFP7, the kinetics of weight loss and death were essentially the same as those for the same dose of rPVM (Fig. 3B).

In order to determine the efficiency of replication of the recombinant viruses in vivo, BALB/c mice were inoculated intranasally with 500 PFU of rPVM, rPVM-GFP7, or the biological virus PVM15. Mice were sacrificed on days 3 and 6 after infection, lungs and nasal turbinate were harvested, and virus titers were determined by a plaque assay. For biologically derived PVM15, the mean titer in the lungs was approximately $1 \times 10^6$ PFU/g of tissue on day 3 (Fig. 4A) and approximately $4 \times 10^6$ PFU/g on day 6 (Fig. 4B). In comparison to PVM15, the mean titer of rPVM in the lungs was reduced threefold (approximately $4 \times 10^6$ PFU/g) and twofold ($2 \times 10^6$ PFU/g) on days 3 and 6, respectively, reflecting the delayed onset of

FIG. 2. In vitro analysis of recovered rPVM and rPVM-GFP7. (A) RT-PCR and restriction analysis of viral RNA from BHK-21 cells infected with the biological wild-type virus PVM15 (lanes 1 and 2), the wild-type virus rPVM (lanes 3 and 4), or rPVM-GFP7 (lanes 5 and 6). The RT-PCR products were made using primers that hybridized within the N and F genes, spanning PVM positions 3312 to 5981. The amplified fragments were digested with AgeI (lanes marked +) or left untreated (lanes marked −) and electrophoresed on a 1% agarose gel. The predicted sizes of the fragments are given on the left. The last lane represents a 1-kbp DNA ladder. (B) Multistep growth kinetics of biological and recombinant PVMs in vitro. Replicate monolayers of BHK-21 cells were infected with the biological parental virus PVM15, rPVM, or rPVM-GFP7 at an input MOI of 0.01 PFU per cell. At the indicated time points, the cells and medium were harvested, freeze-thawed, clarified, and flash frozen. Virus titers were determined by a plaque assay. Error bars, standard errors of the means. (C) Cells from the experiment for which results are shown in panel B were examined for GFP expression on days 1 to 4 using an inverted fluorescence microscope.
disease and lethality observed in Fig. 3. In the nasal turbinates on day 3, virus was detectable in three out of five mice infected with PVM15 and in just one mouse infected with rPVM (Fig. 4C), likewise indicating a delayed replication of rPVM. In contrast, on day 6, both viruses replicated to approximately the same titer in the nasal turbinates, reaching average titers of approximately $2 \times 10^5$ PFU/g of tissue (Fig. 4D).

In animals infected with rPVM-GFP7, virus was detected on day 3 in the lungs of only half of the animals and was not detected in the nasal turbinates of any of the animals (Fig. 4A and B). On day 6, the mean titer of rPVM-GFP7 in the lungs was approximately sevenfold reduced compared to that for its parent, rPVM (Fig. 4B), whereas there was no significant difference in the nasal turbinates. In a second experiment, we compared the replication of rPVM and rPVM-GFP7 following intranasal infection with a higher dose, 5,000 PFU (data not shown). At this higher dose, virus was detectable in the nasal turbinates and lungs of all animals on days 3 and 6: the mean viral titer of rPVM-GFP7 was threefold reduced compared to that of rPVM on day 3, whereas the titers were essentially the same on day 6. Thus, these data confirm that rPVM-GFP7 is modestly more attenuated than its parent, rPVM, which in turn is modestly more attenuated than its biological parent, PVM15. These differences were greater on day 3 than on day 6.

**Construction and replication of rPVM-GFP-ΔG and pPVM-GFP-Gt.** We constructed two derivatives of pPVM-GFP7 in which either the complete G gene was deleted (ΔG) or the sequence encoding the G cytoplasmic tail was deleted (Gt) (Fig. 5B). The second construct was designed based loosely on the predicted G gene of the nonpathogenic isolate of strain 15 (Fig. 5A). However, instead of introducing a nucleotide insert and frameshift, we deleted 167 nt spanning the beginning of the ORF so as to move the AUG that normally is located at codon 34 (nt 182 to 184) into position as the first AUG in the G ORF. The resulting viruses, rPVM-GFP-ΔG and rPVM-GFP-Gt, were readily recovered, and the deletions were confirmed in the viral RNA of the final virus stocks by RT-PCR amplification and sequencing. In addition, an antiserum specific for the PVM G protein confirmed the expression of G by rPVM-GFP-Gt and the lack of G expression in cells infected with rPVM-GFP-ΔG (Fig. 5C).

