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Bovine Parainfluenza Virus Type 3 (BPIV3) Fusion and Hemagglutinin-Neuraminidase Glycoproteins Make an Important Contribution to the Restricted Replication of BPIV3 in Primates

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This study examines the contribution of the fusion (F) and hemagglutinin-neuraminidase (HN) glycoprotein genes of bovine parainfluenza virus type 3 (BPIV3) to its restricted replication in the respiratory tract of nonhuman primates. A chimeric recombinant human parainfluenza type 3 virus (HPIV3) containing BPIV3 F and HN glycoprotein genes in place of its own and the reciprocal recombinant consisting of BPIV3 bearing the HPIV3 F and HN genes (rBPIV3-F_HHN_H) were generated to assess the effect of glycoprotein substitution on replication of HPIV3 and BPIV3 in the upper and lower respiratory tract of rhesus monkeys. The chimeric viruses were readily recovered and replicated in simian LLC-MK2 cells to a level comparable to that of their parental viruses, suggesting that the heterologous glycoproteins were compatible with the PIV3 internal proteins. HPIV3 bearing the BPIV3 F and HN genes was restricted in replication in rhesus monkeys to a level similar to that of its BPIV3 parent virus, indicating that the glycoprotein genes of BPIV3 are major determinants of its host range restriction of replication in rhesus monkeys. rBPIV3-F_HHN_H replicated in rhesus monkeys to a level intermediate between that of HPIV3 and BPIV3. This observation indicates that the F and HN genes make a significant contribution to the overall attenuation of BPIV3 for rhesus monkeys. Furthermore, it shows that BPIV3 sequences outside the F and HN region also contribute to the attenuation phenotype in primates, a finding consistent with the previous demonstration that the nucleoprotein coding sequence of BPIV3 is a determinant of its attenuation for primates. Despite its restricted replication in the respiratory tract of rhesus monkeys, rBPIV3-F_HHN_H conferred a level of protection against challenge with HPIV3 that was indistinguishable from that induced by previous infection with wild-type HPIV3. The usefulness of rBPIV3-F_HHN_H as a vaccine candidate against HPIV3 and as a vector for other viral antigens is discussed.

Bovine parainfluenza virus type 3 (BPIV3) is restricted in replication in the respiratory tract of rhesus monkeys, chimpanzees, and humans, and it is being evaluated as a vaccine against human PIV3 (HPIV3) (8, 10, 12, 26, 27). HPIV3 and BPIV3 are closely related enveloped, nonsegmented, negative-strand RNA viruses within the *Respirovirus* genus of the *Paramyxoviridae* family (2, 10). The two viruses are 25% related antigenically by cross-neutralization studies (8), and they share neutralization epitopes on their fusion (F) and hemagglutinin-neuraminidase (HN) surface glycoproteins (9, 30). HPIV3 and BPIV3 are essentially identical in genome organization (2). Both viruses encode nine proteins: the nucleoprotein (N), phosphoprotein (P), and large polymerase protein (L) are nucleocapsid-associated proteins; the C, D, and V accessory proteins are proteins of unknown function encoded by the P mRNA or by an edited version thereof; the M protein is an internal matrix protein; and the F and HN glycoproteins are protective antigens of the virus that induce neutralizing antibodies (10, 14). The amino acid sequence identities of the HN and F proteins of HPIV3 and BPIV3 are 79 and 75%, respectively (2).

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A study to define the genetic basis of the host range restriction of replication of BPIV3 in the respiratory tract of primates was previously initiated by constructing and characterizing a recombinant HPIV3 (rHPIV3) in which the N open reading frame (ORF) was replaced by that of its BPIV3 counterpart (1). The resulting chimeric virus, here referred to as rHPIV3-N_B, replicated efficiently in vitro but was restricted in replication in the upper respiratory tract of rhesus monkeys, identifying the N protein as an independent determinant of the host range restriction of BPIV3 in rhesus monkeys (1). In this study, the contribution of the F and HN genes to the attenuation of BPIV3 for rhesus monkeys was examined by generating and characterizing two reciprocal BPIV3/HPIV3 chimeras. In one chimera, the F and HN genes of HPIV3 were replaced with their BPIV3 counterparts, resulting in a recombinant designated rHPIV3-F_BHN_B. The reciprocal chimeric recombinant (rBPIV3-F_HHN_H) was constructed by replacing the F and HN genes of a recombinant BPIV3 (rBPIV3) with their HPIV3 counterparts. The F and HN genes were exchanged as pairs because of the known requirement for the presence of homologous F and HN proteins of PIVs for full functional activity (13, 21, 41). The replication of the two chimeric PIV3 recombinants was evaluated in vitro and also in vivo in the respiratory tract of rhesus monkeys. The findings of this study identify the BPIV3 F and HN genes as major contributors to the restricted

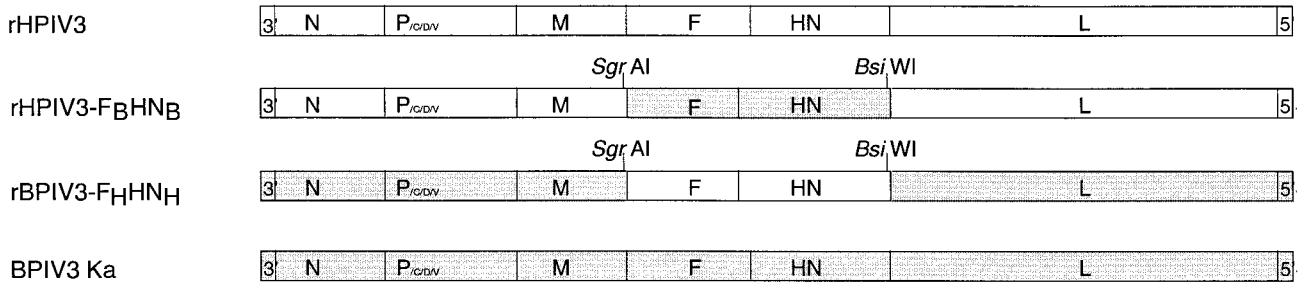

FIG. 1. Genomes of the rHPIV3-F_BHN_B and rBPIV3-F_HHN_H chimeras and of the parent viruses, rHPIV3 JS and BPIV3 Ka, shown schematically (not to scale). The F and HN genes were exchanged as a single restriction fragment between rHPIV3 and rBPIV3, using *Sgr*AI and *Bsi*WI sites that had been introduced preceding the M and HN gene end sequences, respectively.

replication of the BPIV3 in nonhuman primates, demonstrate that one or more additional BPIV3 genes contribute to this host range phenotype, and identify rBPIV3-F_HHN_H, which possesses attenuating BPIV3 sequences as well as the antigenic specificity of HPIV3, as a promising candidate for a vaccine against HPIV3.

