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Effects of Human Metapneumovirus and Respiratory Syncytial Virus Antigen Insertion in Two 3′ Proximal Genome Positions of Bovine/Human Parainfluenza Virus Type 3 on Virus Replication and Immunogenicity

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A live attenuated bovine parainfluenza virus type 3 (PIV3), harboring the fusion (F) and hemagglutinin-neuraminidase (HN) genes of human PIV3, was used as a virus vector to express surface glycoproteins derived from two human pathogens, human metapneumovirus (hMPV) and respiratory syncytial virus (RSV). RSV and hMPV are both paramyxoviruses that cause respiratory disease in young children, the elderly, and immunocompromised individuals. RSV has been known for decades to cause acute lower respiratory tract infections in young children, which often result in hospitalization, while hMPV has only been recently identified as a novel human respiratory pathogen. In this study, the ability of bovine/human PIV3 to express three different foreign transmembrane surface glycoproteins and to induce a protective immune response was evaluated. The RNA-dependent RNA polymerase of paramyxoviruses binds to a single site at the 3′ end of the viral RNA genome to initiate transcription of viral genes. The genome position of the viral gene determines its level of gene expression. The promoter-proximal gene is transcribed with the highest frequency, and each downstream gene is transcribed less often due to attenuation of transcription at each gene junction. This feature of paramyxoviruses was exploited using the PIV3 vector by inserting the foreign viral genes at the 3′ terminus, at position 1 or 2, of the viral RNA genome. These locations were expected to yield high levels of foreign viral protein expression stimulating a protective immune response. The immunogenicity and protection results obtained with a hamster model showed that bovine/human PIV3 can be employed to generate bivalent PIV3/RSV or PIV3/hMPV vaccine candidates that will be further evaluated for safety and efficacy in primates.

Despite control of many infectious diseases in the industrialized world, acute viral respiratory tract infections remain a leading cause of illness and reason for hospitalization. Two paramyxoviruses, respiratory syncytial virus (RSV) and human parainfluenza virus type 3 (hPIV3), are the causative agents of acute respiratory diseases of infancy and early childhood, resulting in 20 to 25% of pneumonia and 45 to 50% of bronchiolitis in hospitalized children (8). In addition, a recently identified human metapneumovirus (hMPV) appears to be associated with lower respiratory tract infections in children (25). Preliminary epidemiological reports have estimated an hMPV disease incidence rate of 7 to 10% in young children (5, 11, 16, 18, 25). The symptoms of hMPV infections are similar to those caused by RSV and hPIV3, and hospitalizations of young children with acute lower respiratory tract infections are necessary in some cases (17). Recently, Greensill et al. reported the detection of hMPV in bronchoalveolar lavage fluids from 21 of 30 infants (70%) ventilated for RSV bronchiolitis (7).

RSV remains one of the most common respiratory pathogens afflicting infants, the elderly, and immunocompromised individuals. Hospitalization and immunoglobulin treatment are often necessary to alleviate serious RSV infections. For decades, approaches to generate an RSV vaccine employing virus subunits or inactivated virus vaccines have failed either due to lack of immunogenicity or the potential for causing enhanced pulmonary disease upon reinfection with naturally occurring wild-type RSV. The advent of reverse genetics for negative-strand RNA viruses resulted in a plethora of genetically designed RSV vaccine candidates that harbor mutations in essential RSV genes or deletions of nonessential RSV genes in an effort to attenuate virus replication without compromising immunogenicity (3). The results from these studies clearly demonstrated that an RSV vaccine candidate must be properly fine tuned to balance immunogenicity and attenuation for administration to 1- to 2-month-old infants. If the RSV candidate vaccine is insufficiently attenuated, the children will show signs of disease. If the vaccine virus does not replicate enough, the level of immunogenicity elicited in the immunized children will be low and ineffective (4). Two subgroups of RSV (A and B) circulate at any given time. The F proteins of the two RSV subgroups are highly conserved (98% amino acid identity), unlike the RSV attachment glycoproteins (G), which display only 53% identity at the amino acid level.

hPIV3 is the causative agent of croup, an acute lower respiratory disease most often observed in young children (6, 8, 29).

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Two live attenuated hPIV3 vaccine candidates have been evaluated so far in human clinical trials. A live attenuated bovine PIV3, a Jennerian-type vaccine, was administered to children between 2 and 6 months of age in human clinical trials and was shown to be safe and immunogenic (12, 14). A cold-passaged hPIV3 strain that displayed a temperature-sensitive phenotype (cpts-hPIV3) was also shown to be immunogenic in human clinical trials (13, 23).

hMPV causes clinical symptoms similar to those of RSV, ranging from mild respiratory problems to severe cough, bronchiolitis, and pneumonia (2, 25). Two subgroups of hMPV (clades A and B) were identified based on sequence comparison of the F and G genes derived from a number of different clinical isolates. The F genes of clades A and B are highly conserved and display >95% identity at the amino acid level. In contrast, the hMPV G proteins are variable and show only a 35% amino acid identity (R. A. M. Fouchier, personal communication). On the basis of electron microscopy and comparison of gene constellations and sequences, hMPV has been assigned to the *Metapneumovirus* genus of the *Paramyxoviridae* family. hMPV displays a unique gene organization (3′ N-P-M-F-M2-SH-G-L 5′) that is similar to but unlike that of either PIV3 or RSV (24, 25). Efforts to prepare neutralizing hMPV monoclonal and polyclonal antisera and to generate live attenuated hMPV vaccines have been initiated by a number of research laboratories in the United States, Canada, Europe, and Australia.

Food and Drug Administration-approved vaccines for RSV, hPIV3, or hMPV are not currently available. However, a desirable and marketable vaccine product would likely contain virus vaccines effective against all three respiratory pathogens, RSV, hPIV3, and hMPV, since all of these viruses are responsible for extensive morbidity and mortality in the same population, young children.

In this study, we investigated the suitability of a chimeric bovine/human PIV3 (b/h PIV3), itself a vaccine candidate for hPIV3, to serve as a live, attenuated virus vaccine vector to express the RSV F, RSV G, or hMPV F proteins. The b/h PIV3 virus vector harbors the F and HN genes of hPIV3 (9). Bivalent vaccine candidates were generated that expressed RSV or hMPV surface glycoproteins. Antibodies produced in response to expression of the surface glycoproteins of RSV, the fusion protein (F) and G, or the F protein of hMPV by b/h PIV3 are expected to result in cross-neutralizing and cross-protection against infection by all strains of RSV or hMPV, respectively, because the RSV F genes are highly conserved between subgroups A and B of RSV, and the hMPV F genes of clades A and B show a high degree of conservation. The foreign antigens were inserted in the two 3′-most proximal positions of the b/h PIV3 RNA genome. Position 1 is located between the native bPIV3 leader and the bPIV3 N gene, and position 2 is juxtaposed between the bPIV3 N and P genes. Since the paramyxovirus RNA-dependent RNA polymerase enters at a single site at the very 3′ end of the genomic RNA to initiate sequential transcription of viral genes, a transcription gradient was generated in which genes located proximal to the 3′ terminus of the viral RNA are transcribed at a higher frequency, yielding higher levels of protein expression (15). In order to achieve maximum levels of protein expression, the positions at the 3′ end that were selected for gene insertion should not compromise the replication efficiency of the resulting chimeric viruses.

Recombinant b/h PIV3 expressing RSV or hMPV surface glycoproteins from position 1 or 2 in the PIV3 genome was analyzed for levels of antigen expression, replication in tissue culture, replication in a small animal model, and the ability to elicit an immune response. All of these viruses expressed the RSV or hMPV proteins efficiently in both of the positions used for gene insertion into the PIV3 genome. This study further evaluated whether structurally similar antigens would result in chimeric viruses with similar phenotypes and whether the foreign glycoproteins derived from hMPV or RSV were functionally incorporated into the b/hPIV3 envelope. The RSV F, RSV G, or hMPV F gene products display structural features that identify them as integral membrane proteins. These proteins possess functionally similar domains such as transmembrane regions and signal peptides that enable them to be incorporated into PIV3 envelope and serve as targets for the host immune response. Animals immunized with the chimeric b/h PIV3/RSV or b/h PIV3/hMPV were protected in challenge studies from both RSV or hMPV and PIV3 and produced neutralizing RSV or hMPV antibodies and PIV3 HAI antibodies. The most promising b/h PIV3/RSV and b/h PIV3/hMPV F vaccine candidates will be evaluated for safety and efficacy in primates and humans.