In a multistep growth study with BHK-21 cells, the kinetics of replication and the final virus yield for the ΔG and Gt mutants were essentially indistinguishable from those of their immediate parent, rPVM-GFP7, and also were very similar to those of rPVM (Fig. 5D). Thus, the G protein is completely dispensable for PVM replication in BHK-21 cells. In addition, the N-terminal truncation did not detectably interfere with viral replication, which is consistent with previous findings for similar HRSV mutants in Vero cells (40, 41).

**Analysis of G protein expression by rPVM.** To investigate whether the G protein of PVM is expressed in a secreted form analogous to that of HRSV, BHK-21 cells were infected with rPVM-GFP7, rPVM-GFP-Gt, or rPVM-GFP-ΔG. As a positive control for detecting a soluble G protein, HEp-2 cells were infected with HRSV and treated in parallel. Virus particles and soluble proteins in the medium supernatants were separated and subjected to SDS-PAGE and Western blot analysis as described in Materials and Methods. HRSV proteins were detected using polyclonal antibodies directed against the complete virus (Fig. 6C). In the gel pattern representing purified HRSV, this antiserum reacted with most of the major structural proteins, including the G protein (Fig. 6C, lane V). In the pattern representing the medium supernatant from which the virus had been removed by centrifugation, this antiserum reacted with a single band that migrated slightly more rapidly than virion-associated G protein and thus represented secreted
HRSV G protein (Fig. 6C, lane S). The absence of other HRSV structural proteins in this lane confirmed that this band was not virion associated. The PVM G protein was detected using a G-specific rabbit antiserum (Fig. 6A) and an antiserum from a mouse that had recovered from PVM infection (Fig. 6B). In the pattern representing purified virus from rPVM-GFP, the G-specific rabbit serum detected a pair of G protein bands (Fig. 6A) that appear to correspond to the two G-specific bands described by Ling and Pringle (25, 26). The convalescent-phase mouse antiserum detected the same two bands as well as several other structural proteins (Fig. 6B). Similar species were detected for the Gt mutant, for which the pair of G-related species migrated slightly more rapidly than that of rPVM-GFP. The two species of G protein were absent from purified \( \mu \text{H9004} \) virus, confirming their identities. However, we were not able to detect the PVM G protein in virus-depleted medium from cells infected with the parental virus or with rPVM-GFP-Gt. Thus, in contrast to HRSV, the PVM G protein does not appear to be expressed significantly in a secreted form.

Replication and virulence of rPVM-GFP-\( \Delta \)G and rPVM-GFP-Gt in vivo. In order to evaluate the effect of deletion or truncation of the G protein on PVM replication in vivo, BALB/c mice in groups of five were infected intranasally with 500 PFU or 5,000 PFU of the parental virus rPVM-GFP, a dose corresponding to the LD\(_{50}\). The mice were sacrificed on day 6, the lungs and nasal turbinates were removed, and virus titers were determined by a plaque assay. The mean virus titers and the percentages of animals with detectable virus are given below the corresponding graphs. Neighboring groups (indicated with brackets) were compared using the Mann-Whitney test. Asterisks indicate statistically significant differences \((P < 0.05)\). The results of two independent experiments were combined in each graph.

Although deletion of the complete G gene had no effect on virus replication in vitro, no virus was detected in the nasal turbinates and lungs of mice 6 days after infection with 500 PFU or 5,000 PFU of the \( \Delta \)G virus (Table 1). In contrast, the Gt mutant replicated with an efficiency that was indistinguishable from that of its parent, rPVM-GFP, resulting in virus titers of \( 10^{6.1} \) PFU/g and \( 10^{6.4} \) PFU/g in the lungs of mice infected with 500 PFU and 5,000 PFU of rPVM-GFP-Gt, respectively, compared to \( 10^{6.0} \) PFU/g after infection with 500 PFU of rPVM-GFP (Table 1). Similarly, the Gt virus replicated to the same high titer as its parent, rPVM-GFP, in the nasal turbinates (Table 1).

