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A Single Amino Acid Substitution in the Viral Polymerase Creates a Temperature-Sensitive and Attenuated Recombinant Bovine Parainfluenza Virus Type 3

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Bovine parainfluenza virus type 3 (bPIV3) is under development as a live virus vaccine vector. The RNA genome of a recombinant bPIV3 harbored four nucleotide changes, one of which resulted in a mutation of the viral polymerase (A. A. Haller *et al.,* 2000, *J. Virol.* 74, 11626–11635). The contribution of this conservative amino acid substitution (I1103V) in the polymerase to the temperature-sensitive and attenuation phenotypes of r-bPIV3 was investigated by creating a new virus, r-bPIV3(I), that expressed the wild-type polymerase. r-bPIV3(I) was not temperature-sensitive for growth *in vitro* and the replication of r-bPIV3(I) was no longer restricted in hamsters. The effect of the amino acid substitution in the polymerase was also studied in a chimeric bovine/human PIV3, a virus that displayed temperature-sensitive and attenuated phenotypes (A. A. Haller *et al.,* 2000, *J. Virol.* 74, 11626–11635). It was not clear whether these defects were due to the impaired polymerase or the replacement of the bPIV3 surface glycoproteins with those of hPIV3. The results showed that the altered polymerase was indeed responsible for the temperature-sensitive phenotype of bovine/human PIV3 but did not appear to play a role in the attenuation phenotype.

Key Words: bovine parainfluenza type 3; polymerase mutation; temperature sensitivity; attenuation; live virus vaccine vector.

INTRODUCTION

The paramyxovirus family comprised a great number of human pathogens such as human parainfluenza virus types 1, 2, and 3 (hPIV1, 2, 3), measles, mumps, and human respiratory syncytial virus (hRSV) (Dolin, 1994; Welliver *et al.,* 1986; Johnson and Goodpasture, 1935; Griffin and Bellini, 1996). All of these viruses cause serious respiratory illnesses in young infants and the elderly, often resulting in hospitalization or death (Falsey *et al.,* 1995). Attempts to create effective vaccines to protect from these infectious agents have been ongoing for at least three decades. Live, attenuated viruses are preferable to killed virus or viral subunit preparations for vaccines because live viruses can stimulate a balanced humoral and cellular immune response at a mucosal location (Crowe, 1998).

Bovine parainfluenza virus type 3 (bPIV3) (Kansas/15626/84) was isolated from cattle and has gained increased importance in recent years as a vaccine candidate for hPIV3, an infectious agent causing acute respiratory disease in infants. Bovine and human PIV3 are antigenically related and animals immunized with bPIV3 were protected from infection by hPIV3 (van Wyke Coelingh *et al.,* 1987; Karron *et al.,* 1996). Clinical studies showed that infants as young as 2 months old immunized with bPIV3 exhibited protective immunity to hPIV3 (Karron *et al.,* 1996). Thus, although replication of bPIV3 in humans is somewhat restricted, it elicits an immune response. The precise genetic determinants of bPIV3 that result in host restriction in humans are not yet known (Bailly *et al.,* 2000; Schmidt *et al.,* 2000). Due to the promising results obtained from the clinical trials evaluating bPIV3 as a hPIV3 vaccine candidate, bPIV3 was further developed as a virus vaccine vector to deliver foreign viral antigens (Haller *et al.,* 2000).

bPIV3 is an enveloped, negative-sense, single-strand RNA virus. The bPIV3 genome is an uncapped RNA of 15,456 nucleotides (Haller *et al.,* 2000). The 3′ half of the viral genome encodes the nucleoprotein (NP) that encapsidates the viral RNA and the phosphoprotein (P), which is associated with the functional ribonucleoprotein (RNP) (Collins *et al.,* 1996). In addition, the P open reading frame encodes three smaller proteins, C, D, and V. The functions of these bPIV3 proteins are not known. Results from other paramyxoviruses suggest that the C protein may inhibit the action of interferon (Garcin *et al.,* 2000) and that the V protein slows progression of the cell cycle (Lin and Lamb, 2000). The genes encoding the matrix (M) protein, an integral component of virion particle formation, and the fusion (F) and hemagglutinin-neuraminidase (HN) proteins are also located on the 3′ half of the viral RNA (Collins *et al.,* 1996). The majority of the 5′ half of the viral RNA encodes the 2233 amino acid large (L) protein. The L protein is a multifunctional protein

