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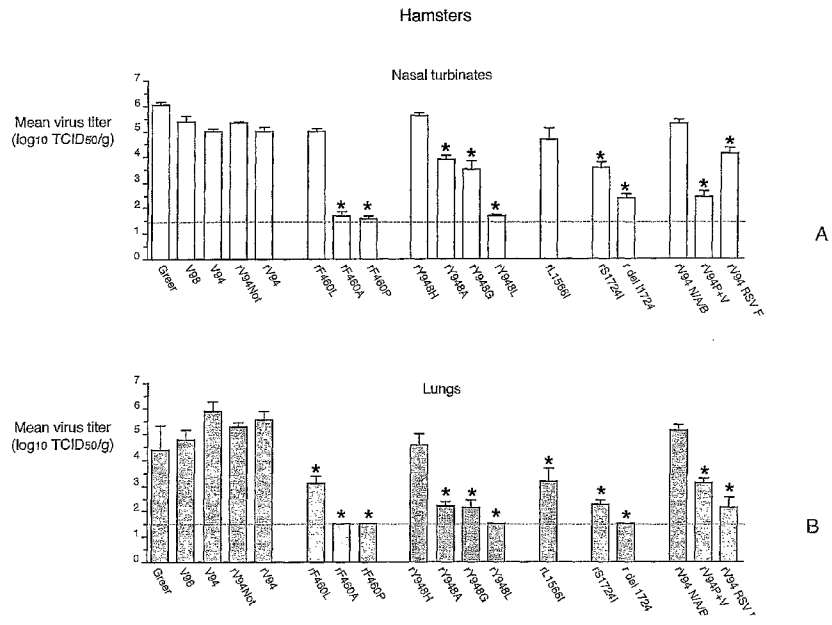

(57) Abstract: The invention provides self replicating infectious recombinant paramyxoviruses. The recombinant paramyxovirus preferably have one or more attenuating mutations. In some embodiments, the recombinant paramyxovirus has a separate variant polynucleotide encoding a P protein and a separate monocistronic polynucleotide encoding a V protein. In some embodiments, recombinant paramyxovirus have at least one temperature sensitive mutation and one non-temperature sensitive mutation. Also provided are compositions and methods for using the recombinant paramyxoviruses as described herein.

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ATTENUATED HUMAN PARAINFLUENZA VIRUS, METHODS AND USES THEREOF

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

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BACKGROUND OF THE INVENTION

Human parainfluenza viruses (HPIVs) are members of the *Paramyxovirinae* subfamily of the *Paramyxoviridae* family of viruses. Paramyxoviruses are enveloped viruses that replicate in the cytoplasm and bud at the plasma membrane and have a single-stranded negative-sense non-segmented RNA genome of approximately 13-19 kb. HPIVs are important pathogens in human populations, causing severe lower respiratory tract infections in infants and young children. Human parainfluenza virus type 1 (HPIV1) and type 2 (HPIV2) are the principal etiologic agents of laryngotracheobronchitis (croup) and also cause pneumonia and bronchitis (Chanock et al., 2001, Parainfluenza Viruses 4th Ed., Knipe et al. eds., Lippincott (Philadelphia, PA) 1341-1379). Human parainfluenza virus type 3 (HPIV3) is a leading cause of hospitalization for viral lower respiratory tract disease in infants and young children (Chanock et al., 2001, *supra*). HPIVs are also important causes of respiratory tract disease in adults. Collectively, HPIV1, HPIV2, and HPIV3 have been identified as the etiologic agents responsible for approximately 18% of hospitalizations for pediatric respiratory disease (Murphy et al., 1988, Virus Res., 11:1-15). HPIVs have also been implicated in a significant proportion of cases of virally induced middle ear effusions in children with otitis media (Heikkinen et al., 1999, N. Engl. J. Med., 340:260-264).

Despite considerable efforts, there are currently no parainfluenza virus vaccines available. Attenuated paramyxoviruses suitable for use in vaccines are currently under development. Two live attenuated HPIV3 vaccine candidates, a temperature sensitive (ts) derivative of the wild type HPIV3 JS strain and a bovine PIV3 strain, are currently being tested. (Karron et al, Pediatric Infectious Dis. J., 15:650, 1996; Karron et al, J. Infect. Dis., 171:1107, 1995; Karron et al., J. Infect. Dis., 172:1445, 1995). A chimeric PIV1 vaccine candidate has been generated by replacing the PIV3 HN and F open reading frames with those of PIV1 in a

PIV3 full length cDNA (Tao et al., 2000a). A chimeric HPIV3 bearing the glycoproteins of HPIV2 was also generated previously (Tao et al., 2000b). Attenuated HPIV2 strains have previously been made by introducing mutations into the L protein (WO 04/027037). Recombinant viruses include HPIV3 recombinant viruses having three identified mutations in the L gene. (Skiadopoulos et al, J Virol. 72:1762, 1998; Tao et al, J Virol. 72:2955, 1998; Tao et al, Vaccine, 17:1100, 1999). These live attenuated vaccine candidates can induce protection against HPIV infection in some experimental animal models. (Karron et al, J Infect. Dis., 172:1445, 1995b; Skiadopoulos et al, Vaccine 18:503, 1999; Skiadopoulos, Virology, 297: 136, 2002). However immunity to previous HPIV3 infection could limit the use of chimeric HPIV3 vaccines bearing heterologous HPIV1 or HPIV2 glycoproteins. Strategies to develop live viral vaccines are important in the design of safe and stable viral vaccine candidates.

In addition to providing possible vaccine candidates for protection against parainfluenza virus infection and disease, candidate vaccines may also be useful in expressing heterologous antigens. Studies demonstrate that foreign genes may be inserted into a paramyxovirus genome and are well expressed. (Bukereyev et al, J. Virol., 70:6634, 1996; Hassan et al, Virology, 237:249, 1997; Jin et al, Virology 251:206, 1998; Schmidt et al., 2001; Skiadopoulos et al., 2002). However, in order to develop vectors for vaccine use, more than a high level of protein expression is required. Factors in the design of a vector for delivery of heterologous antigens include viral host range, immunogenicity, and pathogenicity. Some negative strand viruses are undesirable as vectors because of their pathogenicity, such as measles and rabies virus.

Thus, there remains a need to develop effective immunogenic compositions to alleviate health problems associated with HPIV viruses and other pathogens, and to immunize against multiple HPIV serotypes. There is also a need to develop immunogenic compositions to deliver heterologous antigens.