Whereas infection with 500 PFU of rPVM-GFP resulted in dramatic weight loss and 50% mortality (Fig. 3B and 7A), and infection with 5,000 PFU of rPVM-GFP-Gt was uniformly lethal.
FIG. 5. rPVM-GFP7 viruses with mutations in the G gene. (A) Schematic diagrams (not drawn to scale) of the G genes of the biologically derived virulent and nonpathogenic isolates of PVM strain 15 (23, 34). Open rectangles, ORFs. The amino acid length of the encoded protein is given in each rectangle. Translational start (filled triangles pointing right) or stop (filled vertical bars) codons are identified by their nucleotide positions in the respective G gene. The GS and GE signals are indicated, and nontranslated gene regions are shown as thin horizontal lines. The nucleotide sequence (negative sense) of positions 166 to 190 of the G gene is given, and the inserted U residue that results in a frameshift is shown in parentheses. The complement of the translational stop codon (nucleotides 188 to 190) that is accessed by the frameshift is italicized, and the complement to the alternative start codon at nucleotides 183 to 185 (Met-34) is boldfaced. (B) Schematic representations (not drawn to scale) of rPVM-GFP-ΔG and rPVM-GFP-Gt. The diagram in the middle represents the rPVM-GFP7 genome (filled box, GFP gene). The box above the diagram illustrates the deletion of the entire G gene to create rPVM-GFP-ΔG. The nucleotide sequence (negative sense) shows the GFP/F gene junction and the AgeI site (italicized) left following the 1,350-nt deletion. The sequence is numbered according to the sequence of rPVM-GFP7, and the GFP GE and F GS signals are underlined. The box below the diagram illustrates the deletion of the cytoplasmic tail of G to create rPVM-GFP-Gt. The unmodified G gene is depicted as in panel A, with the nucleotide positions of its ends numbered according to the complete rPVM-GFP7 sequence. The two potential translational start codons at positions 83 (Met-1) and 182 (Met-34) relative to the G gene sequence are indicated. The nucleotide sequence (negative sense) shows the G GS signal (underlined), the 167-nt deletion that deletes the cytoplasmic tail, and the UAC triplet at positions 182 to 184 that is the complement of the new translational initiation site (Met-34 in the original G ORF). (C) Characterization of rPVM-GFP7, rPVM-GFP-ΔG, and rPVM-GFP-Gt with respect to the expression of G protein. Vero cell monolayers in six-well plates were infected with serial dilutions of the indicated viruses and incubated at 32°C under a methylcellulose overlay for 5 days. (Upper panels) GFP-expressing foci were photographed without further treatment by using an inverted fluorescence microscope. (Lower panels) This was followed by fixation and staining using a G-specific antiserum as described for the plaque assay procedure. Each pair of upper and lower panels depicts approximately the same field of view. (D) Multistep growth kinetics of rPVM, rPVM-GFP7, rPVM-GFP-ΔG, and rPVM-GFP-Gt in BHK-21 cells. Duplicate monolayers were infected at an input MOI of 0.01 PFU per cell. At the indicated days postinfection, the medium supernatants were harvested and flash frozen, and fresh medium was added. Viral titers were determined by a plaque assay. Means from two independent experiments were used to generate the diagram. Error bars, standard errors of the means.
(Fig. 3B), the same doses of the ΔG or Gt mutant appeared to be benign. Specifically, infection with 500 PFU of the Gt mutant did not result in any loss of body weight (Fig. 7A) or in the appearance of other disease signs (data not shown). The lack of virulence associated with G was not surprising given the lack of detectable virus replication, but the lack of virulence associated with Gt was unexpected given that it appeared to replicate as efficiently as its parent, rPVM-GFP7. After infection with 5,000 PFU of the ΔG or Gt mutant, the mice also appeared to be mostly disease free (Fig. 7B). However, in the case of the Gt mutant, there appeared to be a minor weight loss of less than 10% on days 7 and 8 after infection (Fig. 7B).