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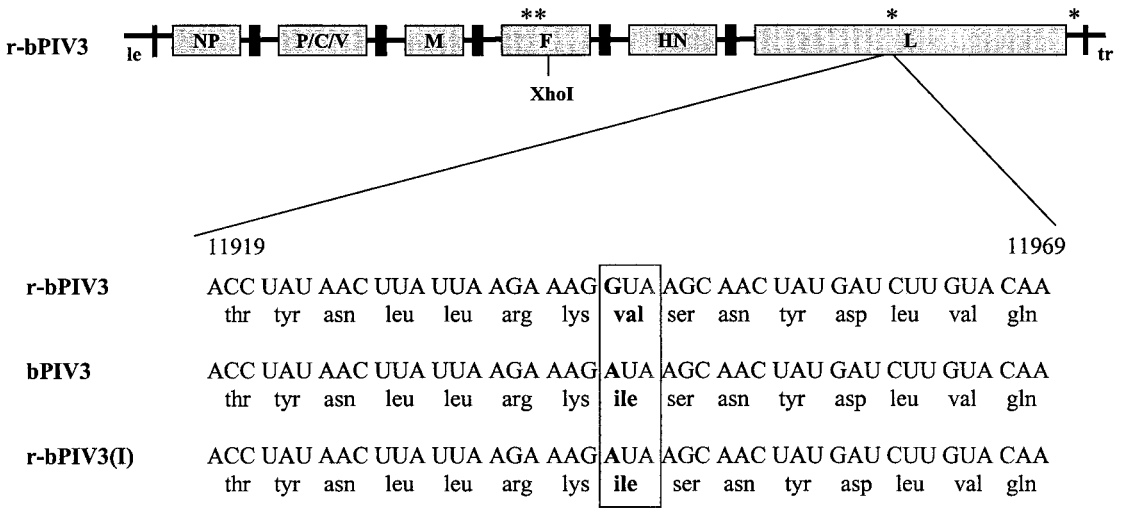

FIG. 1. A schematic representation of the recombinant bPIV3 genome. The bPIV3 genome is flanked by untranslated leader (le) and trailer (tr) regions. The solid boxes between genes indicate the conserved gene stop and gene start sequences of bPIV3 required for viral transcription. The genetic marker, an *Xho*I restriction enzyme site in the F gene, is indicated. The asterisks indicate the location of the four sequence changes in the recombinant viral RNA (in the F gene at nt 6460 and 6463, in the L gene at nt 11,946 and 15,354). The coding sequence of the polymerase gene from nt 11,919 to 11,969 encompassing the point mutation of r-bPIV3, bPIV3, and r-bPIV3(I) is shown in detail. The single nucleotide that is altered in r-bPIV3 is shown in bold print and the substituted amino acid of the polymerase is boxed in.

comprising multiple domains. The L protein binds P and functions as the RNA-dependent RNA polymerase, which has a role in RNA transcription, RNA replication, and RNA polyadenylation (Collins *et al.,* 1996). The template for viral RNA synthesis is the NP encapsidated genomic RNA. Transcription starts at the 3′ end of the genomic RNA. mRNAs are synthesized according to the viral gene order such that a transcription gradient is formed from the 3′ to the 5′ end of the genome. Genome replication directed by the viral polymerase yields full-length positive and subsequently negative-strand genomic RNAs that are encapsidated with NP protein to form viral nucleocapsids (Lamb and Kolakofsky, 1996). Alignment of a number of viral polymerase genes derived from related paramyxoviruses, including Sendai virus, Newcastle disease virus, and rhabdoviruses such as rabies and vesicular stomatitis virus, showed that the viral polymerases contain six colinear and highly conserved motifs important for the catalytic activity of the protein. Three of these conserved motifs are present in other RNA-dependent RNA polymerases and RNA-dependent DNA polymerases. These motifs can be modeled spatially to form the catalytic as well as the RNA template binding sites (Poch *et al.,* 1990; Stec *et al.,* 1991).

An infectious cDNA of bPIV3 was constructed at Aviron and infectious recombinant virus was rescued by reverse genetics (Haller *et al.,* 2000). The linear gene organization of the 15,456-nucleotides-long RNA genome of r-bPIV3 is shown in Fig. 1. Upon sequencing of the bPIV3 cDNA, four sequence changes were identified that differed from the sequence of the biologically derived bPIV3 (Haller *et al.,* 2000). Two of these point mutations (nucleotides 6460 and 6463) were genetically engineered to generate a genomic marker in the viral RNA, an *Xho*I restriction enzyme site in the F gene (Fig. 1). Neither of these nucleotide changes resulted in an amino acid substitution in the F protein. The third nucleotide change was located in the noncoding region of the L gene at position nucleotide 15,354 (Fig. 1). This nucleotide change was not expected to yield an altered virus phenotype as it did not change the consensus gene stop sequences of the L gene or the trailer regions of the bPIV3 genome. The fourth single nucleotide alteration was identified in the L gene of r-bPIV3 at position 11,946 in the viral polymerase (Fig. 1). This nucleotide change resulted in a single amino acid substitution, an isoleucine to a valine at amino acid 1103, in the L protein of r-bPIV3 (Fig. 1). In this paper, we examine the impact of this amino acid change on the replication of r-bPIV3 *in vitro* and in hamsters. This change confers a temperature-sensitive and attenuated phenotype to r-bPIV3 that can be exploited in future vectored vaccine designs.

RESULTS

Minigenome CAT expression by wild-type and mutant bPIV3 polymerases at different temperatures

The r-bPIV3 described previously had a temperature-sensitive phenotype *in vitro* (Haller *et al.,* 2000). A CAT reporter assay was used to determine whether the single amino acid substitution (I1103V) present in the L polymerase of r-bPIV3 had an impact on viral RNA synthesis. Both the wt (I1103) and mutant (V1103) forms of L polymerase were inserted in an expression plasmid (L/pCITE). DNA sequencing of the corrected L gene confirmed that no additional nucleotide alterations were