SUMMARY OF THE INVENTION

The invention provides self-replicating, infectious, recombinant paramyxoviruses (PIV), methods of making the paramyxoviruses of the invention, and uses thereof. The PIV of the invention can have one or more amino acid or nucleic acid mutations that confer an attenuated phenotype. In some embodiments, the number of nucleotides inserted or deleted is such that the total number of nucleotides in the variant viral genome is divisible by six (known as the "rule of six"). The mutation can be stabilized by at least two changes in the codon specifying the mutation. The PIV of the invention can be human parainfluenza virus (HPIV), such as for example HPIV2. In an embodiment, the PIV of the invention comprise a nucleotide sequence having at least 80% sequence identity with SEQ ID NO:1.

The PIV of the invention can include a P protein, V protein, major nucleocapsid (N) protein, and/or large polymerase (L) protein. The proteins may be variant or naturally occurring. In an embodiment, P protein has an amino acid sequence having at least 80% sequence identity with SEQ ID NO:15. In an embodiment, V protein has an amino acid sequence having at least 80% sequence identity with SEQ ID NO:45. In an embodiment, N protein has an amino acid sequence having at least 80% sequence identity with SEQ ID NO:16. In an embodiment, L protein has an amino acid sequence having at least 80% sequence identity with SEQ ID NO:17.

In some embodiments, the PIV of the invention comprise a partial or complete polyhexameric genome or antigenome comprising a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein. Preferably, PIV comprising a monocistronic polynucleotide encoding a V protein have an attenuated phenotype. The variant polynucleotide encoding a P protein and the monocistronic polynucleotide encoding a V protein can be separated by a non-coding polynucleotide spacer sequence and optionally, can be on separate vectors. In some embodiments, the non-coding spacer comprises a gene end transcription signal, intergenic transcription signal, and gene start transcription signal. The non-coding spacer can be upstream of the V ORF.

The polynucleotide encoding the V protein can encode a variant V protein containing a mutation that inhibits the ability of the V protein to interrupt production of interferon in an infected host or signaling though its receptor. The mutation can be amino acid or nucleic acid substitution(s) or deletion(s). Preferably, the number of nucleotides inserted or deleted is such that the total number of nucleotides in the variant viral genome is divisible by six. The mutation can be stabilized by at least two changes in the codon specifying the mutation. In

some embodiments, the V protein comprises one or more amino acid substitutions at or between amino acid residues corresponding to a position 67, 68, 69, 70, 71,72, 105, 106, 107,108, 121, 122, 123, 124, 125, 126, 127, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 167, 168, 169, 170, 171, 172, or mixtures thereof, or any one of the amino acids of 174-225 of SEQ ID NO:45. The polynucleotide encoding the V protein can include a mRNA editing site having a heptaguanosine run. The heptaguanosine run can be substituted such that editing of V gene mRNA is inhibited.

The variant polynucleotide encoding the P protein can comprise a P ORF having one or more nucleotide substitutions wherein the substitution(s) introduces one or more stop codons in an overlapping V ORF reading frame but does not alter the amino acid sequence of P protein encoded by the variant polynucleotide encoding the P protein. The polynucleotide encoding the P protein can include a mRNA editing site having a heptaguanosine run. One or more nucleotides can be inserted into the mRNA editing site such that the nucleotide sequence encoding a carboxy-terminal of P protein is in frame. The heptaguanosine run can be substituted such that editing of P gene mRNA is inhibited.

The invention also includes polynucleotides and methods of using polynucleotides. In some embodiments, an isolated nucleic acid comprises a polynucleotide having at least 80% sequence identity to a polynucleotide of SEQ ID NO:1. In other embodiments, an isolated nucleic acid comprises a polynucleotide having a sequence of SEQ ID NO:2. In further embodiments, an isolated nucleic acid comprises a polynucleotide having a sequence of SEQ ID NO:3. The invention also includes an isolated nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a V polypeptide of SEQ ID NO:45. Other embodiments include an isolated nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a polypeptide of SEQ ID NO:17. The invention also includes vectors including any of the polynucleotides as well as a partial or complete genome or antigenome. Also provided are methods of producing a paramyxovirus polypeptide by culturing a host cell comprising any of the polynucleotides described herein.

Another aspect of the invention includes paramyxovirus polypeptides. In some embodiments, an isolated V polypeptide comprises at least one mutation at an amino acid residue corresponding to a position 67, 68, 69, 70, 71, 72, 105, 106, 107, 108, 121, 122, 123, 124, 125, 126, 127, 130, 131, 133, 134, 135, 136, 137, 138, 139, 140, 167, 168, 169, 170, 171, 172, or mixtures thereof of SEQ ID NO:45. In other embodiments, an isolated L polypeptide

comprises at least one mutation at one amino acid residue corresponding to a position 460, 948, 1566, 1724 or mixtures thereof of an L protein having an amino acid sequence of SEQ ID NO:17. In some embodiments, the L protein comprises one or more of the following substitutions: position 460 is substituted with A or P, position 948 is substituted with A, L or G or position 1724 is substituted with I.

In some embodiments, the PIV of the invention comprise one or more attenuating mutations. The attenuating mutations(s) can be temperature sensitive. Replication of PIV of the invention comprising one or more temperature sensitive mutations is attenuated in vitro at about 37°C or greater, as compared to wild type PIV. Temperature sensitive mutations can comprise amino acid substitution or deletion of one or more amino acid residues corresponding to position 460, 948, 1566, 1724, or 1725 of an L protein having an amino acid sequence of SEQ ID NO:17. In an embodiment, the substitution comprises F460L, F460A, or F460P. In an embodiment, the substitution comprises Y948A, Y948L, or Y948G. In an embodiment, the substitution comprises L1566I. In an embodiment, the substitution comprises S1724I. In an embodiment, amino acid residues at positions 1724 and 1725 are deleted.

The attenuating mutation(s) can be non-temperature sensitive. Non-temperature sensitive mutations can comprise a nucleic acid substitution at a position corresponding to position 15 of a 3' leader sequence having an nucleic acid sequence of SEQ ID NO:18. In an embodiment, the substitution comprises T15C. In preferred embodiments, the recombinant paramyxovirus comprises a polynucleotide having a C at position 15 and has little or no detectable virus with a T at that position. Non-temperature sensitive mutations can be host range restricted. In an embodiment, the PIV of the invention replicates in hamsters but not African green monkeys.