MATERIALS AND METHODS

Cells and viruses. The RSV A2 strain, b/h PIV3, the hMPV/NL/1/00 strain, b/h PIV3/RSV, and b/h PIV3/hMPV viruses were grown in Vero cells in Opti-MEM (minimal essential medium) (Gibco/BRL) in the presence of gentamicin. Modified vaccinia virus Ankara (MVA-T7) or fowlpox-T7 virus (FP-T7), which expressed the phage T7 RNA polymerase, was grown in chicken embryonic kidney cells (SPAFAS). Vero, HeLa, and HEp-2 cells were maintained in MEM (JRH Biosciences) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, nonessential amino acids, and antibiotics.

Construction of b/h PIV3/RSV and b/h PIV3/hMPV full-length cDNAs. To insert foreign genes into the b/h PIV3 cDNA, *Avr*II restriction enzyme sites were introduced in the b/h PIV3 cDNA plasmid (9, 10) by site-directed mutagenesis with the QuikChange kit (Stratagene). One *Avr*II site was introduced at nucleotide (nt) 104 in the b/h PIV3 genome, altering 4 nt with the oligonucleotide 5′GAAATCCTAAGACCCTAGGCATGTTGAGTC3′ and its complement. This restriction enzyme site was used to insert the RSV genes in the first (3′ proximal) position in the viral genome. Another *Avr*II site was introduced in the N-P intergenic region at nt 1774, changing 2 nt by using the oligonucleotide 5′CCACAACTCAATCAACCTAGGATTCATGGAAGACAATG3′ and its complement. This restriction site was used (designated F1 or G1) to insert the RSV genes in the second position between the N and P genes of b/h PIV3 (designated F2 or G2) (Fig. 1). Viruses harboring the *Avr*II sites at nt 104 and 1774 were recovered from full-length b/h PIV3 cDNAs.

Construction of the RSV G cassette. A DNA fragment was generated that contained the bPIV3 N-P intergenic region as well as the 3′-end sequences of the RSV G gene, using the b/h PIV3 cDNA as PCR template. This fragment was generated by PCR with the oligonucleotides 5′CCCAACACACCACGCCAGT AGTCACAAAGAGATGACCACTATCAC3′ and 5′CCCAAGCTTCCTAGG TGAATCTTTGGTTGATTGAGTTGTGG3′. This fragment was then used in overlapping PCR to add the bPIV3 N-P intergenic region to the RSV G gene. The resulting PCR fragment containing the RSV G open reading frame (ORF) with the bPIV3 N-P intergenic region flanked by *Avr*II restriction enzyme sites was cloned into pGEM3.

Construction of the hMPV F cassette. A plasmid (pRF515) carrying the hMPV F gene (strain NL/1/00) was obtained from R. Fouchier. The bPIV3 N-P intergenic region was added at the 3′ end of the hMPV F gene by using overlapping PCRs, and the hMPV F gene cassette with flanking *Avr*II sites was generated in the same manner as described for the RSV G cassette.

Construction of the RSV F cassette. The RSV F gene fragment was isolated by PCR from a full-length bPIV3/RSV F+G cDNA plasmid by using oligonucleo-

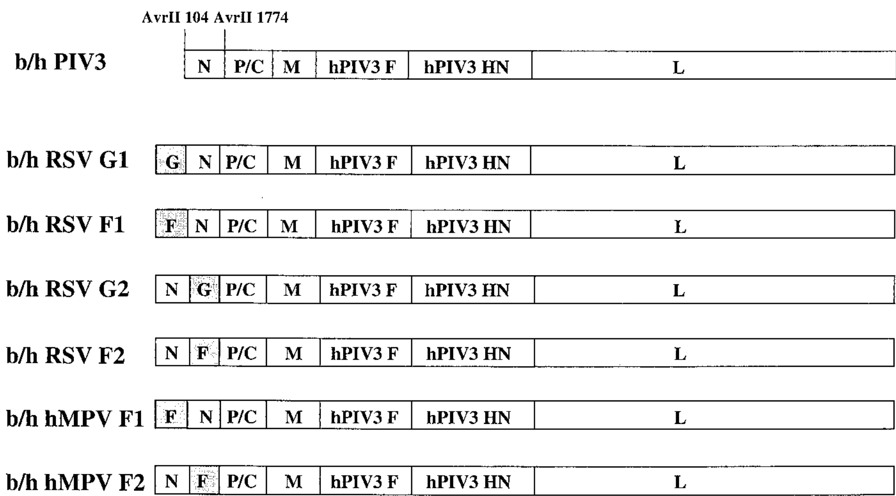

FIG. 1. Schematic representation of the b/h PIV3 RNA genome, which serves as a vector backbone for insertion of RSV or hMPV surface glycoprotein genes. The RSV F, RSV G, and hMPV F gene insertions at position 1 or 2 of the PIV3 genome are indicated by gray boxes. The foreign genes were inserted at position 1 by using an *Avr*II restriction enzyme site introduced at nt 104 in the PIV3 genome or at position 2 at an *Avr*II site at nt 1774. The inserted transcriptional units contain the bPIV3 N-P intergenic region with the gene stop and start sequences utilized for transcription of the gene downstream of the inserted cassette.

tides that added *Avr*II sites at the 5′ and 3′ ends of the RSV F gene. The RSV F gene was introduced into the 1-5 bPIV3 plasmid, containing the first 5,200 nt of the bPIV3 genome and the *Avr*II site at nt 1774, which was linearized with *Avr*II. The bPIV3 N-P intergenic region was isolated by PCR. The oligonucleotide 5′GACGCGTCGACCACAAAGAGATGACCACTATCACC3′ and an oligonucleotide annealing in the bPIV3 F ORF were used to generate a PCR fragment containing the bPIV3 N-P intergenic region, *Avr*II site, and bPIV3 sequences up to nt 5200. The PCR fragment was added to the 1-5 bPIV3 plasmid harboring the RSV F gene at position 2 using *Sal*I and *Nhe*I sites.

The RSV G, RSV F, and hMPV F gene cassettes were sequenced to confirm the presence of an intact ORF, the predicted amino acid sequences, and to verify the rule of six. The RSV G, RSV F, and hMPV F transcriptional units were inserted into the first or second position by using the *Avr*II restriction enzyme sites into a subclone of 1-5 bPIV3. After confirming the proper orientation by restriction enzyme mapping, the plasmids with the RSV genes in the first position were digested with *Sph*I and *Bss*HII and 4-kb (1-5 RSV G1) or 4.8-kb (1-5 RSV F1 or 1-5 hMPV F1) DNA fragments were isolated. The rest of the b/h PIV3 genome was ligated as a 15.1-kb *Sph*I-*Bss*HII DNA fragment, yielding full-length cDNAs. The bPIV3 subclones harboring the RSV genes in the second position were cut with *Sph*I and *Nhe*I, and 5.8-kb (bPIV3/RSV G2) and 6.5-kb (bPIV3/RSV F2 or bPIV3/hMPV F2) DNA fragments were isolated. The rest of the b/h PIV3 genome was ligated as an *Nhe*I-*Sph*I DNA fragment of 14 kb in size to generate full-length cDNA plasmids.

Recombinant virus recovery by reverse genetics. Infectious virus was recovered by reverse genetics in HeLa or HEp-2 cells by transfection methods described previously (9). Briefly, HEp-2 or Vero cells at 80 to 90% confluency in six-well tissue culture dishes were infected with FP-T7 or MVA-T7 at a multiplicity of infection (MOI) of 0.1 to 0.3 or 1 to 5, respectively. Following infection with FP-T7 or MVA-T7, cells were washed once with phosphate-buffered saline (PBS) and transfected with the following amounts of plasmid DNA: 2.0 μg of full-length b/h PIV3 cDNA, 0.4 μg of pCITE/N, 0.4 μg of pCITE/P, and 0.2 μg of pCITE/L. Transfections were performed in the presence of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfection reaction mixtures were incubated at 33°C for 5 to 12 h, after which the medium containing Lipofectamine 2000 was replaced with 2 ml of fresh Opti-MEM containing gentamicin. The transfected cells were incubated further at 33°C for 2 days. Cells were stabilized with 1× SPG (10× SPG is 2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, and 0.054 M L-glutamate) and lysed with one freeze-thaw cycle at −80°C. The crude cell lysate was used to infect a new Vero cell monolayer in order to amplify rescued viruses. The chimeric viruses were cloned by limiting dilutions in Vero cells and stocks of 10^6 to 10^8 PFU/ml were generated. The RSV or hMPV genes of the chimeric viruses were isolated by reverse transcription-PCR (RT-PCR), and the sequences were confirmed. Expression of the RSV proteins was confirmed by immunostaining infected Vero cell monolayers with RSV goat polyclonal antiserum (Biogenesis). Expression of the hMPV F protein was confirmed by immunostaining with polyclonal hMPV guinea pig antiserum (R. Fouchier).