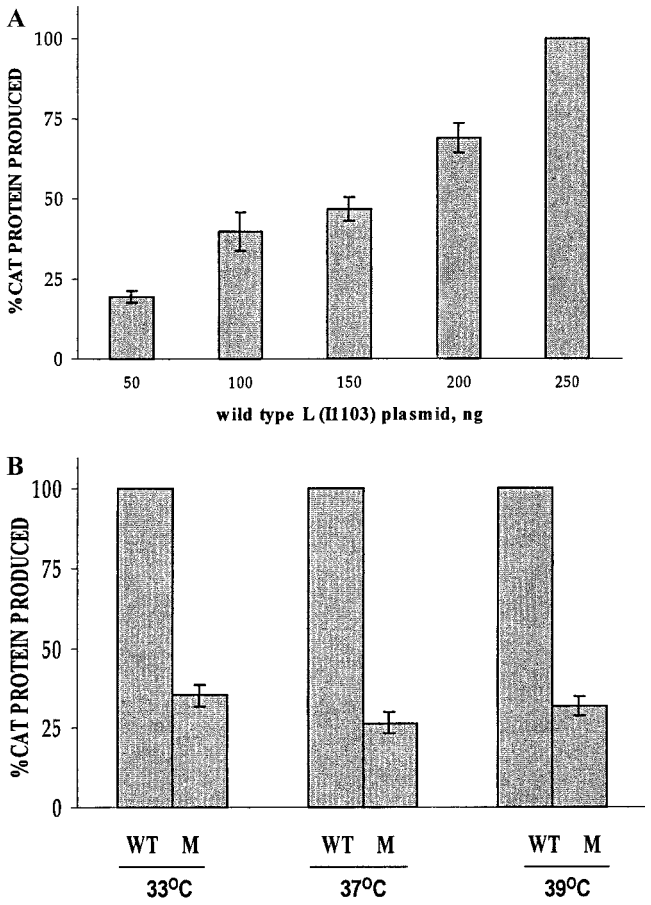

FIG. 2. BPIV3 CAT minigenome assay using wild-type (I1103) and mutant (V1103) L genes. (A) Transient transfections of increasing concentrations of wild-type (I1103) L plasmid in the minigenome assay system is described in detail under Materials and Methods. Increasing amounts of wild-type L plasmid (from 50–250 ng DNA) were transfected together with NP, P, and bPIV3/CAT gene plasmids into HeLa cells infected with MVA-T7. Forty hours posttransfection, the cells were harvested and the amount of CAT protein produced was determined by CAT ELISA. (B) The amount of CAT protein produced in the minigenome assay system at 33, 37, or 39°C in the presence of 200 ng of wild-type (WT) (I1103) or mutant (M) (V1103) L gene plasmids.

introduced during the cloning process. MVA-T7-infected HeLa cells were transfected with plasmids encoding the T7 promoter expressed bPIV3 P, NP, and wt (I1103) or mutant (V1103) L proteins along with the bPIV3/CAT minigenome plasmid, and the cells were incubated at 33, 37, or 39°C. The CAT protein produced was quantified by ELISA.

Different amounts of the wild-type L (I1103) expression plasmid were transfected with constant amounts of NP, P, and minigenome to determine the range of CAT production in HeLa cells (Fig. 2A). At 200 to 250 ng of wild-type L (I1103), a high level of minigenome transcription and replication was obtained as measured by the amount of CAT protein produced. The amount of CAT protein produced at 250 ng of wild-type L plasmid (I1103) was arbitrarily set at 100%. CAT protein was not observed when bPIV3/CAT was transfected alone or in transfections in which the L, NP, or P plasmids were omitted (data not shown). In the presence of similar amounts of the mutant L gene (V1103) plasmid, approximately 30% of wild-type activity at 33, 37 and 39°C was obtained (Fig. 2B). The amounts of CAT protein produced using the wild-type L plasmid (I1103) were arbitrarily set at 100%. The CAT protein production by the mutant bPIV3 polymerase relative to the wild-type bPIV3 polymerase remained the same with increasing temperature (Fig. 2B). The mutant L (V1103) enzyme was less active than wt L (I1103) at all temperatures tested. The mutant bPIV3 polymerase exhibited a temperature-independent defect in RNA synthesis *in vitro*, in contrast to the temperature-sensitive defect observed in the replication of r-bPIV3 in tissue culture. The temperature-sensitive defect observed in the r-bPIV3 virus may be expressed only in the context of other viral or cellular components involved in the viral life cycle.

Replication of r-bPIV3(I) is not temperature-sensitive in tissue culture

The wt L gene was introduced into the full-length bPIV3 cDNA and r-bPIV3(I) virus was recovered by reverse genetics. r-bPIV3(I) still harbored the nucleotide changes specifying an *Xho*I restriction enzyme site in the F gene and the single nucleotide change at 15,354 in the L gene untranslated region. To determine whether r-bPIV3(I) exhibited a temperature-sensitive phenotype, plaque formation on Vero cells at permissive and restrictive temperatures was tested (Table 1). Cells infected with bPIV3, r-bPIV3, and r-bPIV3(I) were incubated at 37, 38, 39, and 40°C for 4 days and plaques were visualized by immunoperoxidase staining with bPIV3 goat polyclonal antiserum. The results showed that r-bPIV3 was temperature-sensitive at 39°C as reported previously (Haller *et al.,* 2000). The titer of r-bPIV3 decreased by approximately 1 $\log_{10}$ at 39°C and was reduced by >3 $\log_{10}$ at 40°C compared to the biologically derived bPIV3 (Table 1). The biologically derived bPIV3 did not change significantly in titer with increasing temperature (Table 1). Plaque formation by r-bPIV3(I) was similar to wild-type virus at all temperatures (Table 1). This data demonstrated that the V1103 in the L protein of r-bPIV3 was the sole determinant of the temperature-sensitive phenotype of r-bPIV3 and that the other nucleotide changes present in the r-bPIV3 genome did not contribute to this phenotype.