The PIV of the invention can comprise at least one temperature sensitive mutation and at least one non-temperature sensitive mutation. In an embodiment, at least one of the temperature sensitive mutations comprises an amino acid substitution or deletion of one or more amino acid residues corresponding to position 460, 948, 1566, 1724, or 1725 or mixtures thereof of an L protein having an amino acid sequence of SEQ ID NO:17 and at least one of the non-temperature sensitive mutations comprises a nucleic acid substitution at a position corresponding to position 15 of a 3' leader sequence having an nucleic acid sequence of SEQ ID NO:18. In preferred embodiments, the recombinant paramyxovirus comprises a polynucleotide having a C at position 15 and has no detectable virus with a T at that position.

In some embodiments, the PIV of the invention comprise one or more supernumerary heterologous polynucleotides or genome segments encoding one or more antigenic determinants of a heterologous pathogen. The heterologous pathogen can comprise HPIV1, HPIV3, measles virus, subgroup A or subgroup B respiratory syncytial virus, mumps virus, human papilloma virus, type 1 or type 2 human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rabies virus, Epstein Barr virus, filovirus, bunyavirus, flavivirus, alphavirus, human metapneumovirus, or influenza virus. In an embodiment, the antigenic determinant comprises measles HA, HPIV1 HN, or HPIV1 F. Preferably, PIV of the invention containing one or more determinants of a heterologous pathogen have an attenuated phenotype.

Another aspect of the invention includes methods of making the PIV and polynucleotides of the invention. In some embodiments, the methods of the invention comprise removing a bicistronic polynucleotide encoding P and V proteins from viral genome or antigenome of a PIV and inserting a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein into a full length or partial genome or antigenome of a PIV. The polynucleotide encoding a variant P protein and the monocistronic polynucleotide encoding a V protein can be on the same vector or separate vectors. The variant polynucleotide encoding a P protein can comprise a mutated mRNA editing site such that editing of mRNA encoding P protein is inhibited. The monocistronic polynucleotide encoding a V protein can comprise a mutated mRNA editing unit such that editing of mRNA encoding V protein is inhibited. In some embodiments, the variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein are separated by a non-coding polynucleotide spacer sequence comprising a gene end transcription signal, intergenic transcription signal, and gene start transcription signal.

In an embodiment, the removing step comprises introducing unique restriction enzyme recognition sequences into the genome or antigenome such that the recognition sequences flank the bicistronic polynucleotide, and digesting the genome with one or more restriction enzymes that cut the genome at the restriction sites flanking the bicistronic polynucleotide. In an embodiment, the inserting step comprises inserting the variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein at the cleaved restriction sites, and religating the genome or antigenome.

In some embodiments, the methods of the invention comprise coexpressing in a cell an expression vector comprising a partial or complete polyhexameric genome or antigenome encoding a PIV of the invention and one or more polynucleotides encoding N protein, P

protein, and L protein and incubating the cell under conditions that allow for viral replication. The cells can be, for example, Hep-2 cells, Vero cells, or LLC-MK2 cells.

Another aspect of the invention is a composition comprising PIV of the invention. The PIV of the invention are useful, for example, in immunogenic compositions for eliciting an immune response in an animal, including live virus vaccines and vectors for expressing heterologous antigens. PIV of the invention can be combined with viruses of other PIV serotypes, strains, or genera in a composition to elicit an immune response against multiple genera, serotypes, and strains.

The compositions of the invention comprise an immunogenic effective amount of a PIV of the invention and a physiologically acceptable carrier. The compositions of the invention can also comprise an adjuvant. In an embodiment, the composition of the invention comprises PIV from two or more serotypes. Preferably, at least one or more of the serotypes is HPIV1, HPIV2, HPIV3, or HPIV4. The HPIV2 can be strain V94, V98, or Greer. In an embodiment, the composition of the invention comprises PIV from two or more genera. Preferably, at least one genus is Rubulavirus genus.

Another aspect of the invention is methods of eliciting an immune response in an animal. The methods generally comprise administering an immunogenic effective amount of a composition of the invention to the animal. Preferably the immune response produces anti-PIV antibodies that are protective. In an embodiment, the antibodies are IgA. In an embodiment, the immune response produces antibodies that bind one or more antigenic determinants of a heterologous pathogen encoded by a supernumerary gene or genome segment of the PIV of the invention. The heterologous pathogen can be HPIV1, HPIV3, measles virus, subgroup A or subgroup B respiratory syncytial virus, mumps virus, human papilloma virus, type 1 or type 2 human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rabies virus, Epstein Barr virus, filovirus, bunyavirus, flavivirus, alphavirus, human metapneumovirus, or influenza virus. In an embodiment, the antigenic determinant is measles HA, HPIV1 HN, or HPIV1 F.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-B show the level of replication of the biologically derived HPIV2 V94 strain and recombinant derivatives(r) thereof in the upper (nasal turbinates;1A) and lower (lungs;1B) respiratory tract of hamsters. The mean level of replication for each group is shown ± standard error (error bars). Values that are significantly different ($P<0.05$; Tukey-Kramer test (Winer, 1971; Dunnet, 1980)) than the parent rV94Not virus are indicated with an asterisk (*).The lower limit of detection of virus replication, which is indicated by the dashed line, is 1.5 $\log_{10}$ $TCID_{50}$/g. Greer, V94 and V98 are wild-type HPIV2 strains.

Figures 2A-D show the level of replication of recombinant and biologically derived V94 and recombinant derivatives thereof in the upper (nasal turbinates) and lower (lung) respiratory tract of African green monkeys. Nasopharyngeal (NP) swab samples are shown in Figures 2A and 2C. Tracheal lavage (TL) samples are shown in Figures 2B and 2D. The mean of the peak virus titer for each animal irrespective of sampling day is shown in Figures 2A and 2B ± standard error (error bars). The lower limit of detection (dashed line) of virus titer in Figures 2A and 2B was 0.5 $\log_{10}$ $TCID_{50}$/ml. The mean sum of the viral titers obtained for each animal on all sampling days is shown in Figures 2C and 2D ± standard error (error bars). The lower limit of detection (dashed line) is 5.0 $\log_{10}$ $TCID_{50}$/ml for NP swab (Figure 2C) and 2.5 $\log_{10}$ $TCID_{50}$/ml for TL samples (Figure 2D). Mean titers and mean of sum titers with an asterisk are statistically different ($P< 0.05$; Tukey-Kramer test) than the parent V94 virus. τ, indicates values where the difference as compared to rV94Not ($P< 0.05$; Tukey-Kramer test) are statistically significant. The number of animals in each group is indicated in Table 5.