To further investigate possible residual virulence, additional mice in groups of five were infected with 5,000 or 50,000 PFU of the Gt mutant (Fig. 7C). This confirmed that infection with 5,000 PFU resulted in a slight dip in body weight, occurring at day 7 in this case, whereas the 50,000-PFU dose resulted in

**TABLE 1. Replication of rPVM-GFP-ΔG and rPVM-GFP-Gt in the upper and lower respiratory tracts of BALB/c mice**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dosea (PFU/animal)</th>
<th>No. of animals</th>
<th>% with detectable virus</th>
<th>Mean virus titerb (log_{10} PFU/g of tissue ± SD)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPVM-GFP7</td>
<td>500</td>
<td>3</td>
<td>100</td>
<td>5.3 ± 0.8 (A) 6.0 ± 0.1 (A)</td>
</tr>
<tr>
<td>rPVM-GFP-ΔG</td>
<td>500</td>
<td>5</td>
<td>0</td>
<td>±2.1 (B) ±2.8 (B)</td>
</tr>
<tr>
<td>rPVM-GFP-Gt</td>
<td>5,000</td>
<td>5</td>
<td>100</td>
<td>5.8 ± 0.7 (A) 6.7 ± 0.2 (A)</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>5</td>
<td>100</td>
<td>5.2 ± 0.3 (A) 6.4 ± 0.2 (C)</td>
</tr>
</tbody>
</table>

a BALB/c mice were inoculated intranasally with 500 or 5,000 PFU of the indicated virus per mouse on day 0.

b Animals were sacrificed on day 6 postinoculation, and virus titers in the lungs and nasal turbinates were determined by a plaque assay. For infection with 500 PFU, data from one of two independent experiments providing comparable results are shown. URT, upper respiratory tract; LRT, lower respiratory tract.

c The Tukey-Kramer test was used to assign mean virus titers to similar groups (A, B, and C). Mean titers with different letters are statistically different (P < 0.01) within each column.
significant weight loss that started on day 5 after infection and reached a maximum loss in excess of 20% on day 9. The mice showed ruffled fur and reduced activity, although all of the mice recovered.

To estimate the difference in virulence between rPVM-GFP7 and its Gt derivative, we noted that 500 PFU of the former resulted in weight loss approaching 30%, with only 50% of the mice surviving (Fig. 3 and 7A), whereas 50,000 PFU of the latter caused weight loss approaching 30% but did not result in any deaths. This comparison indicates that the Gt mutant was more than 100-fold attenuated compared to its immediate parent, rPVM-GFP7. This was not associated with any difference in replication efficiency or virus load.

DISCUSSION

We recently showed that the isolate of PVM strain 15 that is available from the ATCC is highly virulent in mice and thus resembles the description of the original strain 15 isolate of Horsfall and Hahn (21–23). We determined the complete consensus sequence of the genome of this virus (24) and, in the present study, used it to develop a reverse genetic system. This system was used to investigate the contribution of the viral G protein to replication and virulence in the BALB/c mouse, a convenient experimental animal that is thought to be a natural host and is highly permissive to PVM replication and disease.

Although rPVM retained a high level of virulence in mice, it was slightly attenuated compared to its biological parent with respect to replication and virulence. Specifically, rPVM was reduced twofold in replication in the lower respiratory tract and was fourfold less virulent as measured by the LD50. However, it should be noted that all of the fatalities after infection with 50 PFU of PVM15 were euthanized in response to severe weight loss as required by the German regulations for animal rights, and thus, none of the animals died as a direct consequence of infection. If nature had been allowed to take its course, some of these animals might have survived, and the difference between the biological and recombinant versions might thus have been reduced (although there still would be a difference).

There are several possible explanations for the modest reduction in the virulence of rPVM compared to its biological parent. The recombinant virus differed from its biological parent by the addition of two marker restriction sites, an AgeI site in the SH–G intergenic region and a BstBI site in the M2–L intergenic region, which in each case changed the length of the intergenic region in addition to introducing nucleotide substitutions. While the pneumovirus intergenic regions usually appear to be nonspecific spacers, modest effects on virus gene expression and replication due to manipulation of these sequences cannot be ruled out. A second possibility is that the consensus sequence that we determined might be a composite of more than one virus present in the preparation rather than a single predominant virus, a composite that is slightly suboptimal. It is also possible that this preparation of rPVM acquired one or more adventitious mutations during recovery and passage, although we note that the virus preparation used in this study had been propagated only three times in BHK-21 cells and thus was not a high-passage stock. In any event, the difference in virulence was marginal, and further work will help determine the basis for this modest difference.