Multicycle replication of bPIV3, r-bPIV3, and r-bPIV3(I) was also evaluated in Vero cells (Fig. 3). Briefly, subconfluent Vero cells were infected at an m.o.i. of 0.01 and incubated at 37, 39, and 40°C. Infected cells and supernatants were harvested at 0, 24, 48, 72, and 96 h postinfection and the amount of virus at each time point was determined by $TCID_{50}$ assays.

TABLE 1

Determination of the Shut-Off Temperature of r-bPIV3(I) and Bovine/Human PIV3(I)

Virus	Mean virus titer (log_{10} PFU/ml) at indicated temperature[a] (°C)				Temperature-sensitive phenotype
	37	38	39	40	
bPIV3	9.1	8.9	8.8	8.4	−
r-bPIV3	8.6	8.3	6.8	5.3	+
r-bPIV3(I)	8.9	8.9	8.5	8.5	−
Bovine/human PIV3(I)	7.5	7.7	7.5	7.6	−
hPIV3	7.7	7.8	7.5	7.6	−

[a] Plaques were enumerated by immunoperoxidase staining after incubation for 4 days at the indicated temperature.

At 37°C, all three viruses replicated to similar levels with similar kinetics (Fig. 3A). All three viruses reached peak titers of 8 log_{10} $TCID_{50}$/ml by 48 h postinfection and sustained these titers up to 96 h postinfection at 37°C.

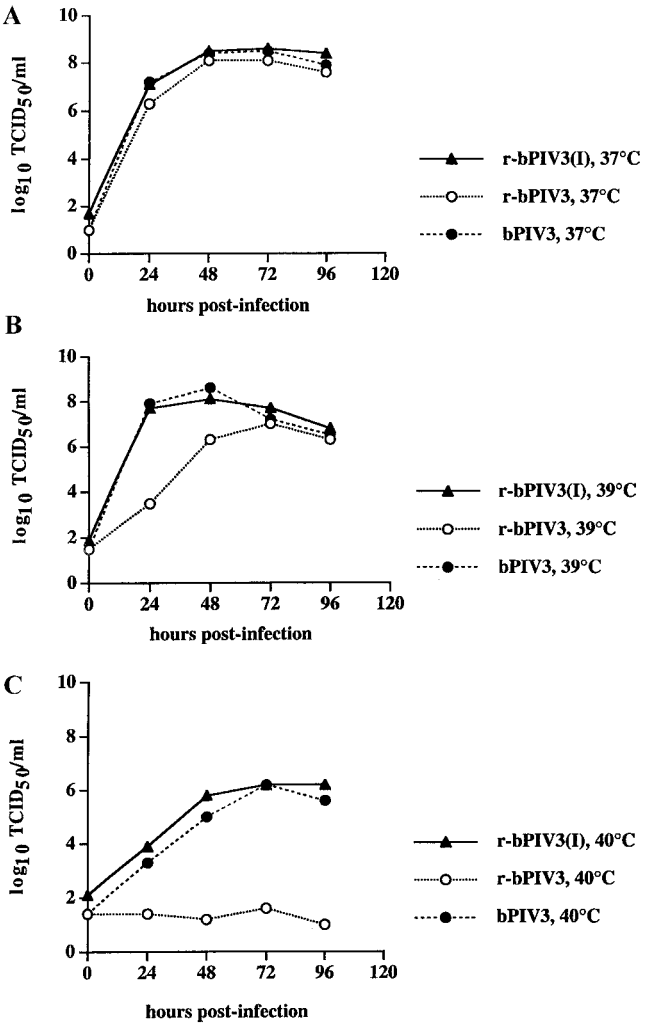

FIG. 3. Multicycle growth curves of bPIV3, r-bPIV3, and r-bPIV3(I) were carried out at 37°C (A), 39°C (B), and 40°C (C) in Vero cells. Time points were collected at 0, 24, 48, 72, and 96 h postinfection. The virus samples were analyzed for virus titers by $TCID_{50}$ assays in Vero cells.

r-bPIV3(I) and bPIV3 yielded similar titers at 39°C (Fig. 3B). Their peak titers were 8.1 and 8.6 log_{10} $TCID_{50}$/ml at 48 h postinfection, respectively. However, at 39°C, r-bPIV3 displayed a delayed onset of replication and a reduced peak titer (7.0 log_{10} $TCID_{50}$/ml) compared to bPIV3 (Fig. 3B); this virus was temperature-sensitive as described previously (Fig. 3B) (Haller *et al.,* 2000). All viruses had reduced titers at 40°C; however, the temperature-sensitive phenotype of r-bPIV3 was accentuated. r-bPIV3 displayed dramatic 7 and 5 log_{10} reductions in virus titers at 40°C compared to its peak titers at 37 and 39°C, respectively (Figs. 3A, 3B, and 3C). The biologically derived bPIV3 was also reduced by 2.3 log_{10} at 40°C compared to its peak titer at 37°C (Figs. 3A and 3C). r-bPIV3(I) replicated as efficiently as the biologically derived bPIV3 at 40°C, reaching peak titers at 72 h postinfection. This data further substantiated that a single amino acid change in the L protein at position 1103 is the major determinant of the temperature-sensitive phenotype observed in r-bPIV3.