Figure 3A shows a partial nucleotide sequence comparison (antigenomic–sense) of the 3' leader (nt 1-30) of a biologically derived, low-passage HPIV2 V98 (SEQ ID NO:18) strain, two different preparations (V94(a) (SEQ ID NO:19) and V94(b) (SEQ ID NO:20) of the biologically derived V94 strain of HPIV2, and the recombinant V94 consensus cDNA sequence. Nucleotide position 15 (bolded and underlined) is the only position found to vary in these virus preparations.

Figure 3B shows sequence electropherograms from uncloned RT-PCR products derived from primary isolates of virus recovered from the lower respiratory tract of four African green monkeys infected with V94 (preparation a). The sequence obtained from V94(a) passaged in LLC-MK2 cells was used as a control.

Figures 4A-B show a diagrammatic representation of the genomic organization of wild type HPIV2 V94. In Figure 4A, individual genes are shown as boxes separated by gene-start

(GS) and gene-end (GE) signals for each gene, which are shaded in gray and black, respectively. The 3′ extragenic leader and 5′ extragenic trailer regions are indicated. The exploded view in Figure 4A shows the nucleotide sequence in the region of the editing site in unedited mRNA encoding V protein (top sequence; SEQ ID NO:46) and the nucleotide sequence in the edited mRNA that includes two inserted G residues and encodes the P protein (bold, bottom sequence; SEQ ID NO:47). The sequence is in antigenomic sense and is arranged by codon triplets. Codons 164 and 165 are numbered. Codon 164 encodes the last common amino acid in the N-terminal half of the P and V polypeptides. Codon 165 and all subsequent codons encode amino acids of the distinct C-terminal portions of the P and V proteins. Figure 4B shows a diagrammatic representation of the P and V polypeptides including the common amino-terminal domains (white box) and distinct carboxy-terminal domains (hatched boxes), numbered according to the amino acid sequence.

Figure 5A shows the introduction of unique restriction enzyme recognition sequences at four positions in the recombinant V94 genome for use in both alteration of the P/V gene and introduction of a supernumerary gene encoding the V protein.

Figure 5B shows a diagram of the genome of the HPIV2 rV94 P+V virus, which includes a polynucleotide engineered to express the P protein separate from a polynucleotide engineered to express only the V protein. Gene-start and gene-end signals for each polynucleotide are shaded in gray and black, respectively. The exploded view of the P and V proteins shows their common amino acid domains (white box) and distinct carboxy-terminal domains (hatched boxes).

Figure 5C shows the details of the construction of separate polynucleotides encoding P protein and V protein in the mutant rV94 P+V genome. Sequences are in antigenomic sense and numbered according to their position in the rV94 P+V antigenomic sequence. The exploded view of the polynucleotide encoding P protein (top) shows the P ORF and flanking sequence showing the positions of the introduced *AscI*, *Bst*EII and *Age*I sites, a 4-nucleotide insert introduced to maintain the "rule of six", and the naturally-occurring P gene-end signal. The exploded view of the polynucleotide encoding the V protein (bottom) shows the V ORF and flanking sequence showing the positions of the introduced *Bst*EII and *Sac*II sites, as well as a gene junction including a gene-end signal, intergenic, and gene-start signal positioned with 6n+1 phasing. The "rule of six" refers to the finding that replication of the genomes of most or all members of Paramyxovirinae is efficient only if the nucleotide length of the genome is an

even multiple of six, a requirement that is thought to reflect the intimate association of each N protein monomer with exactly six nucleotides (Kolakofsky et al, J.Virol. 72, 891-899, 1998)

Figure 6 shows the common amino acid domains (white box) and distinct carboxy-terminal domains (hatched boxes) of P protein and V protein. The exploded view shows the sequence modifications introduced into the wild type P/V ORF to create a V ORF that expresses only V protein and a P ORF that expresses only P protein. Silent third base codon substitutions in the editing site are underlined and in bold type. The top sequence (V ORF in rV94 P+V) shows modifications introduced into the editing site of the modified V gene that inactivate the editing site and allow for expression of V protein only (SEQ ID NO:9). The middle sequence (P/V ORF in wt V94) shows unedited wild type P/V sequence, including the wild type editing site (SEQ ID NO:8). The bottom sequence (P ORF in V94 P+V) shows modifications introduced into the wild type P/V ORF that allow for expression of P protein and not the V protein. The 2 nucleotides (TG) inserted into the editing site to access the 3'-terminal half of the P ORF are indicated by the arrows (; SEQ ID NO: 10). Proposed third base codon substitution mutations (underlined and in bold type) that introduce stop codons in the V ORF reading frame (V ORF codon positions 167, 176, 184, and 186 are boxed) but do not alter the P protein sequence are shown. Dashed lines show the relative positions of the silent third codon mutations in P protein. Solid lines show the relative positions of the stop codons in V protein.

Figure 7A shows agarose gel electrophoresis analysis of RT-PCR products obtained from vRNA purified from V94 or rV94 P+V infected cells, or PCR product generated from the antigenomic V94 P+V cDNA (pFLC V94 P+V) using a sense oligonucleotide (V94, nucleotides 395-425) and an antisense oligonucleotide (V94, nucleotides 3567-3598). The predicted size for the PCR product obtained from wild type V94 is 3203 bp. The predicted size of the PCR product obtained from the P+V gene rearrangement for both rV94 P+V and pFLC V94 P+V is 3899 bp.

Figure 7B shows a Western blot analysis demonstrating the production of V protein in wild type V94 or rV94 P+V infected Vero cells.

Figure 8 shows the growth of recombinant V94 in Vero cell culture. Virus titers are shown as mean $\log_{10}$ $TCID_{50}$/ml of triplicate samples. Error bars indicate standard error.