MATERIALS AND METHODS

Viruses and cells. HEp-2 and simian LLC-MK2 monolayer cell cultures were maintained in minimal essential medium (Life Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (Summit Biotechnology, Fort Collins, Colo.), 50 μg of gentamicin sulfate per ml, and 4 mM glutamine (Life Technologies).

The wild-type BPIV3 strain Kansas/15626/84 (clone 5-2-4, lot BPI3-1) (BPIV3 Ka) was previously described (4, 27). The HPIV3 JS wild type, its recombinant version (rHPIV3), and rHPIV3 containing the BPIV3 Ka N ORF in place of the HPIV3-N ORF (rHPIV3-N_B) were also described previously (1, 15). PIVs were propagated at 32°C in LLC-MK2 cells (ATCC CCL-7) as previously described (20). The modified vaccinia virus strain Ankara (MVA) recombinant that expresses bacteriophage T7 RNA polymerase (MVA-T7) was generously provided by L. Wyatt and B. Moss (44).

Construction of antigenomic cDNAs encoding BPIV3/HPIV3 recombinants. (i) Construction of a cDNA to recover rBPIV3. A full-length cDNA was constructed to encode the complete 15,456-nucleotide (nt) antigenomic RNA of BPIV3 Ka (GenBank accession no. AF178654), with the exception of nt 21 (T to G) and 23 (C to T) (2). The nucleotides differing at each position were both observed in wild-type BPIV3 Ka virus populations with similar frequencies. The cDNA was assembled from four subclones derived from reverse transcription (RT) of viral RNA (2), using the SuperScript II preamplification system (Life Technologies) and PCR amplification with a High Fidelity PCR kit (Clontech Laboratories, Palo Alto, Calif.). The RT-PCR products were cloned into modified pUC19 plasmids (New England Biolabs, Beverly, Mass.), using the following internal restriction enzyme recognition sites: *Sma*I (BPIV3 Ka sequence position nt 186), *Pst*I (nt 2896), *Mlu*I (nt 6192), *Sac*II (nt 10452), and *Bsp*LU11I (nt 15412). Multiple subclones of the antigenomic cDNA were sequenced using a Perkin-Elmer ABI 310 sequencer with dRhodamine terminator cycle sequencing (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom), and only those matching the consensus sequence of BPIV3 Ka (2) were used for assembly of the full-length clone. The 3′ and 5′ ends of BPIV3 Ka had been cloned previously (2). Assembly of the full-length cDNA took place in the previously described p(Right) vector (15), which we modified to contain a new polylinker with restriction enzyme recognition sites for *Xho*I, *Sma*I, *Mlu*I, *Sac*II, *Eco*RI, *Hin*dIII, and *Rsr*II. The full-length cDNA clone pBPIV3(184) contained the following elements in 3′-to-5′ order: a T7 promoter followed by two nonviral guanosine residues, the complete antigenomic sequence of BPIV3 Ka, a hepatitis delta virus ribozyme, and a T7 polymerase transcription terminator, as previously described (1, 15).

(ii) Construction of rHPIV3-F_BHN_B and rBPIV3-F_HHN_H. Unique restriction enzyme recognition sites were introduced into the BPIV3 antigenomic cDNA and into the previously described HPIV3 antigenomic cDNA p3/7(131)2G (15) to facilitate the exchange of the F and HN genes between BPIV3 and HPIV3 cDNAs. Using the transformer site-directed mutagenesis protocol from Clontech, *Sgr*AI restriction sites were introduced in the downstream noncoding region of the M gene at position 4811 of the rBPIV3 sequence and position 4835 of the rHPIV3 JS sequence (GenBank accession no. Z11575). The sequence was changed from TCCAACATTGCA to TCCACCGGTGCA in rBPIV3 and from CGGACGTATCTA to CGCACCGGTGTA in rHPIV3 (recognition sites underlined). *Bsi*WI restriction sites were introduced in the downstream noncoding region of the HN gene at nt 8595 of the rBPIV3 sequence and at nt 8601 of the rHPIV3 JS sequence. The sequence was changed from GATATAAAGA to GACGTACGGA in rBPIV3 to give pBPIVs(107) and from GACAAAAGGG to GACGTACGGG in rHPIV3 to give pHPIVs(106). The F and HN genes were exchanged between pBPIVs(107) and pHPIV3s(106) by digestion of each with *Sgr*AI and *Bsi*WI, gel purification of the fragments, and assembly of the appropriate fragments into the two full-length cDNAs. The HPIV3 backbone bearing the BPIV3 F and HN genes, designated pHPIV(215), encoded 15,480 nt of viral sequence, of which nt 4835 to 8619 came from BPIV3, and it was used to derive rHPIV3-F_BHN_B (Fig. 1). The BPIV3 backbone bearing the HPIV3 F and HN genes, designated pBPIV(215), encoded 15,438 nt of viral sequence, of which nt 4811 to 8577 came from HPIV3, and it was used to derive rBPIV3-F_HHN_H (Fig. 1).