Growth curves of b/h PIV3/RSV or b/h PIV3/hMPV chimeric viruses. Vero cells were grown to 90% confluence and infected at an MOI of 0.01 or 0.1 with b/h PIV3, b/h PIV3/RSV F1, b/h PIV3/RSV G1, b/h PIV3/RSV F2, b/h PIV3/RSV G2, b/h PIV3/hMPV F1, or b/h PIV3/hMPV F2. The infected monolayers were incubated at 37°C. At 0, 24, 48, 72, 96, and 120 h postinfection (hpi), cells and media were harvested and stored at −70°C. Virus titers for each time point harvest were determined by 50% tissue culture infective dose ($TCID_{50}$) or plaque assays in Vero cells. $TCID_{50}$ assays were inspected visually for cytopathic effect (CPE) following incubation at 37°C for 6 days, while plaque assays were immunostained with RSV or hMPV polyclonal antisera for quantification after 5 days of incubation at 35°C.

Western blotting of RSV proteins expressed by b/h PIV3/RSV chimeric viruses. Chimeric viruses were used to infect (70 to 80%) subconfluent Vero cells at an MOI of 0.1. Forty-eight hours postinfection, the medium overlay was removed, and infected monolayers were washed once with 1 ml of PBS. The cells were subsequently lysed in 400 μl of Laemmli buffer (Bio-Rad) containing 5% β-mercaptoethanol (Sigma). Fifteen microliters of each sample was separated on 12% Tris-HCl Ready Gel (Bio-Rad) and transferred to nylon membranes by using a semidry transfer cell (Bio-Rad). Nylon membranes were rinsed in PBS (pH 7.6) containing 0.5% (vol/vol) Tween 20 (Sigma) (PBST) and blocked with PBST containing 5% (wt/vol) dry milk (PBST-M) for 20 to 30 min at room temperature. Membranes were incubated with either a mixture of RSV F monoclonal antibodies (MAbs WHO 1269, 1200, 1153, 1112, 1243, and 1107) (1) at a 1:1,000 dilution in PBST-M or RSV G 10181 polyclonal antibody (Orbigen) at a 1:2,000 dilution in PBST-M for 1 h at room temperature. Following four washes with PBST, the membranes were incubated with a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Dako) at a 1:2,000 dilution in PBST-M for 1 h at room temperature. Membranes were washed four times with PBST, developed with a chemiluminescence substrate (Amersham Pharmacia), and exposed to Biomax Light Film (Kodak) for visualization of protein bands.

Immunoprecipitation of hMPV F proteins expressed by b/h PIV3/hMPV. For immunoprecipitation of the hMPV F protein expressed by b/h PIV3/hMPV, Vero cells were infected with b/h PIV3 or b/h PIV3/hMPV F1 or F2 at an MOI of 0.05. Twenty-four hours postinfection, the cells were incubated for 30 min in Dulbecco's modified Eagle's medium (DME) lacking cysteine and methionine (ICN). The medium was removed, and 0.5 ml of DME lacking cysteine and methionine containing 100 μCi of [^{35}S]Pro-Mix (Amersham) was added to the cells. The infected cells were incubated in the presence of ^{35}S isotopes for 5 h at 37°C. The medium was removed, and the infected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 0.15 M NaCl. The cell lysate was incubated with guinea pig or human polyclonal antisera against hMPV and

bound to recombinant protein G agarose (Invitrogen) overnight at 4°C. The agarose beads were pelleted and washed once with 0.3 M NaCl RIPA buffer and twice with 0.15 M NaCl RIPA buffer. After a final wash with a buffer containing 0.05 M NaCl, 2.5 mM EDTA, and 0.05 M Tris-HCl (pH 7.5), the samples were fractionated on a 10% protein gel. The gel was dried and exposed to MR film (Kodak).

Genetic stability studies of b/h PIV3/RSV and b/h PIV3/hMPV. Subconfluent Vero cells in T25 flasks were infected with b/h PIV3/RSV or b/h PIV3/hMPV at an MOI of 0.1 and incubated for 4 days at 33°C or until CPE was visible. At the end of the incubation period, the infected cells and media were harvested and frozen and thawed two times, and the resulting cell lysate was used to infect a new T25 flask of Vero cells. This cycle was repeated 10 times. All cell lysates from P1 to P10 (P signifies passage number) were analyzed by plaque assay and immunostaining for expression of RSV or hMPV proteins and virus titers. The RSV F, RSV G, and hMPV F gene cassettes were isolated by RT-PCR from P10 lysates and sequenced to identify possible nucleotide alterations.

Replication of b/h PIV3/RSV and b/h PIV3/hMPV chimeric viruses in Syrian Golden hamsters. Five-week-old Syrian Golden hamsters (six animals per group) were infected intranasally with 10^6 PFU of b/h PIV3, b/h PIV3/RSV, RSV A2, b/h PIV3/hMPV, hMPV/NL/1/00, or placebo medium in a 100-μl volume. The different groups were maintained separately in microisolator cages. Four days postinfection, the nasal turbinates and lungs of the animals were harvested, homogenized, and stored at −70°C. The titers of virus present in the tissues were determined by $TCID_{50}$ assays in Vero cells. For the challenge studies, the animals were inoculated on day 28 intranasally with 10^6 PFU of hPIV3, RSV A2, or hMPV/NL/1/00 per ml. Four days postchallenge, the nasal turbinates and lungs of the animals were isolated and assayed for challenge virus replication by plaque assays on Vero cells. Plaques were visualized for quantitation by immunostaining.

Neutralization assay. Neutralization assays were performed for b/h PIV3, b/h PIV3/RSV chimeric viruses, or RSV on Vero cells. Serial twofold dilutions of RSV polyclonal antiserum (Biogenesis, Poole, England), RSV F 1200 MAb obtained from Judy Beeler and the WHO Reagent Bank (1), and hPIV3 F (C191/9) and HN (68/2) MAbs (26, 28), were incubated with approximately 100 PFU of either b/h PIV3, b/h PIV3/RSV chimeric viruses or RSV in 0.5 ml of Opti-MEM at room temperature for 60 min. Following the incubation, virus-serum mixtures were transferred to Vero cell monolayers, incubated at 35°C for 1 h, overlaid with 1% methylcellulose in EMEM/L-15 medium (JRH Biosciences; Lenexa, Kans.) containing 2% FBS and 1% antibiotics, and incubated at 35°C. Six days postinoculation, the infected cell monolayers were immunostained. Neutralization titers were expressed as the reciprocal of the highest serum dilution that caused a 50% inhibition of virus titer.

Neutralization assays were also carried out for serum obtained on day 28 postinfection from hamsters immunized with b/h PIV3, b/h PIV3/RSV, or b/h PIV3/hMPV chimeric viruses, RSV A2, or hMPV. The hamster sera were twofold serially diluted and incubated with 100 PFU of RSV A2 or hMPV for 1 h. The virus-serum mixtures were transferred to Vero cell monolayers and overlaid with methylcellulose. After 5 or 10 days of incubation at 35°C, the monolayers were immunostained with RSV or hMPV polyclonal antiserum, respectively, for quantitation.

HAI assay. Hemagglutination-inhibition (HAI) assays were performed by incubating serial twofold dilutions of day 28 hamster sera at 25°C for 30 min with hPIV3 in V-bottom 96-well plates. Following the addition of guinea pig erythrocytes, the incubation was continued for an additional 90 min, and the presence or absence of hemagglutination in each well was recorded. Titers were expressed as the mean reciprocal $\log_2$ of the highest serum dilution that inhibited hemagglutination.