The reverse genetics system was used to generate rPVM-GFP7, which expresses GFP. Insertion of this additional gene, which increased the length of the genome by 752 nt (5%), resulted in sevenfold and threefold attenuation of in vivo replication and virulence, respectively, compared to its immediate parent, rPVM. A comparable GFP insert in HRSV was not attenuating in vitro (47), and multiple gene insertions into HMPV that increased its size by 30% only moderately attenuated replication efficiency (5). However, the replication efficiencies of those viruses have not been evaluated in vivo. It was somewhat surprising to find that the addition of another gene to PVM had such a small effect on replication in a highly permissive host. The GFP backbone will represent our “wild-type” virus in future studies, permitting direct fluorescence tracking and histological characterization of pulmonary infection in the natural host.

Subsequently, we used the GFP-bearing backbone to recover rPVM lacking the G protein altogether or encoding a G protein lacking its N-terminal cytoplasmic tail. The latter mutation was based on the G protein of the cell culture-adapted nonpathogenic variant of PVM strain 15 described by Rhandhawa and colleagues (34). The G ORF of this biologically derived variant contained one added nucleotide, causing a frameshift such that the main ORF presumably initiated at the internal AUG at position 183 (Met-34). In that study, expression of a truncated protein of the predicted size was confirmed in a cell-free translation system programmed with synthetic mRNA, and surface expression was confirmed by immunofluorescence of cells expressing a cDNA of the gene (34). In the present study, we simplified the situation by making a deletion that placed Met-34 as the first AUG in the sequence.

Neither mutation—deletion of the entire G gene or deletion of the cytoplasmic tail of G—affected viral replication in cell culture. This is consistent with previous studies in which deletion of the G protein of HMPV had no effect on replication in vitro (6). Somewhat different results were obtained with avian metapneumovirus, in which deletion of the G gene (in combination with the SH gene) attenuated the virus in vitro (30), and HRSV, in which deletion of the G gene was attenuating in human HEP-2 cells but not in African green monkey Vero cells (41).

In contrast to the situation in vitro, rPVM-GFP-ΔG was severely attenuated for replication in mice, such that no infectious virus was detected in the lungs or nasal turbinates 6 days after intranasal infection with 500 PFU or 5,000 PFU. However, following infection with the latter (but not the former) dose, mice were protected against challenge 28 days later with a lethal dose of biological wild-type PVM (data not shown). This suggests that a low level of replication occurred, since we have previously observed that inoculation of rodents with 5.7 log10 50% tissue culture infectious doses of HMPV was not immunogenic unless the virus was capable of replication (8). The high degree of attenuation due to the deletion of G is comparable to that observed with HRSV ΔG in mice (41), whereas a comparable HMPV ΔG mutant appeared to be less attenuated in rodent and nonhuman primate models (4). How-
ever, the latter models involved infection with significantly (up to 103-fold) higher doses, making a direct comparison difficult.

The Gt virus appeared to replicate as efficiently as its parent, rPVM-GFP7, both in the nasal turbinates and in the lungs, reaching titers in excess of 5.0 log10 and 6.0 log10 PFU/g in the respective tissues. Despite this efficient growth, infection with the Gt virus was asymptomatic at doses that were highly pathogenic and often lethal with its parent, rPVM-GFP7. The Gt virus appeared to be at least 100-fold attenuated compared to its parent, rPVM-GFP7, with respect to weight loss; attenuation based on the LD50 was not available, because none of the animals infected with this virus died under the conditions of these experiments. Thus, the magnitude of virulence was largely dissociated from the magnitude of replication. This dissociation was not complete, since increasing the dose of the Gt virus to 50,000 PFU resulted in weight loss, and it seems likely that a higher dose would result in deaths. Nonetheless, whereas the parent, rPVM-GFP7, induced weight loss at doses of 500 and 5,000 PFU and was 50% and 100% lethal, respectively, at these doses, its Gt derivative was essentially non-pathogenic at the same doses. To our knowledge, this is the first example of an attenuating mutation in a pneumovirus whose effect is not mediated primarily by reduced viral replication. This is offered with the caveat that we did not determine virus titers on every day between inoculation and day 10, when deaths began to occur with the rPVM-GFP7 virus. Nonetheless, the equivalency between rPVM-GFP-Gt and pPVM-GFP7 at the peak of replication on day 6 suggests that the former virus replicated with an efficiency similar to that of its parent.