BPIV3 support plasmids for recovery of virus from cDNA. Support plasmids encoding the BPIV3 Ka N, P, and L genes were assembled in modified pUC19 vectors and then cloned into the previously described pTM vector (15). To place the individual genes immediately downstream of the T7 promoter in the pTM vector, an *Nco*I site was introduced at the start codon of the N, P, and L ORFs by site-directed mutagenesis. The *Nco*I restriction site and a naturally occurring restriction site downstream of each ORF (*Spe*I for N, *Hin*cII for P, and *Bsp*LU11 for L) was used for cloning into pTM. After cloning, the *Nco*I site in pTM(N) was mutagenized back to the original sequence to restore the correct amino acid assignment in the second codon. In pTM(P) and pTM(L), the amino acid sequence encoded by the ORF was not altered by the introduction of *Nco*I sites.

Transfection. HEp-2 cells (approximately 1.5×10^6 cells per well of a six-well plate) were grown to 90% confluence and transfected with 0.2 μg of the BPIV3 support plasmids pTM(N) and pTM(P), and 0.1 μg of pTM(L), along with 5 μg of the full-length antigenomic cDNA and 12 μl LipofectACE (Life Technologies). Each transfection mixture also contained 1.5×10^7 PFU of MVA-T7, as previously described (15). The cultures were incubated at 32°C for 12 h before the medium was replaced with minimal essential medium (Life Technologies) containing 10% fetal bovine serum. The supernatants were harvested after incubation at 32°C for an additional 3 days, passaged onto LLC-MK2 cell monolayers in 25-cm^2 flasks, and incubated for 5 days at 32°C. Virus present in the supernatant was plaque purified sequentially three times prior to amplification and characterization.

Molecular characterization of recovered chimeric recombinants. The presence of the heterologous F and HN genes in the bovine or human PIV3 backbone was confirmed in plaque-purified recombinant viruses by RT-PCR of viral RNA isolated from infected cells as previously described (2), using a primer pair that recognized conserved sequences in rBPIV3 and rHPIV3. The generation of each PCR product was dependent on the inclusion of reverse transcriptase, indicating that each was derived from viral RNA and not from contaminating cDNA (data not shown). This yielded similarly sized fragments (nt 4206 to 9035 in rBPIV3, nt 4224 to 9041 in rHPIV3, nt 4206 to 9017 in rBPIV3-F_HHN_H, and nt 4224 to 9059 in rHPIV3-F_BHN_B) which were then digested with *Eco*RI and analyzed by electrophoresis on a 1% agarose gel as previously described (2). The nucleotide sequence flanking the introduced *Sgr*AI and *Bsi*WI restriction sites in each virus was confirmed by sequencing the corresponding RT-PCR product.

Replication of HPIV3/BPIV3 chimeras in cell culture. The multicycle growth kinetics of BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, rHPIV3-N_B, and rHPIV3 in LLC-MK2 cells were determined by infecting cells in triplicate at a multiplicity of infection (MOI) of 0.01 and harvesting samples at 24-h intervals over a 6-day period, as previously described (34). Samples were flash-frozen and titered in a single assay on LLC-MK2 cell monolayers in 96-well plates at 32°C, as described elsewhere (16).

Monkey studies. Rhesus monkeys, which were seronegative for PIV3 as determined by hemagglutination inhibition (HAI) assay (8), were inoculated intranasally and intratracheally in groups of two or four animals with 10^5 50% tissue culture infectious doses ($TCID_{50}$) of BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, rHPIV3-N_B, or rHPIV3 per ml. Nasopharyngeal swabs were collected daily on days 1 to 11 and on day 13. Tracheal lavage samples were collected on