RESULTS

Recovery of chimeric PIV3 harboring the RSV F, RSV G, or hMPV F gene in genome position 1 or 2. bPIV3 had been shown previously to be attenuated for replication in rhesus monkeys and safe and immunogenic in children (12, 27). The genetic determinants specifying bPIV3 attenuation in rhesus monkeys were shown to be located in the N, P, and L genes (22). Based on these data, it is expected that the chimeric b/h PIV3, harboring the hPIV3 F and HN genes, will still be attenuated in humans, and development as a vaccine vector is warranted. An additional degree of attenuation should result from inserting foreign antigens such as RSV or hMPV genes into the b/h PIV3 genome.

The surface glycoproteins of RSV are known to be responsible for eliciting neutralizing antibodies. It is expected that the highly conserved RSV F protein alone will elicit protective immunity against both subgroup A and B RSV strains. Similarly, the hMPV F protein alone should provide protection against all clades of hMPV. To test the immunogenicity of the glycoproteins, the b/h PIV3 vector was engineered to accept insertions of the genes at two positions. Unique *Avr*II restriction enzyme sites were introduced into the PIV3 genome upstream of the N gene translational start codon (position 1) and between the bPIV3 N and P genes (position 2). The RSV F or G genes and the hMPV F gene coupled to the bPIV3 N-P intergenic region, were inserted as transcriptional cassettes in positions 1 and 2 of the PIV3 genome to generate bivalent PIV3/RSV or PIV3/hMPV vaccine candidates (Fig. 1). The 3′-most proximal gene positions in the PIV3 viral RNA genome are transcribed at a higher frequency than genes located near the 5′ end. Therefore, foreign antigens inserted into PIV3 position 1 or 2 should be expressed at high levels eliciting a strong immune response. The shifting of the PIV3 N gene from genome position 1 to 2 rendered recombinant virus recovery less efficient, perhaps due to smaller amounts of N protein and/or delayed N protein production. A greater number of transfections were necessary to recover chimeric viruses harboring gene insertions in position 1 than gene insertions in position 2.

Comparison of replication kinetics of chimeric viruses harboring RSV or hMPV genes in position 1 or 2 of the PIV3 genome. In order to study whether gene insertions in position 1 or 2 of the PIV3 genome had an effect on virus replication, multicycle growth curves were carried out for b/h PIV3/RSV F1, F2, G1, and G2 as well as b/h PIV3/hMPV F1 and F2 in Vero cells at an MOI of 0.1 (Fig. 2). b/h PIV3/RSV G2 and F2 replicated to peak titers of 8.3 and 7.7 $\log_{10}$ PFU/ml at 72 hpi, which was only 0.5 to 1.0 $\log_{10}$ lower than the peak titers observed for the vector b/h PIV3 (Fig. 2A). b/h PIV3 F1 replicated to a peak titer of 6.9 $\log_{10}$, which is ~1 $\log_{10}$ lower than that observed for b/h PIV3 harboring the RSV F gene in position 2. b/h PIV3/RSV G1 displayed a delayed replication kinetics compared to b/h PIV3/RSV G2 (Fig. 2A). This delay in replication observed for b/h PIV3/RSV G1 resulted in peak titers of 7.0 $\log_{10}$ at 96 hpi (Fig. 2A).

The chimeric virus harboring the hMPV F gene in position 2 of the b/h PIV3 genome replicated as efficiently as the b/h PIV3 vector. Peak titers observed for b/h PIV3/hMPV F2 at 96 hpi were 8.1 $\log_{10}$ PFU/ml (Fig. 2B). In contrast, the PIV3 expressing hMPV F protein from position 1 displayed a delayed onset of virus replication, and the peak titers were decreased by 1.8 $\log_{10}$ compared to b/h PIV3/hMPV F2 at 96 hpi. The highest achievable titer obtained for b/h PIV3/hMPV F1 in Vero cells was 6.3 $\log_{10}$ PFU/ml (Fig. 2B). The virus replication defect displayed by b/h PIV3/hMPV F1 was more severe than that of b/h PIV3/RSV G1 or b/h PIV3/RSV F1, suggesting that the protein inserted could have an effect on virus replication.

Collectively, the data showed that b/h PIV3 expressing RSV or hMPV proteins in genome position 1 or 2 replicated to peak titers of 10^6 to 10^8 PFU/ml in Vero cells. Viruses harboring the

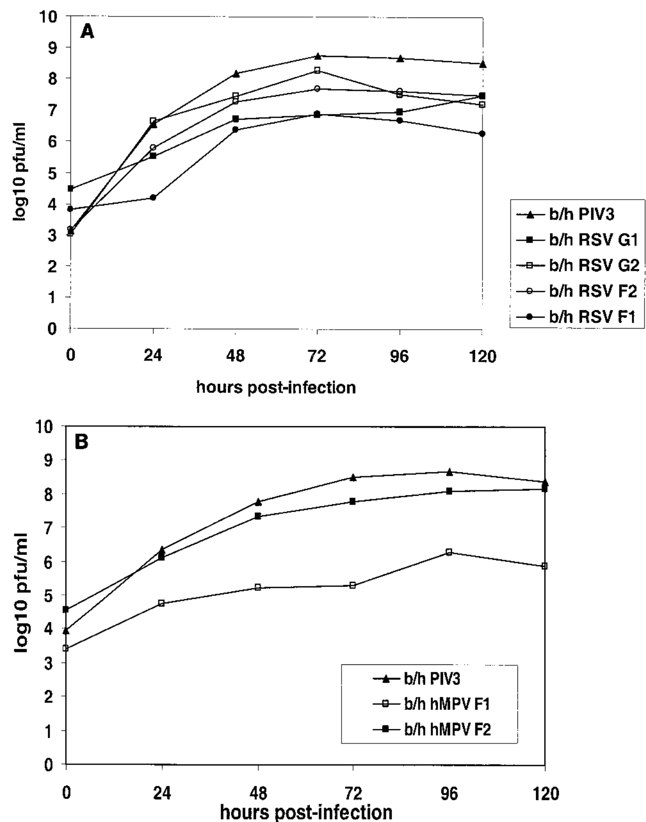

FIG. 2. (A) Multicycle growth curves of b/h PIV3/RSV F1, F2, G1, G2, and b/h PIV3 carried out in Vero cells at an MOI of 0.1. (B) Multicycle growth curves of b/h PIV3/hMPV F1, F2, and b/h PIV3 in Vero cells at an MOI of 0.1. Cells and supernatants were collected at 0, 24, 48, 72, 96, and 120 hpi. The titers of the samples were determined by plaque assays on Vero cells.

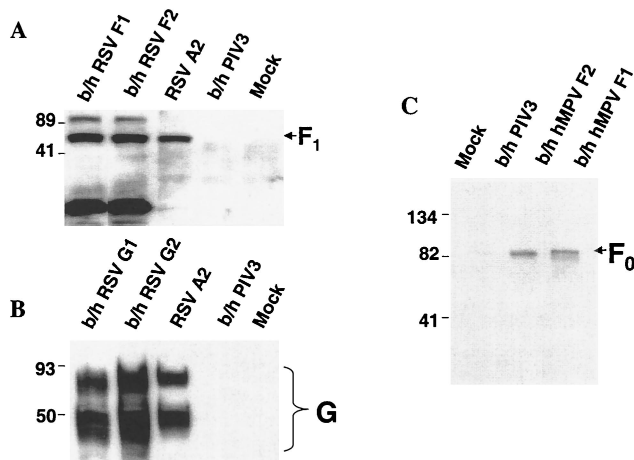

FIG. 3. RSV or hMPV protein expression by the chimeric b/h PIV3 in Vero cells. (A) Immunoblot of Vero cell lysates infected with b/h PIV3/RSV F1 or F2 or wild-type RSV A2, b/h PIV3 (at an MOI of 0.1), or mock-infected cells at 48 hpi probed with RSV F-specific monoclonal antisera. An ~50-kDa band corresponding to the processed RSV F_1 protein was observed for b/h PIV3/RSV F1 and F2 lysates as well as for RSV A2. A larger band of ~90 kDa, the uncleaved F_0 precursor, is also present in relatively smaller quantities in lanes 1 and 2, indicating that RSV F protein processing occurs efficiently in the chimeric viruses. (B) Western blot of cell lysates infected with b/h PIV3/RSV G1 or G2, RSV A2, b/h PIV3 (at an MOI of 0.1) and mock-infected cell lysates at 48 hpi. The Western blot was probed with RSV G polyclonal antisera and displays mature glycosylated and immature forms of the RSV G protein expressed by b/h PIV3/RSV G1 or G2 or RSV A2. As expected, a signal was not observed in lysates derived from Vero cells infected with b/h PIV3 or in mock-infected cells. (C) b/h PIV3/hMPV F1- or F2-, b/h PIV3-, or mock-infected ^{35}S-labeled cell lysates were harvested 48 hpi and immunoprecipitated with hMPV-specific polyclonal guinea pig antisera. A band of ~80 kDa was observed in cell lysates derived from b/h PIV3/hMPV F1 and F2 that was not present in b/h PIV3- or mock-infected lysates. This size is consistent with an uncleaved F_0 hMPV protein.

antigen insertion in position 2 replicated more efficiently in tissue culture than those containing foreign genes in position 1.