Figure 9A shows the primary amino acid sequence of the HPIV2 strain V94 V polypeptide (SEQ ID NO:45). Regions that are potential targets for point or deletion mutagenesis are highlighted: I, similar to a sequence identified in SV5 that is required for RNA

binding. II, similar to a sequence identified in SV5 that is required for STAT binding. III, similar to a sequence identified in SV5 that is required for STAT degradation. IV, putative leucine (bold font) rich nuclear export signal sequence. V, this highly conserved sequence may also form part of a zinc finger binding domain. VI, this region includes 5 of 7 conserved cysteine residues in the cysteine rich domain (CRD). *, indicates Ala-165 (bolded and underlined) the first amino acid unique to the carboxy-terminal half of the V protein.

Figure 9B shows a sequence comparison of the highly conserved carboxy-terminal cysteine-rich domain of 22 members of the Paramyxovirus family. Boxed and bolded sequences are highly conserved. Residues indicated with * may directly interact with one or more zinc ions and can be targets of amino acid deletion or substitution mutagenesis.

Figure 10A-C shows the complete rV94 P+V antigenomic cDNA sequence from nucleotides 1 through 16350 (SEQ ID NO:1).

Figure 11 shows the antigenomic cDNA P ORF sequence (1188 nucleotides) from nucleotides 1997 through 3184 of the rV94 P+V antigenomic cDNA sequence (SEQ ID NO:2).

Figure 12 shows the antigenomic cDNA V ORF sequence (678 nucleotides) from nucleotides 3239 through 3916 of the rV94 P+V antigenomic cDNA sequence (SEQ ID NO:3).

DETAILED DESCRIPTION OF INVENTION

I. Definitions

Paramyxovirus as used herein refers to a paramyxovirus of the *Paramyxovirinae* subfamily of the *Paramyxoviridae* family. Paramyxoviruses are enveloped viruses that have a single strand of negative sense RNA of approximately 13 to 19 kb as a genome. Examples of paramyxoviruses include, but are not limited to, human parainfluenza virus (HPIV) including types 1, 2, 3, 4A, and 4B (HPIV1, HPIV2, HPIV3, HPIV4A, and HPIV4B, respectively), mouse parainfluenza type 1 (Sendai virus, MPIV1), bovine parainfluenza virus type 3 (BPIV3), simian virus 5 (SV5), simian virus 41 (SV41), and mumps virus. HPIV1, HPIV3, MPIV1, and BPIV3 are classified in the genus Respirovirus. HPIV2, HPIV4, SV5, SV41, and mumps virus are classified in the genus Rubulavirus. MPIV1, SV5, and BPIV3 are animal counterparts of HPIV1, HPIV2, and HPIV3, respectively (Chancock et al., *Parainfluenza Viruses*, Knipe et al. (Eds.), pp. 1341-1379, Lippincott Williams & Wilkins, Philadelphia, 2001). HPIV1, HPIV2, and HPIV3 represent distinct serotypes and do not elicit significant cross immunity. HPIVs are etiological agents of respiratory infections such as croup, pneumonia, or bronchitis.

The term "human parainfluenza virus type 2" or "HPIV2" refers to an isolate, clone, recombinant, or variant of human parainfluenza virus type 2 of the *Paramyxovirinae* subfamily. A "naturally occurring" isolate or "wild type" HPIV2 is a virus isolated from a natural source or has the sequence of a HPIV2 isolated from a natural source. Naturally occurring isolates may differ from one another in sequence. In some embodiments, a naturally occurring isolate of HPIV2 of the invention has at least 90% nucleic acid sequence identity to HPIV2 strain V94 (SEQ ID NO:4; Table 6; Genbank Accession No. AF533010). "Recombinant HPIV2" refers to virus derived from a polynucleotide that has been constructed to encode a HPIV genome or antigenome, and may include a sequence of a wild type or variant HPIV2. In some embodiments, the recombinant HPIV2 comprises an expression vector.

The HPIV2 genome encodes at least seven polypeptides. The ribonucleocapsid–associated polypeptides include the nucleocapsid protein (N) (Table 9; SEQ ID NO:16), the phosphoprotein (P) (Table 10; SEQ ID NO:15), and the large polymerase (L) protein (Table 11; SEQ ID NO:17) that carry out transcription and replication. Similar to other Rubulaviruses, the P/V gene of HPIV2 includes an alternative open reading frame (ORF) that is accessed by a shift in reading frame mediated by cotranscriptional editing to generate the mRNA encoding P protein. The unedited mRNA encodes the V protein. The internal matrix protein (M) and the major protective antigens, fusion glycoprotein (F) and hemagglutinin–neuraminidase

glycoprotein (HN), are envelope–associated proteins. The gene order is 3′- N- P/V- M- F- HN- L-5'. A HPIV2 encoding polynucleotide can be isolated from infected humans or cells or can be prepared as described herein.

"Variants" of HPIV refer to a virus that has a genomic sequence that differs from the sequence of a reference virus. In some embodiments, a variant may be prepared by altering or modifying the nucleic acid sequence of the viral genome by addition, substitution, and deletion of nucleotides. As discussed previously, it is preferred that variants that have a modification due to addition or deletion of nucleotides conform to the rule of six. In some embodiments, variants may be obtained by passage of a viral particle or genome *in vitro* in a host cell or *in vivo* in a non-human host. In some embodiments, the number of nucleotides inserted or deleted is such that the total number of nucleotides in the variant viral genome is divisible by six (known as the "rule of six").

In some embodiments, the variants have at least one altered phenotype. The altered phenotypes can include, without limitation, a change in growth characteristics, attenuation, temperature sensitive growth, cold adaptation, plaque size, host range restriction or a change in immunogenicity. In some embodiments, variant HPIV2 can be immunogenic and elicit protective antibodies in a mammal. Preferably, the HPIV2 variants are attenuated.

In some embodiments, the variant HPIV2 genome or antigenome has at least 80% sequence identity, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99% or greater sequence identity to a paramyxovirus reference genomic or antigenomic sequence. The reference sequence may be HPIV2 strain V94 (SEQ ID NO:4; Table 6; Genbank Accession No. AF533010), HPIV2 strain V98 (SEQ ID NO:5; Table 7; Genbank Accession No. AF533011), or HPIV2 strain Greer (SEQ ID NO:6; Table 8; Genbank Accession No. AF533012). Preferably, the reference sequence is strain V94 having a sequence of SEQ ID NO:4.