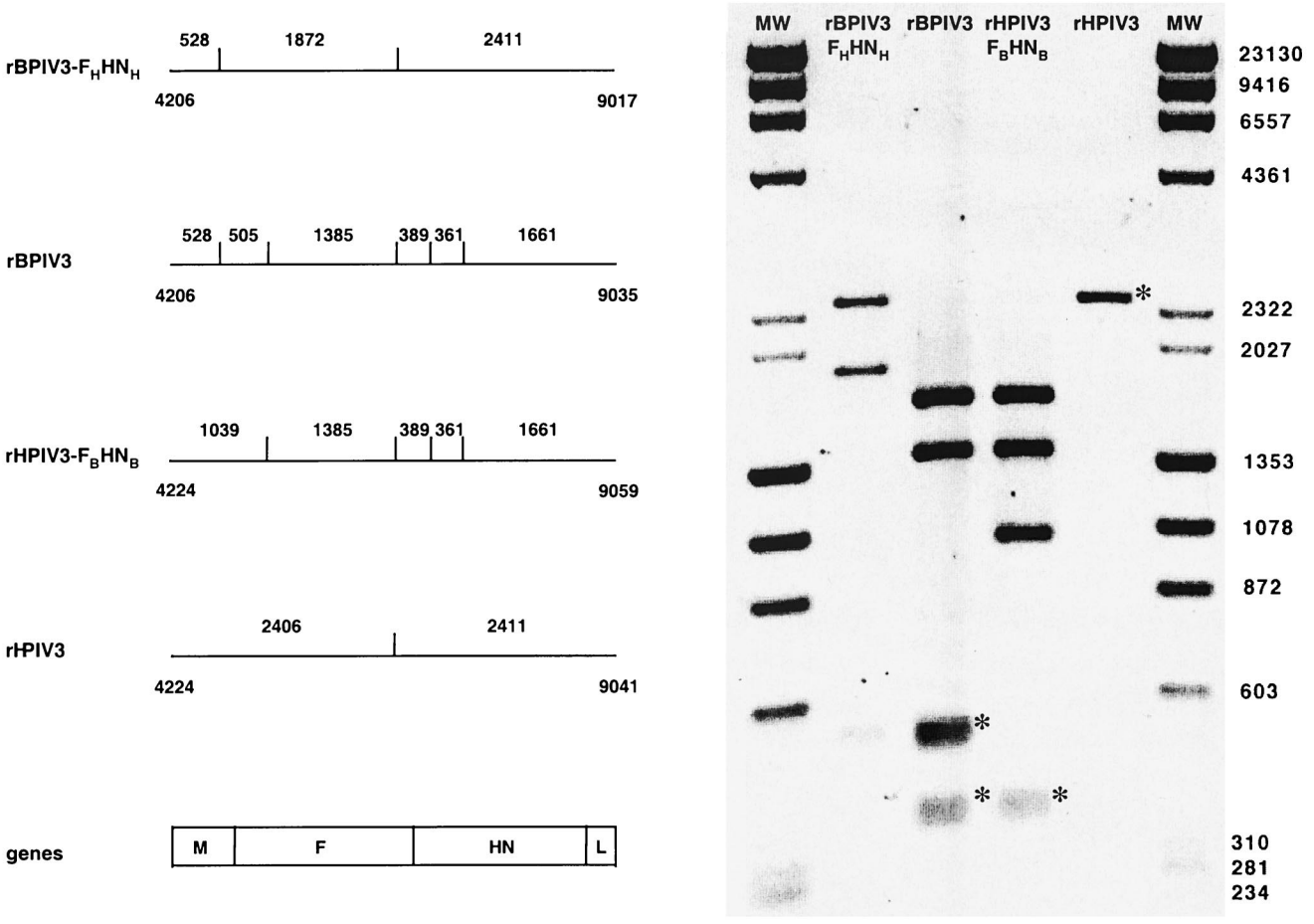

FIG. 2. Confirmation of the identity of recombinant viruses by RT-PCR of viral RNA and *Eco*RI digestion. RT-PCR products of viral RNA were prepared with a primer pair that recognized conserved regions on either side of the F and HN genes in both BPIV3 and HPIV3. Digestion with *Eco*RI resulted in a unique pattern of restriction fragments for each of the four viruses. In the schematic diagrams on the left, horizontal lines symbolize the amplified viral sequences and vertical bars show the positions of *Eco*RI sites. The expected size (in nucleotides) of each restriction fragment is indicated above the line. Numbers below each line correspond to sequence positions in the antigenomic RNA of BPIV3 Ka, HPIV3 JS (GenBank accession no. AF178654 and Z11575), or the indicated chimeric derivative. On the right, a 1% agarose gel of the *Eco*RI digestion of PCR products confirms the identities of parental and chimeric viruses. The asterisks indicate gel bands that contain comigrating restriction fragments. Positions of molecular weight markers (MW) are indicated in nucleotides.

days 2, 4, 6, 8, and 10 postinfection. Individual samples were flash-frozen and stored at −70°C until all samples were available for titration. Virus in the specimens was titered on LLC-MK2 cell monolayers in 24- and 96-well plates as previously described (16). Sera collected from monkeys on days 0 and 28 were tested by HAI assay using HPIV3 JS and BPIV3 Ka as antigens, as previously described (8). On day 28 postinoculation, the monkeys were challenged intranasally and intratracheally with 10^6 $TCID_{50}$ of HPIV3 JS per site. Nasopharyngeal swab samples were collected on days 3, 4, 5, 6, 7, and 8, and tracheal lavage samples were collected on days 4, 6, and 8 postchallenge. Samples were titered in a single assay as described above. Serum was collected on day 28 postchallenge.

RESULTS

Recovery of rBPIV3 and BPIV3/HPIV3 chimeras from cDNA. A complete BPIV3 antigenomic cDNA, designated pBPIV (184), was constructed to encode the consensus sequence of BPIV3 Ka, with the exception of nt 21 (T to G) and 23 (C to T) (2). This BPIV3 antigenomic cDNA was further modified by the introduction of unique *Sgr*AI and *Bsi*WI sites into the downstream noncoding regions of the M and HN genes, respectively. The same restriction sites were introduced into the downstream noncoding regions of the M and HN genes of a previously described complete HPIV3 antigenomic cDNA, p3/7(131)2G (15). The F and HN glycoprotein genes of HPIV3 and BPIV3 were swapped by exchanging this *Sgr*AI-*Bsi*WI restriction fragment. A direct exchange of entire genes was anticipated to be well tolerated because of the high level of sequence conservation between the *cis*-acting signals of BPIV3 and HPIV3 (2). The HPIV3 antigenomic cDNA bearing the BPIV3 F and HN genes was designated pHPIV(215), and the BPIV3 antigenomic cDNA bearing the HPIV3 F and HN genes was designated pBPIV(215).

rBPIV3, rHPIV3-F$_B$HN$_B$, and rBPIV3-F$_H$HN$_H$ chimeras were recovered from the cDNAs pBPIV(184), pHPIV(215), and pBPIV(215) after transfection of HEp-2 cells, and their identities were confirmed by *Eco*RI digestion (Fig. 2). In each case, the predicted unique fragment pattern was observed, confirming the identity of the backbone and the inserted F and HN genes.

In LLC-MK2 cells, the cytopathic effect (CPE) caused by rBPIV3-F$_H$HN$_H$ was indistinguishable from that of HPIV3 JS (condensed, rounded-up cells and small syncytia) but different from that of BPIV3 (large multicellular syncytia), whereas the CPE caused by rHPIV3-F$_B$HN$_B$ was identical to that caused by BPIV3. Although this was not a systematic observation, the differences in the cytopathology of the chimeric PIVs could