Expression of RSV or hMPV proteins by chimeric b/h PIV3. RSV F or RSV G protein expression by the chimeric b/h PIV3/RSV was verified by Western blotting. Levels of RSV F protein expression for b/h PIV3/RSV F1 and b/h PIV3/RSV F2 were compared at 48 hpi at an MOI of 0.1 (Fig. 3A). An ~50-kDa band representing the RSV F_1 proteolytic fragment was detected in cells infected with b/h PIV3/RSV F1 and b/h PIV3/RSV F2 as well as wild-type RSV (Fig. 3A, lanes 1, 2, and 3). The amounts of RSV F_1 expressed by b/h PIV3/RSV F1 and b/h PIV3/RSV F2 at 48 hpi were similar. Only low levels of uncleaved F_0 were detected in cells infected with b/h PIV3/RSV F1 and b/h PIV3/RSV F2, indicating that most of the F_0 precursors were efficiently processed during chimeric virus infection as was observed in wild-type RSV infections. As expected, b/h PIV3 and mock-infected cell lysates did not yield a signal for RSV F protein (Fig. 3A, lanes 4 and 5). A smaller band of ~26 kDa was also observed in the b/h PIV3/RSV F1 and F2 lysates (Fig. 3A, lanes 1 and 2) that was not present in wild-type RSV lysates. Since the RSV F MAbs bind to epitopes in the F_1 fragment, it is likely that this proteolytic fragment is derived from the F_1 protein. The absence of this proteolytic fragment in RSV-infected cells may be due to more authentic processing of RSV F in the presence of the complete set of RSV proteins (Fig. 3A, lane 3).

The relative expression of RSV G in b/h PIV3/RSV G1, b/h PIV3/RSV G2, and wild-type RSV infected cells at a MOI of 0.1 at 48 hpi is shown in Fig. 3B. Both the immature and glycosylated forms of RSV G that migrated at approximately 50 and 90 kDa, respectively, were detected. b/h PIV3/RSV G1-infected cells showed levels of RSV G expression similar to that seen in wild-type RSV-infected cells (Fig. 3B, lanes 1 and 3). However, in b/h PIV3/RSV G2-infected cells, the accumulation of RSV G was reproducibly about two to three times more than that present in wild-type RSV-infected cells (Fig. 3B, lanes 2 and 3). The higher levels of RSV G expression may be due to the more 3′ proximal position of the RSV G gene in the PIV3 genome compared to its native position in the RSV genome. Higher levels of expression were not observed for RSV G at position 1, which did not replicate as efficiently as b/h PIV3/RSV G2. RSV G-specific bands were not observed in cell lysates derived from b/h PIV3- or mock-infected cells (Fig. 3B, lanes 4 and 5).

The recovered b/h PIV3/hMPV viruses were analyzed for hMPV F protein expression by immunoprecipitation, since the available hMPV polyclonal antisera did not recognize fully

TABLE 1. Neutralization of b/h PIV3/RSV with RSV and hPIV3 antisera

Virus used in neutralization assay	Reciprocal 50% neutralizing antibody dilution			
	RSV F 1200 MAb	RSV PAb[a]	hPIV3 F C191/9 MAb	hPIV3 HN 68/2 MAb
RSV	2,000	400.0	62.5	<500
b/h PIV3	<25	<15.6	500.0	16,000
b/h RSV F1	<25	<15.6	500.0	32,000
b/h RSV F2	<25	<15.6	500.0	32,000
b/h RSV G1	ND[b]	<15.6	ND	32,000
b/h RSV G2	ND	<15.6	ND	32,000

[a] PAb, polyclonal antibody.
[b] ND, not determined.

TABLE 2. Replication of b/h PIV3 expressing RSV F or G protein in position 1 or 2 of the PIV3 genome in hamsters

Virus[a]	Mean virus titer on day 4 postinfection ($\log_{10}$ $TCID_{50}$/g of tissue ± SE)[b]	
	Nasal turbinates	Lungs
b/h PIV3	4.8 ± 0.4	4.4 ± 0.3
RSV A2	3.4 ± 0.5	3.3 ± 0.5
b/h RSV G1	4.2 ± 0.7	2.9 ± 0.7
b/h RSV F1	4.6 ± 0.4	3.5 ± 0.2
b/h RSV G2	4.2 ± 0.9	4.3 ± 0.2
b/h RSV F2	4.6 ± 0.6	4.4 ± 0.5

[a] Groups of six hamsters were inoculated intranasally with 10^6 PFU of the virus indicated.
[b] $TCID_{50}$ assays were read for CPE on day 6 postinfection. SE, standard error.

denatured hMPV F protein in a Western blot. The expression of hMPV F protein by b/h PIV3/hMPV F1 and F2 was shown by immunoprecipitation with guinea pig hMPV antisera (Fig. 3C). Interestingly, a specific band in the lysates of b/h PIV3/hMPV F1 and F2 was observed at approximately 80 kDa, which corresponded to the size of uncleaved F precursor protein, F_0. Although hMPV F protein was expressed by both b/h PIV3 F1 and F2, it was difficult to compare the amounts produced by b/h PIV3/hMPV F1 and F2. b/h PIV3/hMPV F1 was expected to express less hMPV F protein, because it also replicated less efficiently than b/h PIV3/hMPV F2.

Collectively, these data showed that the chimeric b/h PIV3/RSV expressed the RSV F and G proteins efficiently at both positions 1 and 2. b/h PIV3/hMPV F expressed the hMPV F protein from both genome positions. Antigen expression levels at position 1 or position 2 of the PIV3 genome were similar, such that either position can be used for gene insertion. However, position 2 gene insertions appear to be better tolerated by b/h PIV3, since virus replication was less compromised than for position 1.

All chimeric b/h PIV3/RSV and b/h PIV3/hMPV viruses maintained the RSV or hMPV gene inserts stably for multiple passages. Large-scale production of live viral vaccines requires multiple passages of the virus in order to achieve high virus titers. Therefore, it was important to determine whether gene insertions at position 1 or 2 in chimeric PIV3 were stably retained following multiple passages in Vero cells. The chimeric viruses were serially blind passaged 10 times in Vero cells. At each passage level, the infected cell lysates were analyzed for RSV or hMPV protein expression by plaque assays with immunostaining using RSV or hMPV polyclonal antisera. After passage 10, the introduced RSV or hMPV transcription units were isolated by RT-PCR. The RSV or hMPV gene sequences, including gene end and gene start sequences, were verified by DNA sequence analysis. All b/h PIV3 viruses vectoring RSV or hMPV glycoproteins stably maintained the RSV and hMPV gene cassettes as well as RSV and hMPV protein expression for 10 passages (data not shown).

The chimeric b/h PIV3/RSV could not be neutralized with RSV antisera. We studied whether the introduced foreign surface glycoproteins were incorporated into the b/h PIV3 virion envelope, which may have an effect on virus tropism. In order to address this, neutralization assays were carried out using b/h PIV3/RSV (Table 1). RSV F 1200 MAbs neutralized 50% of wild-type RSV A2 at a 1:2,000 dilution (Table 1). In contrast, even a dilution of 1:25 did not neutralize any of the chimeric b/h PIV3/RSV. Similarly, a dilution of 1:400 of the polyclonal RSV antiserum neutralized 50% of RSV A2, but even at a dilution of 1:15.6 did not neutralize b/h PIV3 RSV (Table 1). hPIV3 F C191/9 MAb neutralized 50% of b/h PIV3 as well as the b/h PIV3/RSV at a dilution of 1:500 (Table 1). The hPIV3 HN 68/2 MAb neutralized b/h PIV3 at a dilution of 1:16,000 and b/h PIV3/RSV at a dilution of 1:32,000 (Table 1). When these assays were performed under the same conditions, but in the presence of guinea pig complement, neutralization of b/h PIV3/RSV was still not observed (data not shown). The results clearly demonstrated that the RSV proteins were not functional: i.e., the RSV F protein could not functionally substitute for the hPIV3 F protein that was blocked by the hPIV3 F antibody. In addition, these results suggested that the RSV proteins were not present in the virion envelope. There still remains the possibility of misincorporated RSV F or G proteins in the b/h PIV3 envelope, such that the neutralizing epitopes were not accessible to RSV antibodies, or the RSV G or F proteins were present in insufficient amounts to change the original neutralization properties of the b/h PIV3 virion. Although the present experiments cannot distinguish between these possibilities, it is unlikely that b/h PIV3 expressing foreign antigens will result in altered virus tropism.