Replication of r-bPIV3(I) is not attenuated in Syrian golden hamsters

Syrian golden hamsters have been shown to be permissive for infection by hPIV3 and bPIV3, displaying efficient replication in the lower and upper respiratory tracts (Durbin *et al.,* 1999; Haller *et al.,* 2000). To examine whether amino acid 1103 in the polymerase protein had an effect on the attenuation phenotype, we tested the replication of bPIV3, r-bPIV3, and r-bPIV3(I) in Syrian golden hamsters (Table 2). Briefly, hamsters were inoculated intranasally with 5×10^5 PFU of bPIV3, r-bPIV3, or r-bPIV3(I). Four days postinfection the animals were sacrificed. The lungs and nasal turbinates were harvested and virus titers were determined by $TCID_{50}$ assays using Vero cells. r-bPIV3(I) replicated to 5.5 and 5.4 log_{10} $TCID_{50}$/g tissue in the nasal turbinates and lungs of hamsters, respectively, which were similar to the levels observed for the biologically derived bPIV3. r-bPIV3 replicated to 5.0 log_{10} $TCID_{50}$/g tissue in the nasal turbinates but showed a 1.5 log_{10} decrease in titer in the lungs

TABLE 2

Replication of bPIV3, r-bPIV3, r-bPIV3(I), hPIV3, and Bovine/Human PIV3(I) in the Upper and Lower Respiratory Tract of Hamsters

Virus[a]	Mean virus titer on day 4 postinfection (log_{10} $TCID_{50}$/g tissue ± S.E.)[b]	
	Nasal turbinates	Lungs
bPIV3	5.3 ± 0.3	5.3 ± 0.2
r-bPIV3	5.0 ± 0.3	3.5 ± 0.2
r-bPIV3(I)	5.5 ± 0.2	5.4 ± 0.2
hPIV3	4.9 ± 0.2	5.4 ± 0.2
Bovine/human PIV3(I)	4.9 ± 0.2	4.5 ± 0.2

[a] Groups of four hamster were inoculated intranasally with 5×10^5 PFU of indicated virus.

[b] Standard error.

compared to bPIV3. These results demonstrated that the single amino acid substitution (I1103V) in the r-bPIV3 polymerase specified not only the temperature-sensitive phenotype but also the attenuation phenotype of r-bPIV3. Although r-bPIV3 was attenuated weakly, animals immunized with r-bPIV3 were still protected completely from challenge with hPIV3 (Haller *et al.,* 2000).

Effect of the polymerase mutation on a chimeric bovine/human PIV3

We previously described the phenotypes of a chimeric bovine/human PIV3(1103V) in which the bPIV3 F and HN genes were replaced with those of hPIV3. Bovine/human PIV3 displayed a shut-off temperature of 39.5°C and was restricted by 1.5 log_{10} $TCID_{50}$/g tissue for replication in the lungs of hamsters (Haller *et al.,* 2000). To determine whether the observed phenotypes of bovine/human PIV3 were caused by the polymerase mutation (1103V) and/or the substitution of the bPIV3 surface glycoprotein genes with those of hPIV3, a recombinant virus, bovine/human PIV3(I) containing 1103I and the hPIV3 F and HN genes was constructed and tested for temperature-sensitivity in tissue culture and replication in hamsters. The results showed that bovine/human PIV3(I), like its parents, bPIV3(I) and hPIV3, did not display any significant decrease in virus titers at temperatures as high as 40°C (Table 1). Bovine/human PIV3(I) was also evaluated for replication in the lower and upper respiratory tracts of Syrian golden hamsters (Table 2). Bovine/human PIV3(I) replicated to levels similar to its hPIV3 parent in the nasal turbinates (4.9 log_{10} $TCID_{50}$/g tissue). However, there was a 0.9 log_{10} $TCID_{50}$ unit reduction in virus titer of bovine/human PIV3(I) in the lungs of hamsters compared to hPIV3 or r-bPIV3(I). These data show that although the polymerase mutation (I1103V) was responsible for the temperature-sensitive phenotype observed for bovine/human PIV3, it did not specify the restricted replication of bovine/human PIV3 in the lower respiratory tract of hamsters. The replacement of the bovine surface glycoprotein genes with those of hPIV3, a closely related virus, resulted in attenuated replication *in vivo*.

DISCUSSION

Our studies showed that a single nucleotide alteration in the polymerase gene of bPIV3 resulted in a recombinant virus that displayed a temperature-sensitive phenotype as well as restricted replication in the lower respiratory tract of hamsters. Both wild-type (I1103) and mutant (V1103) forms of the L polymerase were examined for activity and function in the bPIV3 minigenome replication assay. The results obtained from this assay showed that indeed the mutant L gene displayed a three- to fourfold reduction in RNA synthesis, according to the levels of CAT protein produced, compared to the wild-type L gene. The observation that the defect in the L protein was not exacerbated by elevated temperatures was in contrast to the temperature-sensitivity observed for r-bPIV3 growth in Vero cells. The temperature-independent defect in the L protein observed *in vitro* was partially suppressed in the virus where the defect manifested itself only at high temperatures.