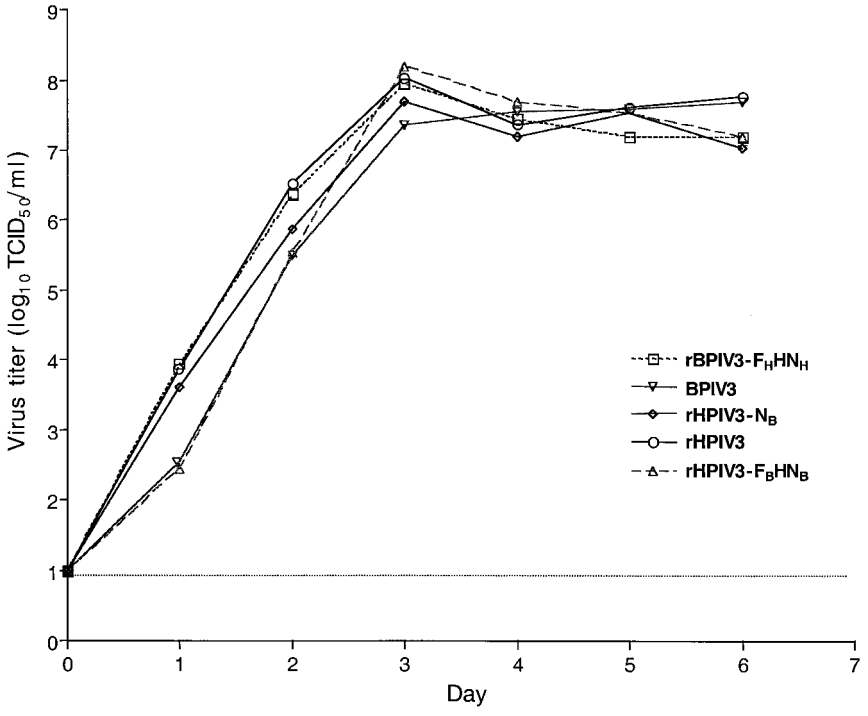

FIG. 3. Multicycle replication of chimeric and parental viruses in simian LLC-MK2 cells. Multicycle replication (MOI of 0.01) of the three chimeras rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, and rHPIV3-N_B is compared with the replication of the BPIV3 Ka and rHPIV3 parents. Virus titers are shown as mean log_{10} $TCID_{50}$ per milliliter ± standard error of triplicate samples. The lower limit of detection of this assay is 10 $TCID_{50}$, as indicated by the dotted horizontal line.

point to a cosegregation of CPE with the parental origin of the F and HN genes.

BPIV3/HPIV3 chimeras replicate efficiently in cell culture. The growth kinetics of rHPIV3-F_BHN_B and rBPIV3-F_HHN_H were compared with those of their parental viruses by infecting LLC-MK2 monolayers at an MOI of 0.01 and monitoring the production of infectious virus. The kinetics and magnitude of replication of the two chimeric viruses were comparable to those of their HPIV3 or BPIV3 parental viruses (Fig. 3). This suggested that BPIV3 and HPIV3 glycoproteins were compatible with the heterologous PIV3 internal proteins.

The F and HN genes of the BPIV3/HPIV3 chimeras are determinants of the host range restriction of replication of BPIV3 Ka in the upper respiratory tract of rhesus monkeys. rHPIV3-F_BHN_B and rBPIV3-F_HHN_H were evaluated for the ability to replicate in the upper and lower respiratory tract of rhesus monkeys. Two questions were specifically addressed. First, did the introduction of the BPIV3 F and HN genes into HPIV3 restrict its replication in rhesus monkeys, as previously shown for the BPIV3 N protein (1)? Second, did the introduction of the HPIV3 F and HN genes into BPIV3 increase its replication in rhesus monkeys? If the predominant attenuating mutations of BPIV3 were in genes other than the F and HN genes, then one would expect little overall effect of the HPIV3-BPIV3 glycoprotein exchange on replication of BPIV3 in rhesus monkeys.

Each chimeric virus was administered intranasally and intratracheally to rhesus monkeys at a dose of 10^5 $TCID_{50}$ per site. The level of replication of the chimeric viruses was compared to that of the rHPIV3 and BPIV3 parental viruses and to that of rHPIV3-N_B (Table 1). Since the rHPIV3 parental virus replicated to a low to moderate level in the lower respiratory tract, meaningful comparisons between groups could be made only for replication in the upper respiratory tract. The level of replication of rHPIV3-F_BHN_B in the upper respiratory tract was similar to that of its BPIV3 parent and substantially lower than that of its HPIV3 parent (Table 1; Fig. 4A). This showed that the BPIV3 glycoprotein genes contained one or more major determinants of the host range attenuation phenotype of BPIV3 for rhesus monkeys. The magnitudes and patterns of replication of rHPIV3-F_BHN_B and rHPIV3-N_B were very similar, indicating that the two bovine genetic elements attenuate HPIV3 to a similar extent.

The rBPIV3-F_HHN_H chimera replicated significantly less well than rHPIV3 in the upper respiratory tract (Table 1), and it grouped with BPIV3 in a Duncan multiple range test. However, inspection of its pattern of replication in Fig. 4B suggested that rBPIV3-F_HHN_H replicated to a level intermediate between that of its HPIV3 and BPIV3 parents. This interpretation is supported by Friedman's test of consistency of ranks (40), which indicates that the median titers of HPIV3, rBPIV3-F_HHN_H, and BPIV3 between days 3 and 8 postinfection are significantly different (df 2 and 8; $P < 0.05$). The observation that the introduction of the HPIV3 F and HN proteins resulted in an increase in the replication of BPIV3 in rhesus monkeys indicates (i) that F and HN contain one or more determinants of host range restriction in the upper respiratory tract and (ii) that one or more genetic elements of BPIV3 that lie outside of the F and HN genes, e.g., the N protein, also attenuate the virus for rhesus monkeys.