In some embodiments, the variant HPIV2 genome is composed of a polynucleotide encoding a V protein having at least 80% sequence identity, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more

preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99% or greater sequence identity to a V gene reference genomic sequence from HPIV2 strain V94 (SEQ ID NO:4; Table 6), HPIV2 strain V98 (SEQ ID NO:5; Table 7), HPIV2 strain Greer (SEQ ID NO:6; Table 8),or a V polypeptide comprising an amino acid sequence of SEQ ID NO:45. In some embodiments, the reference sequence may be encoded by the antigenomic polynucleotide sequence of SEQ ID NO: 3. Preferably, the V protein encoded by the variant HPIV2 has reduced activity as compared to V protein encoded by HPIV2 strain V94, HPIV2 strain V98, or HPIV2 strain Greer.

In some embodiments, the variant HPIV2 genome is composed of a polynucleotide encoding an L protein having at least 80% sequence identity, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99% or greater sequence identity to a reference polynucleotide sequence encoding an L protein from HPIV2 strain V94 (SEQ ID NO:4; Table 6), HPIV2 strain V98 (SEQ ID NO:5; Table 7), HPIV2 strain Greer (SEQ ID NO:6; Table 8) or a L polypeptide comprising a sequence of SEQ ID NO:17. Preferably, the L protein encoded by the variant HPIV2 has reduced activity as compared to L protein encoded by HPIV2 strain V94, HPIV strain V98, or HPIV2 strain Greer.

The term "antigenome" means a viral RNA molecule or DNA molecule complementary to the negative sense single stranded viral RNA genome.

A paramyxovirus that is "attenuated" or has an "*att* phenotype" refers to a paramyxovirus that has decreased replication in a mammal as compared to replication of a reference wild-type paramyxovirus under similar conditions of infection. In some embodiments, a paramyxovirus that is attenuated exhibits at least about 10-fold or greater decrease, more preferably at least about 100-fold or greater decrease, more preferably at least about 1000-fold or greater decrease in virus titer in the upper or lower respiratory tract of a

mammal compared to non attenuated, wild type virus titer in the upper or lower respiratory tract, respectively, of a mammal of the same species under the same conditions of infection. Examples of mammals include, but are not limited to, humans, mice, rabbits, rats, hamsters, such as for example *Mesocricetus auratus*, and non-human primates, such as for example *Ceroptihecus aethiops*. An attenuated paramyxovirus may display different phenotypes including without limitation altered growth, temperature sensitive growth, host range restricted growth or plaque size alteration.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers, which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations, employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

An "infectious clone " of a paramyxovirus as used herein refers to a full-length genome or portion of a genome of a paramyxovirus isolate cloned into a replicable vector that provides for amplification of the viral genome in a cell and in some embodiments, results in viral particles. In some embodiments, a portion of the paramyxovirus genome comprises a polyhexameric nucleic acid sequence encoding at least N protein, P protein, and L protein in a single replicable vector. In other embodiments, the viral genome is a full-length genome. The replicable vector provides for introduction and amplification of the viral genome in a wide variety of prokaryotic and eukaryotic cells.

The term "immunogenic effective amount" of a paramyxovirus, component thereof, or other antigenic determinant refers to an amount of a paramyxovirus, component thereof, or other antigenic determinant that induces an immune response in an animal. The immune response may be determined by measuring a T or B cell response, or by challenging an immunized animal with a virus capable of replicating in the host species. Typically, the induction of an immune response is determined by the detection of antibodies specific for paramyxovirus, a component thereof, or other antigenic determinants.

An "isolated" nucleic acid molecule refers to a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source. Preferably, the isolated nucleic is free of association with all components with which it is naturally associated. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature.

A "monocistronic" polynucleotide refers to a polynucleotide that encodes only one protein. For example, a monocistronic polynucleotide encoding V protein only encodes V protein, it does not encode for both V protein and P protein.

"Percent (%) nucleic acid sequence identity" with respect to the nucleic acid sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference paramyxovirus nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. In some embodiments, the reference paramyxovirus nucleic acid sequence is HPIV2 Strain V94 (SEQ ID NO:4). Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared.

For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence A to, with, or against a given nucleic acid sequence B (which can alternatively be phrased as a given nucleic acid sequence A that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence B) is calculated as follows:

100 times the fraction W/Z

where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Z is the total number of nucleotides in B. It will be appreciated that where the length of nucleic acid sequence A is not equal to the length of nucleic acid sequence B, the % nucleic acid sequence identity of A to B will not equal the % nucleic acid sequence identity of B to A.

"Percent (%) amino acid sequence identity" with respect to the amino acid sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues polypeptide reference sequence, such as for example the amino acid sequence of N protein, P protein, V protein, M protein, F protein, HN,

or L protein, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2, clustal V (DNASTAR) or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared.

For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

As used herein, "stable" paramyxovirus refers to a paramyxovirus that has a low risk of reversion to a reference virus sequence or phenotype after passaging, infection, or selective pressure. In some embodiments, the reference sequence is the sequence from which an altered or variant paramyxovirus is derived. In other embodiments, the reference sequence or phenotype may be that of a wild type strain such as V94, V98 or Greer. Non-wild type phenotypes include without limitation, a change in growth characteristics, attenuation, temperature sensitive growth, cold adaptation, plaque size, host range restriction or a change in immunogenicity, or mixtures thereof. In some embodiments, the mutation is stable if it does not revert to the reference sequence or phenotype after at least 8 *in vitro* cell culture passages. In some embodiments, the mutation is stable if it does not revert to a reference sequence or phenotype when grown at 38-40°C. In some embodiments, the mutation is stable if it does not revert to a reference sequence or phenotype at least 10 days post-infection of a mammal. Generally, genetic stability increases as the number of nucleotide substitutions increases. For example, a codon substitution that would require 3 nucleotides changes to revert to the wild type or wild type-like codon is more stable than a codon substitution that would require only 1 nucleotide change to revert to the wild type or wild type-like codon. Deletion mutations

generally confer a greater level of genetic stability than codon substitutions. For example, deletion of a codon would require insertion of 3 nucleotides to revert to wild type.