In the present study, we were not able to detect a secreted form of the PVM G protein in the supernatants of cells infected with PVM expressing the wild-type or truncated G protein. This is consistent with the surface expression of truncated G observed by Rhandhwa and colleagues, as mentioned above (34), although that study did not address whether a proportion of wild-type or truncated G protein might also be secreted. This is in contrast with HRSV, for which a substantial proportion of G protein expressed from the wild-type gene is secreted. In the case of HRSV, secretion is dependent on translational initiation at the second AUG in the ORF (Met-48), which is located within the transmembrane anchor, followed by proteolytic trimming that completely removes the remaining transmembrane domain and creates a new N terminus at position 66 (19). In the case of PVM, the G ORF contains a second AUG at codon 34, but it is in a sequence context that is not consistent with efficient translational initiation due to the presence of a C in the −3 position, and it is not known whether significant translation occurs. In addition, as already noted, the second AUG in the PVM G ORF is located at the inner face of the transmembrane domain rather than in the middle of this domain. In any event, no secreted form of PVM G was observed. Thus, while the immunomodulatory function of the HRSV G protein appears to depend in large part on its secretion (32), the effects of PVM G presumably are not mediated by a comparable species.

These findings suggest that the cytoplasmic tail of G has characteristics of a true virulence factor, that is, a factor whose pathogenic effect can be dissociated from pathogen load. The mechanism of attenuation for the Gt virus remains unknown. Comparisons between the biologically derived nonpathogenic version of strain 15 and the virulent strain J3666 indicated that the pathogenesis observed with the latter was associated with enhanced pulmonary inflammation that was paralleled by increased induction of proinflammatory cytokines, including macrophage inflammatory protein 1α, macrophage chemotactic protein-1, eotaxin, and others (13). Thus, the virulence of strain J3666 appears to be mediated, at least in part, by an overly robust inflammatory response. As already noted, strains 15 and J3666 contain numerous differences involving a number of proteins and potential cis-acting elements, including residues that are conserved within the Pneumovirus genus (24, 42). Therefore, while it seems likely that the truncation of the G protein will prove to be an important factor in the differences between the pathogenic and nonpathogenic phenotypes characterized in the published comparisons between strains 15 and J3666 (42), this will need to be directly evaluated. If the cytoplasmic tail of G indeed is a major contributor to the difference between an “inflammatory” and a “noninflammatory” phenotype, it will be interesting to determine the mechanism for this effect. One possibility is that the cytoplasmic tail directly affects intracellular signaling. As an inexact precedent, the cytoplasmic tail of pathogenic Hantaan virus recently was found to inhibit the induction of type I interferon involving the RIG-I helicase, whereas the cytoplasmic tail of a nonpathogenic strain lacked this ability (1). In the case of PVM, one possibility is that the cytoplasmic tail somehow stimulates activation of NF-κB, leading to the expression of proinflammatory cytokines, and that its deletion ablates this ability. However, further studies are necessary to investigate the mechanistic basis for these effects.

Deletion of the cytoplasmic tail provides a novel attenuating mutation for the pneumoviruses. If the attenuation phenotype of the Gt virus can be reproduced in those pneumoviruses for which a vaccine is needed (HRSV, HMPV, avian metapneumovirus), it would be ideal for developing a live vaccine. A particular problem with live vaccines is that attenuation usually is based on decreased virus replication, which in turn reduces immunogenicity. A viral mutant that replicates efficiently without causing disease would be an ideal vaccine candidate, one that combines safety with a high level of immunogenicity.

In conclusion, we have developed a reverse genetic system for PVM that largely reproduces the virulent phenotype of the biological wild-type virus. The utility of the system was shown by construction of recombinant viruses expressing GFP as marker protein, as well as by construction of gene deletion/truncation mutants that investigated the contribution of the G protein to virulence. The capability of engineering recombinant PVM provides a convenient model for characterizing factors involved in pneumovirus virulence in a natural host.

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