A recombinant virus, r-bPIV3(I) with the corrected polymerase gene, was recovered. r-bPIV3(I) differed from r-bPIV3 only at the amino acid 1103 in the L gene. The other three nucleotide alterations present in the F gene and the untranslated region of the L gene were still present in r-bPIV3(I). r-bPIV3(I) did not express a temperature-sensitive replication phenotype in tissue culture. r-bPIV3(I) showed the same plaque-forming efficiency as the biologically derived bPIV3 at 37, 38, 39, and 40°C. In multicycle growth curves, r-bPIV3(I) and bPIV3 displayed similar kinetics of virus replication. Both viruses reached peak titers at the same time points and replication was comparable at all temperatures tested. The ability of r-bPIV3(I) to replicate in the upper and lower respiratory tracts of Syrian golden hamsters was similar to the biologically derived bPIV3. Thus, unlike r-bPIV3, r-bPIV3(I) was not restricted for replication in hamsters.

Sequence alignment of the polymerase genes of a number of parainfluenza viruses was performed to examine the degree of conservation of the altered amino acid residue at position 1103 in the L protein and the region surrounding it (Fig. 4). Interestingly, amino acid 1103 was located in a 20 amino acid stretch of the parainfluenza polymerase gene that was highly conserved among bPIV3, hPIV3, hPIV1, and Sendai virus, albeit this region is not part of the four conserved polymerase motifs A, B, C, or D (Poch *et al.,* 1990). The amino acid at position 1103 of these naturally occurring isolates was either isoleucine (bPIV3, hPIV3) or leucine (hPIV1, Sendai). A valine (r-bPIV3) was found at a similar position in hPIV2 and mumps virus; however, the overall similarity

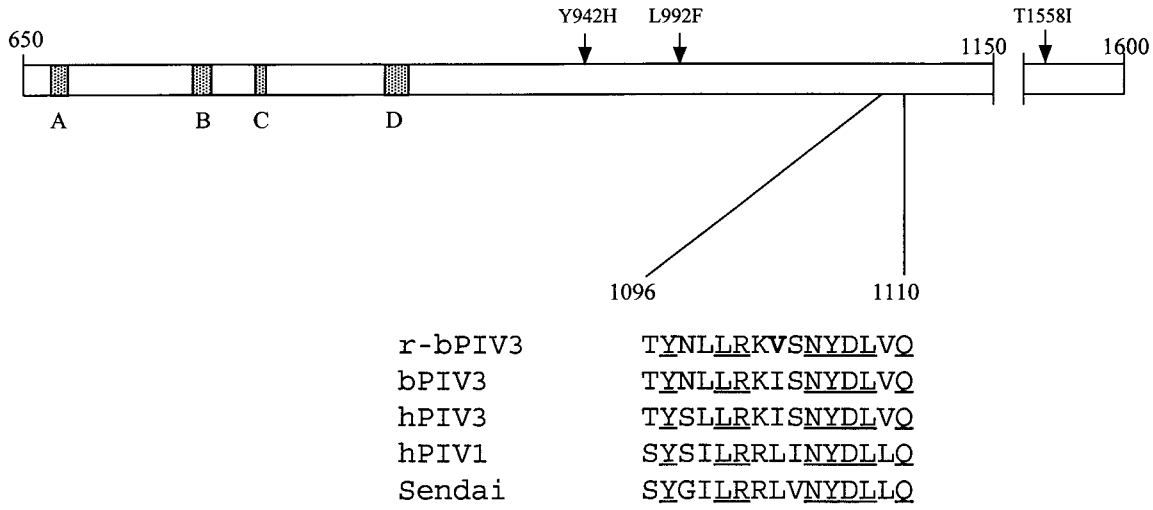

FIG. 4. Comparison of polymerase genes among selected paramyxoviruses. The diagram shows the location of the amino acid substitution observed for r-bPIV3 compared to the wild-type sequence of bPIV3, hPIV3, hPIV1, and Sendai virus. The four shaded boxes (A, B, C, D) indicate conserved polymerase motifs (Poch *et al.,* 1990; Stec *et al.,* 1991). The three amino acid changes, Y942H, L992F, and T1558I, present in the polymerase of cp45 hPIV3 responsible for its temperature-sensitive phenotype are shown. The conserved region harboring the I1103V substitution and alignment of this region of the L protein in r-bPIV3, bPIV3, hPIV3, hPIV1, and Sendai virus is shown (GenBank Accession Nos. NC002161, BAA32575, AAD23456, AAB48690, P27566). The amino acid residues that are conserved among all five viruses are underlined. The valine 1103 present in r-bPIV3 is shown in bold print.