"Recombinant" in reference to a polynucleotide refers to a polynucleotide that has been isolated and/or altered by the hand of man and includes recombinant molecules and recombinant viruses. "Recombinant" in reference to a paramyxovirus refers to a virus that is encoded or has been produced from such a polynucleotide. "Recombinant HPIV2 genome or antigenome" or "rHPIV2" refers to a polynucleotide that has been constructed to encode a HPIV strain or variant, and may include a sequence of a wild type or variant HPIV2. In some embodiments, the recombinant HPIV2 genome or antigenome is in the form of a cDNA. In some embodiments, a polynucleotide sequence encoding all or a portion of a paramyxovirus viral genome or antigenome may be isolated and combined with other control sequences in a vector. The other control sequences may be those that are found in the naturally occurring gene or from other sources. The vector provides for amplification of the recombinant molecule(s) in prokaryotic or eukaryotic cells. It also can provide for introduction into host cells and expression of the polynucleotide. The vectors described herein for recombinant paramyxovirus sequences are introduced into eukaryotic cells and propagated under suitable conditions as known to those of skill in the art, and are introduced into animal cells and expressed under suitable conditions as known to those of skill in the art.

The term " replicable vector," as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked into a cell and providing for amplification of the nucleic acid. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional nucleic acid segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. In some embodiments, the vector is a vector that can replicate to high copy number in a cell.

The term "shut-off temperature" refers to a temperature at which the reduction of virus titer compared to its titer at a reference temperature is 100-fold greater than the reduction of

wild type virus at the same temperature. In some embodiments, the reference temperature is about 32°C, and the shutoff temperature is about 39°C, more preferably 38°C or 37°C. A determination of the shut off temperature allows a comparison of the temperature sensitivity of different virus strains or isolates and is often indicative of the level of attenuation. The lower the shutoff temperature the higher the level of attenuation of the paramyxovirus isolate or strain.

The term "transfection" as used herein refers to introducing DNA into a eukaryotic cell so that the DNA is replicable and/or expressed, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transfection is done using standard techniques appropriate to such cells. Methods for transfecting eukaryotic cells include polyethyleneglycol/DMSO, liposomes, electroporation, and electrical nuclear transport.

Polypeptide sequences defined herein are represented by one-letter or three letter symbols for amino acid residues as follows:

A	ala, alanine	L	leu, leucine
R	arg, arginine	K	lys, lysine
N	asn, asparagine	M	met, methionine
D	asp, aspartic acid	F	phe, phenylalanine
C	cys, cysteine	P	pro, proline
Q	gln, glutamine	S	ser, serine
E	glu, glutamic acid	T	thr, threonine
G	gly, glycine	W	try, tryptophan
H	his, histidine	Y	tyr, tyrosine
I	ile, isoleucine	V	val, valine

II. Aspects of the Invention

Strategies to generate attenuated viruses are important in the design of safe and stable viral constructs useful in an immunogenic composition. The phenotype of a viral isolate or strain may be modified to achieve a balance between attenuation of viral replication and immunogenicity of the modified variant. In some embodiments, viral replication may be decreased about 100 to 1000 fold and yet still retain immunogenicity. In some embodiments, it is desirable to generate an attenuated virus that has at least one temperature sensitive attenuating mutation and one non- ts attenuating mutation. Attenuated viruses that have more than one mutation and/or more than one phenotype can have enhanced stability.

The V protein of paramyxoviruses is an attractive target for introduction of one or more mutations. V protein inhibits production of α /β interferons and decreases signaling of α /β interferons through their receptors. A paramyxovirus with a modified V protein should have decreased pathogenicity since it may be defective in counteracting host cell interferon response. Replication of paramyxoviruses expressing V protein with a carboxy terminal deletion has been found to be defective *in vivo* and *in vitro*, including in Vero cells which do not express antiviral interferons α and β (Kato et al., 1997, *Embo J.*, 16(3):578-587; Delenda et al., 1997, *Virology*, 228(1):55-62; Durbin et al., 1999, *Virology*, 261(2):319-330; Kawano et al., 2001, *Virology*, 284(1):99-112; He et al., 2002, *Virology*, 303(1):15-32; Park et al., 2003, *J. Virol.*, 77(17):9522-9532). The V protein might also have other functions during viral infection, and mutations might also achieve an attenuating effect by interfering with additional functions.

V protein is encoded by a bicistronic polynucleotide encoding both the P and V proteins. The bicistronic polynucleotide encodes a nucleocapsid-associated P phosphoprotein from an overlapping reading frame (Ohgimoto et al., 1990). P protein is a structural protein that plays a major role in transcription and replication of the viral genome. The alternative reading frames are accessed by an unusual cotranscriptional editing of the P/V encoding mRNA, a feature unique to most members of the *Paramyxovirinae* subfamily. The polynucleotide encoding the P and V protein includes an alternative open reading frame (ORF) that is accessed by a shift in reading frame mediated by cotranscriptional editing to generate the mRNA encoding a P protein. A V protein is generated from an unedited mRNA encoding P and V. Therefore, the P and V genes have a common amino-terminal sequence and unique carboxy-termini. The overlapping nature of the P and V ORFs, however, greatly restricts the number and types of mutations that can be introduced into the V protein without also affecting the P protein, and vice versa.

One aspect of the invention includes polynucleotides,vectors and a viral construct comprising a polynucleotide encoding a variant P protein and a monocistronic polynucleotide encoding a V protein. Separation of the coding sequence of the V and P protein allows changes to the V protein without affecting the function of the P protein. Insertion of a polynucleotide encoding a V protein into the viral genome provides an attenuating phenotype. Preferably, the polynucleotide encoding the V protein is modified with at least one mutation that decreases the ability of the V protein to inhibit interferon production and/ or signaling. The polynucleotide encoding a variant P protein and the monocistronic polynucleotide encoding the V protein can be on the same vector or separate vectors.

Another aspect of the invention involves a novel attenuating mutation of the L polymerase. In some embodiments, residue positions are selected for substitution based on a comparison to other related viruses and an indication that when an amino acid at the position is substituted in other related viruses an attenuating phenotype is observed. The amino acids selected for substitution at those positions are chosen from those amino acids that are encoded by a codon that differs in at least two nucleotide positions from the wild type amino acid found at that position. In some embodiments, at least two nucleotide changes are made in a codon specifying the changed amino acid. In some embodiments, mutations of the L polymerase have a temperature sensitive phenotype.

In yet another aspect, an attenuating mutation may be made in a noncoding region of the genome including the 3' leader and/or 5'trailer of the viral genome. In some embodiments, a recombinant and infectious parainfluenza virus comprises an attenuating mutation at a position corresponding to nucleotide position 15 of the 3' leader of viral genome or antigenome. In some embodiments, a recombinant parainfluenza virus with a mutation at a position corresponding to position 15 has a host range restriction phenotype.