Replication of b/h PIV3/RSV F1, G1, F2, and G2 in Syrian Golden hamsters. Syrian Golden hamsters represent a permissive small animal model suitable for evaluation of replication and immunogenicity of genetically engineered b/h PIV3 (9). It was expected that the introduction of the RSV antigens would not alter the ability of the chimeric b/h PIV3 to infect and replicate in hamsters, since the foreign antigens were not functionally incorporated into the virion (Table 1). When animals were immunized intranasally, the results showed that all of the chimeric b/h PIV3/RSV replicated to 4.2 to 4.6 $\log_{10}$ $TCID_{50}$/g of tissue in the nasal turbinates of hamsters (Table 2). These levels of replication were similar to those observed for b/h PIV3, which replicated to 4.8 $\log_{10}$ $TCID_{50}$/g of tissue (Table 2). Syrian Golden hamsters are only semipermissive for infection with RSV. The titers of RSV observed in the upper respiratory tract of hamsters were lower by 1.4 $\log_{10}$ $TCID_{50}$/g tissue compared to those of b/h PIV3 (Table 2). The b/h PIV3/RSV harboring the RSV gene in position 1 displayed titers reduced 0.9 to 1.5 $\log_{10}$ in the lungs of hamsters compared to b/h PIV3 (Table 2). In contrast, the b/h PIV3/RSV that con-

TABLE 3. Hamsters immunized with b/h PIV3 expressing RSV F or RSV G proteins are protected from challenge with RSV A2 or hPIV3

Immunizing virus[a]	Mean hPIV3 titer on day 4 postchallenge (log_{10} $TCID_{50}$/g of tissue ± SE)[b]		Mean RSV A2 titer on day 4 postchallenge (log_{10} PFU/g of tissue ± SE)[b]	
	Nasal turbinates	Lungs	Nasal turbinates	Lungs
b/h PIV3	<1.2 ± 0.0	<1.0 ± 0.1	3.3 ± 0.9	2.9 ± 0.4
b/h RSV G1	<1.2 ± 0.1	<1.1 ± 0.1	<1.0 ± 0.3	<0.7 ± 0.1
b/h RSV F1	<1.0 ± 0.0	<1.0 ± 0.0	<0.8 ± 0.1	<0.5 ± 0.0
b/h RSV G2	<1.2 ± 0.2	<1.1 ± 0.2	<0.8 ± 0.1	<0.8 ± 0.3
b/h RSV F2	<1.2 ± 0.1	<1.0 ± 0.1	<1.3 ± 0.6	<1.6 ± 1.0
RSV A2	4.5 ± 0.6	4.8 ± 0.6	<0.6 ± 0.2	<0.6 ± 0.1
Placebo	4.4 ± 0.1	4.1 ± 0.1	3.6 ± 0.8	3.1 ± 0.7

[a] Virus used to immunize groups of six hamsters on day 0.
[b] On day 28, the hamsters were challenged with 10^6 PFU of hPIV3 or RSV A2. SE, standard error.

TABLE 4. Vaccination of hamsters with b/h PIV3/RSV induces PIV3 serum HAI and RSV neutralizing antibodies

Virus[a]	Neutralizing antibody response to RSV (mean reciprocal log_2 ± SE)[b,c]	HAI antibody response to hPIV3 (mean reciprocal log_2 ± SE)[c]
RSV	7.9 ± 1.0	<2.0 ± 0.0
b/h RSV F1	5.5 ± 0.5	5.5 ± 0.5
b/h RSV G1	3.4 ± 0.5	6.6 ± 0.7
b/h RSV F2	6.9 ± 0.7	6.7 ± 0.8
b/h RSV G2	3.4 ± 0.5	5.2 ± 0.4
b/h PIV3	<2.0 ± 0.0	7.2 ± 0.5

[a] Virus used to immunize hamsters.
[b] The neutralizing antibody titers were determined by a 50% plaque reduction assay.
[c] The neutralizing antibody titers of hamster pre-serum were <2.0, and the HAI antibody titers were <2.0.

tained a gene insertion at position 2 replicated to titers similar to those observed for b/h PIV3 in the lower respiratory tract of hamsters (Table 2). Chimeric PIV3 harboring foreign genes in position 1 or 2 retained the ability to replicate efficiently in the lower and upper respiratory tracts of hamsters. The introduction of an additional gene into the b/h PIV3 genome at position 1 or 2 did not attenuate the virus significantly in vivo.

b/h PIV3/RSV-vaccinated hamsters were protected from challenge with RSV A2 or hPIV3 and produced RSV neutralizing and PIV3 HAI serum antibodies. In order to evaluate whether the levels of replication observed for b/h PIV3/RSV were sufficient to elicit a protective immune response in hamsters, the animals were challenged intranasally with 10^6 PFU of RSV or hPIV3 per animal on day 28 postvaccination. Animals immunized with b/h PIV3/RSV were protected completely from hPIV3 and RSV infection (Table 3). RSV challenge virus was detected at very low levels, and hPIV3 challenge virus was not observed in the upper and lower respiratory tract of hamsters. Only the animals that had received placebo medium displayed 4.4 and 4.1 log_{10} $TCID_{50}$ of hPIV3 per g of tissue, and 3.6 and 3.1 log_{10} PFU of RSV per g of tissue in the upper and lower respiratory tracts, respectively (Table 3). This study also showed that animals immunized with RSV were not protected from challenge with hPIV3. Similarly, animals vaccinated with bh/PIV3 displayed high titers of RSV challenge virus (Table 3).

Prior to administration of the challenge dose, serum samples were obtained on day 28 from the b/h PIV3/RSV-immunized animals. The hamster sera were analyzed for the presence of RSV neutralizing antibodies by using a 50% plaque reduction assay and for PIV3 HAI serum antibodies by carrying out HAI assays (Table 4). The results showed that the viruses expressing the RSV F protein at genome position 1 or 2 displayed RSV neutralizing antibody titers of 5.5 and 6.9 log_2, respectively. These titers were slightly lower than the antibody titers observed for serum obtained from animals vaccinated with wild-type RSV (Table 4). In contrast, the viruses expressing the RSV G protein showed RSV neutralizing antibody titers that were reduced by 4 log_2 compared to wild-type RSV (Table 4). All of the chimeric b/h PIV3/RSV hamster sera showed levels of HAI serum antibodies that were reduced by 0.5 to 2.0 log_2 compared to the levels observed for b/h PIV3 (Table 4). The results showed that the chimeric b/h PIV3/RSV could infect and replicate efficiently in hamsters. A range of neutralizing serum antibody titers against RSV and hPIV3 were observed following immunization with the various b/h PIV3/RSV chimeric viruses. However, all of the animals immunized were protected against both RSV and hPIV3 infection upon challenge with these viruses.