among these viruses is very limited. It is interesting to speculate whether a change of valine to isoleucine at this position in hPIV2 and mumps could result in attenuation of these viruses. Importation of temperature-sensitivity markers has been successfully demonstrated with parainfluenza viruses (hPIV3, Sendai virus) and a pneumovirus (hRSV) (Feller *et al.,* 2000; Skiadopoulos *et al.,* 1999). The apparent prerequisite of isoleucine or leucine at 1103 is interesting, since isoleucine, leucine, and valine have similar side chains differing only by a single methyl group. However, this conservative substitution was sufficient to confer dramatic attenuation on r-bPIV3 replication *in vitro* and *in vivo*. Similar effects on virus replication upon conservative amino acid substitutions have been observed previously. For example, a leucine to isoleucine substitution in the Sendai virus polymerase at position 1558 exerted a dramatic effect on virus replication (Feller *et al.,* 2000). This amino acid substitution did not affect viral transcription at the permissive temperature (33°C), but reduced transcription by 50% at 39.6°C. *In vitro* replication activity of the hPIV1 polymerase was reduced by 85% at the permissive temperature. Surprisingly, the replication activity in tissue culture was better than wild-type L at the permissive temperature, but decreased rapidly at 37°C and was inactive at 39.6°C (Feller *et al.,* 2000). These results underscore the difficulty of predicting the outcome of a change in virus phenotype even in cases where the amino acid substitution is chemically conservative.

The region of the r-bPIV3 L protein harboring the substituted amino acid was also compared to the location of the mutations found in cp45 hPIV3 that are responsible for its temperature-sensitive and attenuation phenotype (Fig. 4). These three amino acid substitutions, Y942H, L992F, T1558I, in the L protein of hPIV3 have been shown to result in temperature-sensitive and attenuation phenotypes when present alone or in combination (Skiadopoulos *et al.,* 1998). None of the three point mutations present in cp45 hPIV3 were close in primary sequence to the I1103V change found in r-bPIV3. In the linear context of the polymerase protein, the I1103V change of r-bPIV3 was not close to any of the temperature-sensitivity markers in cp45 hPIV3. However, the spatial relationships of this mutation to those present in cp45 hPIV3 remain to be seen.

The polymerase mutation studied in this report also conferred the temperature-sensitive phenotype of bovine/human PIV3 in tissue culture, a chimeric virus described previously (Haller *et al.,* 2000). The restoration of the wild-type sequence in the polymerase protein in bovine/human PIV3(I) resulted in a non-temperature-sensitive virus. Thus the introduction of the hPIV3 F and HN genes alone did not result in an observable phenotype *in vitro*. In contrast, bovine/human PIV3(I) still displayed restricted replication in the lower respiratory tract of hamsters. The impaired virus replication *in vivo* is likely due to the introduction of the hPIV3 F and HN surface glycoproteins that display 77 and 80% amino acid identities, respectively. Despite the high degree of similarity in the amino acid sequences of the bovine and human PIV3 surface glycoproteins, it appears that these proteins are not completely interchangeable, resulting in an observable replication defect *in vivo* for bovine/human PIV3(I).

In summary, we have identified a mutation in the bovine parainfluenza virus polymerase that confers replication defects *in vitro* and *in vivo*. The polymerase mutation (I1103V) can be used to modulate the expression levels of genes introduced in bPIV3. In cases where low levels of expression are required, the r-bPIV3 vector can be employed. However, when higher levels of foreign protein are required, r-bPIV3(I) can be used instead. The modulation of expression levels of the gene of interest may be advantageous for a virus vaccine vector depending on its application.

MATERIALS AND METHODS

Viruses and cells

The bPIV3 (Kansas/15626/84), r-bPIV3, r-bPIV3(I), hPIV3 (Washington/478/85/57), and bovine/human PIV3(I) were grown in Vero cells in Opti-MEM medium (Gibco/BRL) containing antibiotics. The modified vaccinia virus Ankara (MVA-T7), which expresses the phage T7 RNA polymerase (a gift from B. Moss), was grown in chicken embryonic kidney cells (SPAFAS) (Wyatt *et al.,* 1995). Vero and HeLa cells were maintained in MEM media (JRH Biosciences) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, nonessential amino acids, and antibiotics.

Construction of the pbPIV3/CAT minireplicon

The pbPIV3/CAT minireplicon was constructed to contain the antisense CAT gene flanked by the bPIV3 leader linked to the hepatitis delta ribozyme (RZ) and T7 polymerase termination (T7T) signals. The bPIV3 trailer sequences were preceded by the T7 RNA polymerase promoter. The DNA fragment containing the T7T, the RZ, and the bPIV3 leader region of pbPIV3/CAT was produced by two overlapping PCR fragments using two pairs of primers encoding a *Kpn*I restriction enzyme site and an *Xba*I site, respectively. The T7T/RBZ fragment was amplified from pRSV/CAT, the RSV minigenome plasmid (H. Jin, personal communication), and the bPIV3 leader region was amplified by PCR from the bPIV3 cDNA (Haller *et al.,* 2000). These two PCR fragments contained overlapping sequences and were ligated by annealing/extension using limited cycles of PCR. The PCR product was cleaved with *Kpn*I and *Xba*I and introduced into the pRSV/CAT plasmid cut with *Kpn*I and *Xba*I, thereby replacing the T7T/RZ/RSV leader in pRSV/CAT. The DNA fragment encoding the T7 RNA polymerase promoter and the bPIV3 trailer region was amplified using two oligos complementary to the bPIV3 trailer region encoding a *Pst*I restriction enzyme site or the T7 RNA polymerase promoter encoding a *Hin*dIII restriction enzyme site. The PCR product was digested with *Pst*I and *Hin*dIII and used to replace the complementary fragment in the pRSV/CAT plasmid to yield the pbPIV3/CAT minireplicon plasmid.