The chimeric BPIV3 bearing HPIV3 glycoprotein genes induces serum HAI antibody to HPIV3 and a high level of resistance to HPIV3 challenge. rBPIV3-F_HHN_H has important features that make it a candidate live attenuated virus vaccine against HPIV3, including attenuating genes from BPIV3 and the antigenic specificity of HPIV3, i.e., the F and HN glycoproteins, which are the major protective antigens. Therefore, its immunogenicity and protective efficacy against challenge

TABLE 1. The F and HN glycoprotein genes of BPIV3 contribute to its restricted replication in the respiratory tracts of rhesus monkeys

Immunizing virus[a]	No. of animals[b]	Mean peak virus titer[c] (log_{10} $TCID_{50}$/ml ± SE) [Duncan grouping][d]		Serum HAI antibody titer (mean reciprocal log_2 ± SE) on day 28[e] [Duncan grouping] for:	
		NP swab[f]	Tracheal lavage[g]	HPIV3	BPIV3
rHPIV3	6	4.7 ± 0.54 [A]	2.4 ± 0.37 [A]	9.5 ± 0.72 [A]	6.8 ± 1.03 [B]
rBPIV3-F_HHN_H	4	3.1 ± 0.58 [B]	1.6 ± 0.05 [A]	6.8 ± 0.63 [BC]	3.8 ± 0.63 [C]
rHPIV3-N_B	6	3.0 ± 0.60 [B]	1.4 ± 0.19 [A]	8.2 ± 0.48 [AB]	6.5 ± 0.62 [B]
rHPIV3-F_BHN_B	4	2.9 ± 0.28 [B]	2.0 ± 0.24 [A]	4.5 ± 0.29 [D]	9.5 ± 0.65 [A]
BPIV3 Ka	6	2.6 ± 0.26 [B]	1.6 ± 0.10 [A]	5.5 ± 0.62 [CD]	9.2 ± 0.60 [A]

[a] Monkeys were inoculated intranasally and intratracheally with 10^5 $TCID_{50}$ of virus in a 1-ml inoculum at each site. Two animals in the rHPIV3 group received rHPIV3 with the *Sgr*AI and *Bsi*WI sites (rHPIV3s). There was no significant difference in the level of replication between rHPIV3 and rHPIV3s.
[b] The groups with six animals contain four animals each from a previous rhesus study (1).
[c] Mean of the peak virus titer for each animal in its group irrespective of sampling day.
[d] Virus titrations were performed on LLC-MK2 cells at 32°C. The limit of detectability of virus titer was 10 $TCID_{50}$/ml. Mean viral titers were compared using a Duncan multiple range test ($\alpha = 0.05$). Within each column, mean titers with different letters are statistically different. Titers indicated with two letters are not significantly different from those indicated with either letter.
[e] The titers on day 0 were <2.0. Day 28 was the day of challenge with wild-type HPIV3.
[f] Nasopharyngeal (NP) swab samples were collected on days 1 to 11 and on day 13 postinfection.
[g] Tracheal lavage samples were collected on days 2, 4, 6, 8, and 10 postinfection.

with HPIV3 were examined. Rhesus monkeys were immunized by infection with BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_H-HN_H, rHPIV3-N_B, or rHPIV3. They were challenged 28 days later with HPIV3 JS wild-type virus. Serum samples were taken prior to the initial infection on day 0 and prior to the challenge (Table 1). BPIV3 and rHPIV3-F_BHN_B induced serum HAI antibodies that reacted more efficiently with BPIV3 than HPIV3, whereas the converse was the case for HPIV3 and rBPIV3-F_HHN_H. Thus, the origin of the glycoprotein genes in each virus determined whether the HAI antibody response was directed predominantly against HPIV3 or against BPIV3. The replication of challenge HPIV3 virus was significantly reduced in the upper and lower respiratory tracts of previously immunized monkeys (Table 2). Although the level of protective efficacy against HPIV3 was not significantly different among the different viruses, viruses bearing HPIV3 F and HN appeared to be slightly more protective in the upper respiratory tract than viruses bearing BPIV3 F and HN. This is in accordance with the higher level of HPIV3-specific serum HAI antibodies induced by viruses bearing HPIV3 F and HN.

DISCUSSION

The Jennerian approach to the development of live attenuated viruses involves the use of a mammalian or avian virus to immunize humans against an antigenically related human virus. The approach is named after Edward Jenner's successful use of vaccinia virus, a virus putatively of bovine origin, to protect against smallpox in humans. Mammalian and avian viruses that are well adapted to their natural host typically do