Syrian Golden hamsters were permissive for infection with chimeric PIV3/hMPV. A small animal model for hMPV has not yet been identified. However, because the chimeric b/h PIV3 expressed only a single hMPV protein, it was expected that the chimeric viruses would replicate efficiently in the respiratory tract of hamsters. Syrian Golden hamsters were infected with b/h PIV3/hMPV F1 and F2 intranasally, and the ability of these viruses to replicate in the respiratory tract was analyzed (Table 5). The results showed that b/h PIV3/hMPV F1 and F2 replicated in the nasal turbinates of hamsters to high levels of 5.3 and 5.7 log_{10} $TCID_{50}$/g of tissue, respectively. These titers were similar to those observed for b/h PIV3 (4.8 log_{10} $TCID_{50}$/g of tissue). For comparison, wild-type hMPV displayed titers of 5.3 log_{10} $TCID_{50}$/g of tissue in the upper respiratory tracts of hamsters (Table 5). b/h PIV3/hMPV F1 and F2 replicated to titers of 5.7 and 4.6 log_{10} $TCID_{50}$/g of tissue in the lungs of hamsters (Table 5). These titers were also similar to those observed for b/h PIV3 (5.6 log_{10} $TCID_{50}$/g of tissue). Wild-type hMPV displayed reduced titers of 3.6 log_{10} $TCID_{50}$/g tissue in the lower respiratory tract of hamsters (Ta-

TABLE 5. Replication of b/h PIV3 expressing hMPV F protein at position 1 or 2 of the PIV3 genome in hamsters

Virus[a]	Mean virus titer on day 4 postinfection (log_{10} $TCID_{50}$/g of tissue ± SE)[b]	
	Nasal turbinates	Lungs
b/h PIV3	4.8 ± 0.2	5.6 ± 0.6
b/h hMPV F1	5.3 ± 0.5	5.7 ± 0.4
b/h hMPV F2	5.7 ± 0.5	4.6 ± 0.3
hMPV	5.3 ± 0.1	3.6 ± 0.3

[a] Groups of six hamsters were inoculated intranasally with 10^6 PFU of the virus indicated.
[b] $TCID_{50}$ assays were read for CPE on day 10 postinfection. SE, standard error.

TABLE 6. Hamsters immunized with b/h PIV3 expressing hMPV F protein are protected from challenge with hMPV or hPIV3

Challenge virus	Mean hPIV3 titer on day 4 postchallenge (log_{10} $TCID_{50}$/g of tissue ± SE)[b]		Mean hMPV titer on day 4 postchallenge (log_{10} PFU/g of tissue ± SE)[b]	
Immunizing virus[a]	Nasal turbinates	Lungs	Nasal turbinates	Lungs
b/h PIV3	<1.3 ± 0.2	<1.1 ± 0.1	ND[c]	ND
b/h hMPV F1	<1.3 ± 0.1	<1.1 ± 0.1	3.5 ± 0.8	<0.5 ± 0.2
b/h hMPV F2	<1.2 ± 0.1	<1.2 ± 0.1	<0.9 ± 0.4	<0.5 ± 0.1
hMPV	ND	ND	<0.8 ± 0.3	<0.4 ± 0.0
Placebo	4.3 ± 0.3	4.5 ± 0.5	6.0 ± 0.3	4.5 ± 1.3

[a] Virus used to immunize groups of six hamsters on day 0.
[b] On day 28, the hamsters were challenged with 10^6 PFU of hPIV3 or hMPV/NL/1/00.
[c] ND, not determined.

ble 5). These data demonstrated that b/h PIV3/hMPV F1 and F2 could efficiently infect and replicate in the upper and lower respiratory tracts of Syrian Golden hamsters and suggested that hamsters are a suitable small animal model to study immunogenicity of hMPV as well as hMPV vaccine candidates.

b/h PIV3/hMPV-vaccinated hamsters were protected from challenge with hMPV or hPIV3 and produced hMPV neutralizing and PIV3 HAI serum antibodies. As predicted, chimeric b/h PIV3 expressing the hMPV F protein in position 1 or 2 replicated to high levels in the respiratory tracts of hamsters (Table 5). On day 28 postimmunization, the vaccinated animals were challenged intranasally with 10^6 PFU of hPIV3 or hMPV. Viruses present in the nasal turbinate and lung tissues 4 days postchallenge were quantified by plaque assays. Animals that had received b/h PIV3/hMPV F1 or F2 were completely protected from the hMPV challenge in the lower respiratory tract. As expected, wild-type hMPV-immunized animals were protected from hMPV challenge. Only animals that had received placebo medium displayed high hMPV challenge virus titers of 4.5 log_{10} PFU/g of lung tissue (Table 6). In the upper respiratory tract, b/h PIV3/hMPV F1 protected only partially from hMPV challenge. A decrease of 2.5 log_{10} was observed in b/h PIV3/hMPV F1-vaccinated animals compared to the hMPV challenge virus titers present in the placebo-treated control, which were 6.0 log_{10} PFU/g of nasal turbinate tissue. In contrast, b/h PIV3/hMPV F2 protected the hamsters completely in the upper respiratory tract, and only trace amounts of hMPV challenge virus were detected (Table 6). All b/h PIV3/hMPV F-immunized animals were completely protected from challenge with hPIV3. Only the animals that had received placebo medium for immunization displayed high levels of hPIV3 in the lung and nasal turbinate tissues, resulting in titers of 4.5 and 4.3 log_{10} $TCID_{50}$/g tissue, respectively (Table 6).

Serum samples were obtained from the hamsters on day 28 prior to administration of the challenge virus and analyzed for the presence of hMPV neutralizing antibodies and HAI serum antibodies (Table 7). High levels of hMPV neutralizing antibodies, 7.4 log_2, were observed for sera derived from wild-type hMPV-infected animals. Sera obtained from b/h PIV3/hMPV F1- or F2-vaccinated hamsters showed neutralizing antibody titers of 7.8 and 7.4 log_2, respectively, which were equivalent to those observed for wild-type hMPV sera (Table 7). The HAI antibody levels were also similar to those observed for b/h PIV3, the virus vector. The chimeric b/h PIV3/hMPV F1 and F2 displayed HAI titers of 5.8 and 6.3 log_2, respectively, which are 1.2 and 0.7 log_2 lower than the HAI titers obtained from b/h PIV3-infected hamster sera (Table 7).

TABLE 7. Vaccination of hamsters with b/h PIV3/hMPV induces PIV3 serum HAI and hMPV neutralizing antibodies

Virus[a]	Neutralizing antibody response to hMPV (mean reciprocal log_2 ± SE)[b,c]	HAI antibody response to hPIV3 (mean reciprocal log_2 ± SE)[c]
hMPV	7.4 ± 1.5	<2.0 ± 0.0
b/h hMPV F1	7.8 ± 1.0	5.8 ± 0.7
b/h hMPV F2	7.4 ± 1.0	6.3 ± 0.5
b/h PIV3	<2.0 ± 0.0	7.0 ± 0.8

[a] Virus used to immunize hamsters.
[b] The neutralizing antibody titers were determined by a 50% plaque reduction assay. SE, standard error.
[c] The neutralizing antibody titers of hamster pre-serum were <2.0, and the HAI antibody titers were <2.0.

In summary, the results showed that b/h PIV3 expressing hMPV F protein in position 1 or 2 of the b/h PIV3 genome can efficiently infect and replicate in Syrian Golden hamsters and induce protective immunity against challenge with hPIV3 and hMPV. The immunization of hamsters with these chimeric viruses also elicited the production of hMPV neutralizing antibodies and HAI serum antibodies with levels similar to those observed for wild-type hMPV or b/h PIV3, respectively.

DISCUSSION

The effect of foreign gene insertion in position 1 or 2 of the b/h PIV3 genome on in vitro and in vivo virus replication and foreign antigen expression was evaluated in this study. The three antigens chosen were the RSV F, RSV G, and hMPV F surface glycoproteins, all of which have the potential to be incorporated into the b/h PIV3 virion. We wanted to study whether all foreign membrane glycoproteins expressed by b/h PIV3 would produce viruses with similar phenotypes or whether the type of protein inserted would yield viruses with different replication characteristics. PIV3 genome positions 1 and 2 were expected to yield the highest levels of foreign protein expression since paramyxoviruses transcribe the 3′-most proximal genes with the highest frequency. Foreign gene insertions in position 1 could interfere with virus replication by delaying and/or reducing bPIV3 N protein expression, which now takes place from genome position 2. Insertion in the first position also has a greater potential for effecting initiation of transcription and replication of the antigenome. In contrast, gene insertions in position 2 should alter the N-P mRNA ratio, which may impair virus replication as well. Therefore, it was of interest to test which of the genome positions would better tolerate foreign gene insertions without compromising virus replication.