Construction of the full-length r-bPIV3(I) and bovine/human PIV3(I) cDNAs

Employing the QuikChange mutagenesis kit (Stratagene), the single G residue at nucleotide position 11,946 in the r-bPIV3 L gene was changed to an A residue in the plasmid L/pCITE described previously (Haller *et al.,* 2000). The nucleotide change was confirmed by DNA sequencing. The altered L/pCITE plasmid was then digested with restriction enzymes *Sac*I and *Pac*I to liberate a 2.5-kb DNA fragment harboring the single nucleotide change. This DNA fragment was then introduced into two previously described plasmids, bPIV3/N/S and the plasmid containing the full-length bovine/human PIV3 cDNA, cleaved with *Sac*I and *Pac*I. The L gene of the full-length plasmids r-bPIV3(I) and bovine/human PIV3(I) were sequenced to verify the presence of the single nucleotide change.

Transfection of full-length r-bPIV3(I) and bovine/human PIV3(I) cDNA plasmids and preparation of recombinant virus stocks

HeLa cells (80% confluent) were infected with modified vaccinia virus Ankara (MVA-T7) at an m.o.i. of 4. One hour postinfection, the full-length r-bPIV3(I) cDNA plasmid (4 μg) or the bovine/human PIV3(I) cDNA plasmid were transfected into the MVA-T7-infected HeLa cells together with the NP/pCITE (0.4 μg), P/pCITE (0.4 μg), and L/pCITE (0.2 μg) expression plasmids in the presence of 9 μl LipofecTACE (Gibco/BRL) in 200 μl Opti-MEM (Gibco/BRL). Forty-eight hours posttransfection, the passage 0 (P0) cells and media were harvested together and subjected to one freeze-thaw cycle. The resulting P0 cell lysate was then used to infect fresh Vero cell monolayers in the presence of 40 μg/μl 1-β-D-arabinofuranosylcytosine (ara C), a replication inhibitor of vaccinia virus, to generate a P1 virus stock. The media and cells from this infection cycle were harvested and freeze-thawed once, and the presence of bPIV3 infection was confirmed by immunostaining of virus plaques using bPIV3-specific antiserum (VMRD) or hPIV3-specific antiserum (Chemi-Con). Following two cycles of plaque purification in Vero cells, high titer P2 (passage 2) virus stock was prepared in Vero cells and stored at $-80°C$.

Genotyping of r-bPIV3(I) and bovine/human PIV3(I)

Viral RNA of r-bPIV3(I) or bovine/human PIV3(I) was isolated from infected Vero cells using RNA STAT-50 LS Reagent (Tel-Test, Inc.). To verify the genotypes of r-bPIV3(I) or bovine/human PIV3(I), viral cDNA of r-bPIV3(I) starting at nt 4500 was prepared using Superscript Reverse Transcriptase (Gibco/BRL). DNA fragments encompassing the region from nt 11,000 to nt 12,700 were amplified by PCR, resulting in a 1.7-kb PCR product. The PCR products were sequenced to confirm

the presence of the single nucleotide change in the viral polymerase gene. The presence of the hPIV3 F and HN genes of bovine/human PIV3(I) was also verified by sequencing of the RT-PCR products as described previously (Haller *et al.,* 2000).

bPIV3 minigenome assay

Subconfluent HeLa cells were infected with MVA-T7 at an m.o.i. of 5 PFU/cell (Wyatt *et al.,* 1995). The infected cells were cotransfected using LipofecTACE (Gibco BRL/Life Sciences) with three plasmids encoding the P, NP, and L bPIV3 genes and a CAT minigenome flanked by the bPIV3 leader and trailer. The plasmids were transfected using the following amounts of DNA: NP/pCITE (200 ng), P/pCITE (200 ng), L/pCITE (50–300 ng), and bPIV3/CAT (500 ng). The cells were transfected for 5 h at 33°C. Following the removal of the transfection mix, the incubation of the cells was continued at 33, 37, or 39°C. Forty hours posttransfection, the cells were lysed and analyzed for the amount of CAT protein produced by CAT ELISAs (Roche Molecular).

Growth curves

Vero cells were grown to 90% confluence and infected at an m.o.i. of 0.01 PFU/cell with bPIV3, r-bPIV3, or r-bPIV3(I). The infected monolayers were incubated at 37, 39, and 40°C. At 0, 24, 48, 72, and 96 h postinfection, cells and media were harvested together and stored at −80°C. Virus titers for each time point harvest were determined by $TCID_{50}$ assay in Vero cells.

Efficiency of plaque formation assay

Plaque assays were carried out on Vero cells with bPIV3, r-bPIV3, r-bPIV3(I), and bovine/human PIV3(I). The infected monolayers were incubated at 37, 38, 39, and 40°C. Four days postinfection, the infected monolayers were immunostained using bPIV3-specific antisera (VMRD) or hPIV3-specific monoclonal antibodies (Chemicon). The plaques were quantified and titers at the different temperatures were determined to identify the shut-off temperatures of the viruses.

Small animal studies

Five-week-old Syrian golden hamsters (four animals per group) were infected intranasally with 5×10^5 PFU of bPIV3, r-bPIV3, r-bPIV3(I), or bovine/human PIV3(I) in a 100 μl volume. The three 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 −80°C. The titers of virus present in the tissues was determined by $TCID_{50}$ assays in Vero cells.

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