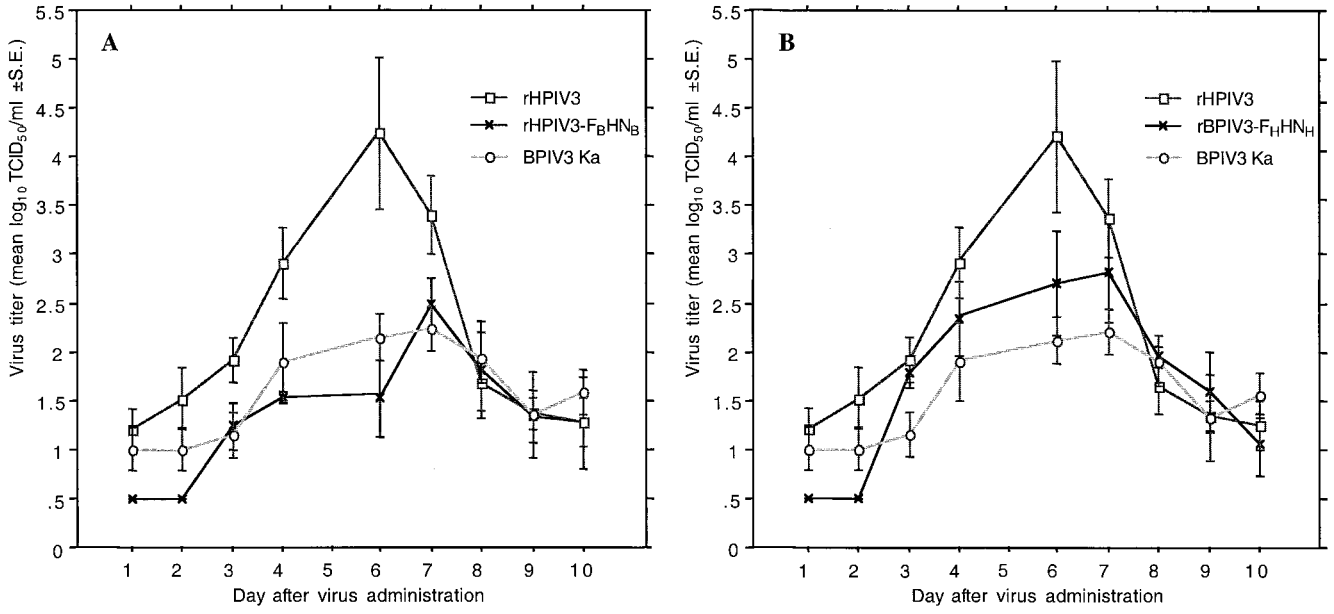

FIG. 4. Mean titers of chimeric and parental viruses in nasopharyngeal swabs of infected rhesus monkeys over the course of infection. Virus titers are shown as mean $TCID_{50}$ per milliliter in LLC-MK2 cells ± standard error for groups of four or six monkeys infected with the same virus. Data are from the same experiment as shown in Table 1. (A) Mean titers of rHPIV3-F_BHN_B compared to rHPIV3 and BPIV3 Ka titers; (B) mean rBPIV3-F_HHN_H titers compared to those of BPIV3 Ka and rHPIV3, which are the same values in panel A but are presented separately to facilitate comparison. Day 5 titers are not shown because they were much lower than day 4 and day 6 titers, most likely due to technical problems during the sample collection.

TABLE 2. Immunization of rhesus monkeys with BPIV3/HPIV3 chimeric recombinants induces resistance to challenge with wild-type HPIV3 28 days later

Immunizing virus[a]	No. of animals[b]	Mean peak virus titer[c] following challenge (log_{10} $TCID_{50}$/ml ± SE) [Duncan grouping][d]		Serum HAI antibody titer (mean reciprocal log_2 ± SE) for HPIV3 [Duncan grouping] for:	
		Nasopharyngeal swab[e]	Tracheal lavage[f]	On the day of challenge	28 days after challenge
None	4	4.5 ± 0.33 [A]	4.5 ± 0.19 [A]	<2	12.0 ± 0.58 [A]
rHPIV3	6	2.3 ± 0.14 [B]	1.2 ± 0.20 [B]	9.5 ± 0.72 [A]	11.7 ± 0.21 [A]
rBPIV3-$F_H$$HN_H$	4	2.5 ± 0.25 [B]	1.0 ± 0.48 [B]	6.8 ± 0.63 [BC]	10.5 ± 0.29 [AB]
rHPIV3-N_B	6	2.3 ± 0.41 [B]	1.4 ± 0.08 [B]	8.2 ± 0.48 [AB]	11.5 ± 0.22 [A]
rHPIV3-$F_B$$HN_B$	4	3.0 ± 0.14 [B]	1.0 ± 0.0 [B]	4.5 ± 0.29 [D]	9.5 ± 0.87 [B]
BPIV3 Ka	6	2.9 ± 0.26 [B]	1.3 ± 0.20 [B]	5.5 ± 0.62 [CD]	9.3 ± 0.76 [B]

[a] Each previously immunized monkey and nonimmunized controls were challenged with 10^6 $TCID_{50}$ of HPIV3 JS in a 1-ml inoculum at each site.
[b] The groups with six animals contain four animals each from a previous rhesus study (1).
[c] Mean of peak virus titer for each animal in its group irrespective of sampling day.
[d] Virus titrations were performed on LLC-MK2 cells. The limit of detectability of virus titer was 10 $TCID_{50}$/ml. Mean viral titers were compared using a Duncan multiple range test ($\alpha = 0.05$). Within each column, mean titers with different letters are statistically different. Titers indicated with two letters are not significantly different from those indicated with either letter.
[e] Nasopharyngeal swab samples were collected on days 3 to 8 postchallenge.
[f] Tracheal lavage samples were collected on days 4, 6, and 8 postchallenge.

not replicate efficiently in humans and hence exhibit an attenuation phenotype based on host range restriction. At present, we lack a thorough understanding of the genetic basis of this form of host range restriction. However, animal viruses such as vaccinia virus or bovine rotavirus that manifest host range restriction in humans exhibit significant divergence of nucleotide sequence from that of the corresponding human virus (32, 38), and it is reasoned that extensive sequence divergence of this nature should lead to the genetic stability of the host range attenuation phenotype following replication of the vaccine in the foreign human host. The Jennerian approach to the development of live attenuated viruses has been successfully employed to develop rotavirus vaccines. The rhesus rotavirus was found to be attenuated in humans and protective against human serotype 3, to which it is antigenically related (25). A second Jennerian rotavirus vaccine, based on the UK strain of bovine rotavirus, is also being developed (7). Jennerian vaccines for PIV1 and for hepatitis A virus are attenuated and immunogenic in nonhuman primates (18, 22). Another example involves reassortant viruses that contain two gene segments encoding the hemagglutinin and neuraminidase surface glycoproteins from a human influenza A virus and the six remaining gene segments from an avian influenza A virus that were attenuated in humans (5, 33, 39). This indicated that one or more of the six gene segments of the avian virus attenuated the avian-human influenza A viruses for humans. The genetic determinants of this attenuation were mapped using reassortant viruses possessing a single gene segment from an attenuating avian influenza A virus and the remaining genes from a human influenza A virus strain. It was shown that the nonstructural, polymerase (PB1 and PB2), and M genes contributed to the attenuation phenotype of avian influenza A viruses in humans (6). In another study, the severe host range restriction of bovine respiratory syncytial virus (BRSV) for replication in chimpanzees was only slightly alleviated by replacement of the BRSV F and G glycoproteins with their HRSV counterparts. This indicated that F and G are involved in this host range restriction, but that one or more additional BRSV genes are also involved (3). This illustrates that more than one gene can contribute to the host range restriction phenotype of a mammalian or avian virus in primates. We expect that multiple determinants will typically specify the host range phenotype of Jennerian vaccines, although this has not been well studied.