Our studies showed that b/h PIV3 expressed the RSV F, RSV G, or hMPV F proteins efficiently from genome positions 1 and 2. Curiously, the 3′ proximal location of the foreign antigens did not result in enhanced protein expression compared to that of wild-type RSV, as expected from the transcription gradient known to be generated by nonsegmented negative-strand RNA viruses. Chimeric b/h PIV3/RSV G expressed slightly less RSV G protein

from position 1 than from position 2; however, this effect was not observed for b/h PIV3/RSV F1 or b/h PIV3/hMPV F1. The RSV F_0 protein expressed by PIV3 was processed into F_1 and F_2, like the RSV F protein derived from wild-type RSV. A smaller proteolytic fragment accumulated to large amounts in lysates derived from b/h PIV3/RSV F1 and F2, which was not observed in wild-type RSV lysates. The precise origin of this peptide is not known for certain, but the antibodies used to probe the Western blot recognized epitopes in the N-terminal half of the glycoprotein. While the precursor RSV F_0 and the processed RSV F_1 were observed for b/h PIV3/RSV F1 and F2, F protein processing was not observed for hMPV F expressed by b/h PIV3 from position 1 or 2. b/h PIV3/hMPV F1 or F2 displayed only protein bands corresponding in size to the uncleaved F_0 protein precursor. Analysis of the F protein cleavage site revealed that the hMPV F protein cleavage site consisted of multiple charged amino acid residues (RQSR) but differed from the conserved furin protease cleavage site seen in related viruses like RSV or APV (RKRR and RRRR, respectively). Underlining indicates altered amino acids that are different in hMPV. Sendai virus, a paramyxovirus with a nonconsensus furin protease cleavage site needs exogenous protease for multiple rounds of replication in tissue culture (19). The "weak" cleavage site of the hMPV F protein may therefore be responsible for the presence of only the F_0 protein detected by immunoprecipitation in this study, since the F_1 and F_2 fragments would be present only at low levels. Inefficient F protein cleavage may hinder the cell-to-cell spread of hMPV, accounting for slow replication in tissue culture, and may explain the trypsin requirement of some hMPV strains (25).

PIV3 harboring foreign genes in position 1 was more difficult to recover by reverse genetics than viruses harboring gene insertions in position 2. This may be due to an impairment of bPIV3 N protein levels, since the N gene was moved to genome position 2 in b/h PIV3 harboring foreign genes in position 1. An analysis of the kinetics of virus replication showed that viruses harboring foreign gene insertions in position 2 replicated to peak titers observed for b/h PIV3, the virus vector. In contrast, viruses containing the gene insertion in position 1 of the PIV3 genome displayed a delayed onset of peak virus replication and reduced virus peak titers compared to b/h PIV3. This was most pronounced for b/h PIV3/hMPV F1 and b/h PIV3/RSV G1, which suggested that the type of foreign gene inserted into the PIV3 genome also has an effect on virus replication, even though all of the antigens are surface glycoproteins with similar functions. All chimeric PIV3 viruses with gene insertions in position 2 replicated to high titers of 10^7 to 10^8 PFU/ml in Vero cells, and thus position 2 is a better position for maximum level of foreign gene expression.

To study whether foreign gene insertions had an effect on in vivo virus replication, hamsters were infected intranasally with the chimeric b/h PIV3 expressing RSV or hMPV proteins. Only b/h PIV3 expressing the RSV F or RSV G protein in position 1 displayed restricted replication in the lower respiratory tract of hamsters. However, protection from challenge with hPIV3 or RSV was not affected by the lower levels of replication in the lungs of hamsters observed for b/h PIV3/RSV G1 or F1. Notably, b/h PIV3/RSV F1 displayed lower titers of RSV neutralizing and HAI serum antibodies than b/h PIV3/RSV F2. High levels of replication were observed for b/h PIV3/hMPV F1 and F2, but b/h PIV3/hMPV F1 protected immunized hamsters only partially in the upper respiratory tract. Additional studies are necessary to determine the reason for partial protection displayed by b/h PIV3/hMPV F1. The levels of RSV neutralizing and PIV3 HAI antibody titers of b/h PIV3/hMPV F1 and F2 were not affected by genome position, because both viruses elicited antibody titers equivalent to those observed for wild-type hMPV.

b/h PIV3 had been used previously to express RSV proteins and to demonstrate immune protection in hamsters and primates (20, 21). In these studies, the genes encoding the RSV F or G protein were inserted singly in the 3′ proximal position of the PIV3 genome or inserted in tandem as a combination of RSV G and F. Characterization of RSV protein expression did not reveal a good correlation between genome position and the amount of RSV protein expressed. The data showed that RSV F protein was expressed at smaller amounts from position 1 in the PIV3 genome (b/h PIV3-F_A) than from the RSV F gene located in position 2 in b/h PIV3 G_AF_A, which is not expected in the presence of a transcriptional gradient. Similarly, RSV G protein expression for the single RSV G gene inserted at the 3′ proximal position was higher than that observed for b/h PIV3 RSV G_AF_A, in which the RSV G gene is also located at the 3′-most proximal end of the viral genome (21). These results suggested that PIV3 genome location alone may not be the sole determinant for levels of foreign gene expression. The gene start or gene end sequences and/or RNA elements that direct mRNA stability or translation efficiency may also play a role in determining efficiency of viral mRNA transcription and viral protein expression. Interestingly, the RSV neutralizing antibody titers present in sera derived from hamsters infected with b/h PIV3/RSV F1 or G1 in the study by Schmidt et al. (20) were higher than those in our data, although the neutralization antibody titers for wild-type RSV sera from both studies were identical. This difference in antibody titers may be due to higher replication levels of b/h PIV3/RSV F1 or G1 generated by Schmidt et al. in the respiratory tract of hamsters. b/h PIV3/RSV F1 and G1 displayed titers 0.5 and 1.7 $\log_{10}$ higher, respectively, in the upper respiratory tract and 1.1 and 2.2 $\log_{10}$ higher, respectively, in the lower respiratory tract of hamsters than the replication titers observed in this study (20). The increased levels of viral replication in the respiratory tract of hamsters may result in higher RSV neutralizing antibody titers. The origins of the hPIV3 F and HN genes, the Texas strain for the b/h PIV3 generated in this study and the JS strain for the b/h PIV3 generated by Schmidt et al. (20), may direct the in vivo hamster replication efficiencies observed for the chimeric viruses. The neutralizing antibody titers of b/h PIV3/RSV F1 and G1 hamster sera were also higher than wild-type RSV serum titers (20). In contrast, the neutralizing antibody titers of wild-type RSV-infected rhesus monkeys were higher than those observed for b/h PIV3/RSV F1 or G1 primate sera when the same viruses were evaluated in rhesus monkeys, albeit wild-type RSV replicated to lower titers in rhesus monkeys than chimeric PIV3/RSV (21). In order to resolve these discrepancies, it would be necessary to compare the b/h PIV3/RSV F1 and G1 viruses generated in the two different laboratories directly in the same study to determine whether the origin of the hPIV3 surface glycoprotein genes and/or other genetic components, such as the types of gene start and stop sequences, can influence virus replication and immunogenicity.

In this study, the chimeric b/h PIV3 viruses expressing the RSV or hMPV surface glycoproteins were shown to function as bivalent vaccines in a small animal model. These viruses have characteristics that make them suitable for evaluation as live attenuated virus vaccines for RSV, hMPV, and hPIV3. The chimeric viruses grow to high titers in tissue culture. The inserted antigens of the chimeric b/h PIV3 were genetically stable and maintained up to passage 10 in Vero cells. In vaccinated hamsters, the viruses elicited a protective immune response upon challenge with wild-type hPIV3, RSV, and hMPV. The challenges were carried out with homotypic viruses; however, the degree of amino acid identity is very high for the two subgroups of RSV A and B (89%), as well as the two subgroups of hMPV A and B (95%), and therefore immunological cross protection is expected to occur. Although this remains to be tested directly, RSV F MAbs such as Synagis can neutralize RSV originating from subgroup A as well as subgroup B. The chimeric viruses induced an effective neutralizing antibody response to the vectored non-PIV3 glycoproteins despite not being incorporated into the virion. The latter finding should allay concerns that a change in viral tropism is not likely as a consequence of new virus-cell receptor interactions of the b/h PIV3 expressing glycoproteins from RSV or hMPV. The b/h PIV3/RSV F2 and b/h PIV3/hMPV F2 viruses will be further evaluated for safety and efficacy in a primate model.

This study demonstrated the utility and versatility of b/hPIV3 as a virus vector for expression and delivery of three different foreign viral antigens: RSV F, RSV G, and hMPV F. The ease, speed, and effectiveness of generating potential vaccine candidates for newly discovered viral pathogens for which infectious cDNAs and recombinant virus recovery systems are not available further underscore the importance of developing b/h PIV3 as a virus vaccine vector.

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