The present study sought to further explore the genetic basis of attenuation manifested by the Jennerian BPIV3 vaccine candidate for nonhuman primates. Previously, it was found that introduction of the BPIV3 N ORF into the HPIV3 background resulted in a level of host range restriction nearly equivalent to that of BPIV3. Here, we found that this was also true for the F and HN genes of BPIV3 that were introduced into the HPIV3 backbone as a set of two genes. Unfortunately, we were unable to observe significant differences in replication of chimeric and parental viruses for the lower respiratory tracts of rhesus monkeys due to a low level of replication of HPIV3 wild-type virus at this site. Clearly, the rhesus monkey is limited in its ability to detect differences in replication of BPIV3 and HPIV3 for the lower respiratory tract, but previous studies in humans of BPIV3 and HPIV3 candidate vaccines indicated that the attenuation of these viruses for the upper respiratory tract of rhesus monkeys correlated well with their attenuation in both the upper and lower respiratory tract in seronegative infants and children (8, 20, 27, 28).

The mechanisms responsible for the restricted replication of the BPIV3/HPIV3 chimeras in rhesus monkeys are unknown, but it is not surprising that the N and HN/F proteins have been identified as attenuating elements since in other viral systems these proteins are important determinants of host range (11, 29, 36, 37). There are several possible mechanisms by which HN and F glycoproteins could be determinants of host range. First, the balance of receptor binding and neuraminidase activities of the HPIV3 and BPIV3 glycoproteins could be optimized for the sialoglycoproteins and sialoglycolipids present in the respiratory tracts of the hosts. Such receptors are known to differ among hosts (11, 24). Second, the HN and F glycoproteins are known to interact with host cell proteins such as chaperones and cytoskeletal proteins during transport and folding, and their role in virus assembly could be optimized for their host of origin (34, 35). Third, optimal cleavage activation of F could be host cell specific (19, 23, 43). Fourth, the activity of the neuraminidase can be modified by intracellular halide ion concentration and other factors which could differ between hosts (31).

The importation of BPIV3 genes into a virulent HPIV3 backbone is useful to identify genes that are independent attenuating genetic elements, but this analysis does not provide information on the relative contribution that these genes make to the overall attenuation of BPIV3 for primates. To accomplish this, one needs to start with BPIV3 and replace a single attenuating genetic element, identified as indicated above, with its HPIV3 counterpart. If the resulting BPIV3/HPIV3 chimeric

virus exhibits increased replicative capacity in primates, then one can conclude that the gene makes a contribution to the overall attenuation of BPIV3 for primates. To perform this analysis, rBPIV3 was derived from cDNA and used to construct a BPIV3/HPIV3 chimeric virus in which the F and HN genes of BPIV3 were replaced with their HPIV3 counterparts. The resulting chimeric recombinant rBPIV3-F_HHN_H, like its rHPIV3-F_BHN_B counterpart, replicated in vitro as well as its parental viruses. This observation confirmed our assumption that the highly conserved PIV3 gene-end, intergenic, and gene-start *cis*-acting sequences (2) that were exchanged along with the F and HN ORFs to generate rBPIV3-F_HHN_H and rHPIV3-F_BHN_B would be recognized by the heterologous PIV3 polymerase complexes of the chimeric viruses. We had also thought it likely that the F and HN exchange between BPIV3 and HPIV3 would be compatible since the considerably more divergent HPIV1 F and HN proteins were highly functional in a HPIV3 background (42), and this was confirmed by the undiminished capacity of the chimeric viruses for replication in vitro. rBPIV3-F_HHN_H replicated in the upper respiratory tracts of rhesus monkeys to a level intermediate between that of its HPIV3 and BPIV3 parents, indicating that the BPIV3 F and HN genes make an independent contribution to the overall attenuation of BPIV3 for primates, at least in the upper respiratory tract. The overall attenuation of BPIV3 thus is the sum of two or more genetic elements, one of which is the set of F and HN genes and one of the others is possibly N (1).

Although BPIV3 itself is being evaluated as a vaccine virus for HPIV3 (26, 27), it is only 25% related antigenically to HPIV3 (8). Thus, the immunogenicity of BPIV3 against HPIV3 would be improved if it could be modified to express the protective F and HN antigens of HPIV3. rBPIV3-F_HHN_H represents such a virus; in this study, immunization of rhesus monkeys with rBPIV3-F_HHN_H induced a higher level of antibody to HPIV3 than did immunization with BPIV3. Furthermore, rBPIV3-F_HHN_H conferred a level of protection against replication of HPIV3 challenge in the upper and lower respiratory tract that was statistically indistinguishable from that conferred by a previous infection with rHPIV3. Similarly, rHPIV3-N_B, which is attenuated by the BPIV3 N protein but possesses HPIV3 protective antigens, also induced a high level of resistance to HPIV3 challenge, confirming our previous observations (1). Despite replicating to a similar level in rhesus monkeys, rHPIV3-N_B induced higher levels of antibodies to HPIV3 than rBPIV3-F_HHN_H, but the reasons for this are not understood. Additional animals are being immunized to determine whether this difference in immunogenicity is reproducible.

rBPIV3-F_HHN_H replicates to a higher level in rhesus monkeys than BPIV3, although it is significantly attenuated compared to HPIV3. Since the level of replication of BPIV3 in humans is low (27), this increase might be well tolerated by vaccinees. Alternatively, it is possible that rBPIV3-F_HHN_H might replicate in human infants to a level sufficiently high to cause respiratory tract illness. However, the slight increase in replication of rBPIV3-F_HHN_H in primates offers an opportunity to use rBPIV3-F_HHN_H as a vector for other viral antigens. Recently, it was shown that the importation of a measles virus HA glycoprotein as an additional gene into an attenuated HPIV3 vaccine candidate further attenuated the vaccine in vivo (17). Thus, the slight increase in replication of rBPIV3-F_HHN_H in monkeys over that of BPIV3 might be offset by the addition of one or more foreign glycoprotein genes. The data presented here further define the basis for the host range restriction of BPIV3 for primates and identify rBPIV3-F_HHN_H as a potential vaccine candidate and as a vector that deserves further study.

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