The attenuating mutations and methods of the invention provide recombinant, infectious, self-replicating paramyxoviruses comprising a partial or complete polyhexameric genome or antigenome having a polynucleotide encoding a variant P protein and a monocistronic polynucleotide encoding a V protein, as well as novel attenuating mutations in the L polymerase and 3' leader sequence. In some embodiments, an attenuated paramyxovirus has a temperature sensitive and at least one other attenuating mutation that provides a phenotype including host range restriction, reduced plaque size, or change in immunogenicity. The attenuated infectious virus can be utilized in live virus vaccines and/or in immunogenic compositions to protect against HPIV infection and/or to deliver heterologous antigens. The attenuating mutations can be utilized as part of a menu of attenuating mutations to develop attenuated paramyxovirus strains that may be utilized in vaccines.

A. Mutations

1. L polymerase

One aspect of the invention provides a recombinant and infectious variant of HPIV2 having one or more attenuating mutations in the L polymerase (L protein). In some embodiments, residue positions are selected for substitution based on a comparison to other related viruses and an indication that when an amino acid at a position is substituted in other

related viruses an attenuating phenotype is observed. The amino acids selected for substitution at those positions are chosen from those amino acids that are encoded by a codon that differs in at least two nucleotide positions from the wild type amino acid found at that position. In some embodiments, at least two nucleotide changes are made in a codon specifying the changed amino acid.

In some embodiments, an attenuating mutation comprises a substitution at one or more amino acid residues corresponding to positions 460, 948, or 1724 of SEQ ID NO:17. Preferred amino acid substitutions include F460A, F460P, Y948A, Y948L, Y948G, S1724I, or mixtures thereof.

The attenuating mutations are preferably stable. Amino acid substitutions that require two or three nucleotide substitutions are preferred. For example, F460A requires three nucleotide substitution mutations (wild type TTT, variant GCA). The nucleotide substitutions encoding the preferred amino acid substitutions described above are shown in Table 2.

The attenuating mutation(s) can be temperature sensitive. In an embodiment, the L protein mutations are not attenuating for replication at permissive temperature, such as for example 30-32°C, but are attenuating for replication at restrictive temperatures, such as for example 37°C - 40°C. In an embodiment, replication of the HPIV2 variants is reduced at about 39°C. In some embodiments, the shut-off temperature of the HPIV2 variants is preferably about 39°C, more preferably about 38°C, or about 37°C. Preferably, the paramyxovirus strains with mutations in L polymerase have a lower shutoff temperature than control paramyxovirus. In some embodiements, the control is a wild type virus. In other embodiments, the control is another attenuated paramyxovirus.

Preferably, the HPIV2 variants are attenuated *in vivo*. In an embodiment, the HPIV2 variants exhibit reduced replication in the upper and/or lower respiratory tract of a mammal as compared to wild-type HPIV2 or other attenuated paramyxoviruses. In an embodiment, the replication is reduced at least about 10 fold, 100 fold, more preferably about 500 fold, more preferably about 1000 fold, more preferably about 1500 fold, more preferably about 2000 fold, more preferably about 3000 fold, more preferably about 4000 fold, more preferably about 5000 fold, more preferably about 6000 fold as compared to wild-type HPIV2 or other attenuated paramyxoviruses. In an embodiment, the mammal is a golden Syrian hamster (*Mesocricetus auratus*). In another embodiment, the mammal is an African green monkey (*Cercopithecus aethiops*).

The HPIV2 variants preferably comprise a partial or complete polyhexameric genome or antigenome encoding a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P) and a L polymerase (L) protein. HPIV2 variants may further comprise a genome or antigenome encoding a fusion (F) protein and a hemaglutinin- neuraminidase (HN) protein. HPIV2 variants that further comprise F and HN proteins are useful in immunogenic compositions.

Another aspect of the invention includes an isolated nucleic acid or vector comprising a polynucleotide encoding a polypeptide with at least 80% sequence identity to a L protein having a sequence of SEQ ID NO:17. An isolated polypeptide comprising at least 80% sequence identity to a L polypeptide of SEQ ID NO: 17 and preferably, comprising at least one mutation of amino acid residues corresponding to positions 460, 948, or 1724 of SEQ ID NO:17 is also provided.

An attenuating mutation in the L protein can be generated by PCR mutagenesis and standard molecular cloning techniques. Examples of PCR primers useful for generating the attenuating mutations of the invention are described in Example 1 and Table 2.

2. 3' leader sequence

One aspect of the invention provides recombinant and infectious variants of HPIV2 having an attenuating mutation in the 3' leader of a viral genome. In some embodiments, an attenuating mutation comprises a nucleic acid substitution at a position corresponding to T15 of SEQ ID NO:4. In an embodiment, the nucleotide substitution comprises T15C. In some embodiments, the recombinant and infectious variants predominantly have a C at position 15 and more preferably, have little or no detectable virus with a T at position 15.

In some embodiments, the attenuating mutation may also have a phenotype selected from a change in growth characteristics, attenuation, temperature sensitivity, cold adaption, plaque size, host range restriction or changes in immunogenicity. Preferably, the attenuating mutation has a phenotype of host range restriction. In an embodiment, HPIV2 including a nucleotide substitution at a position corresponding to T15 of SEQ ID NO:5 is attenuated in the respiratory tract of African green monkeys, but not in the respiratory tract of golden Syrian hamsters. In some embodiments, the attenuating mutation does not confer a temperature sensitive phenotype.

An attenuating mutation in the 3' leader of HPIV2 can be generated by PCR mutagenesis and standard molecular cloning techniques. Examples of PCR primers useful for generating the attenuating mutation of the invention are described in Example 2.

3. Separation of bicistronic polynucleotides encoding P and V proteins into a polynucleotide encoding a P or a V protein.

One aspect of the invention provides recombinant, infectious, self-replicating paramyxovirus comprising a partial or complete polyhexameric genome or antigenome having a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein. The polynucleotide encoding a P protein has been altered to no longer encode the V protein. The paramyxovirus of the invention preferably includes a major nucleocapsid protein (N protein), a nucleocapsid phosphoprotein (P protein), and a large polymerase protein (L protein). The N protein, P protein, or L protein can be from a heterologous Rubulavirus, such as HPIV2, HPIV4HPIV2, HPIV4, mumps, SV41 and SV5. Paramyxovirus variants may further comprise a genome or antigenome encoding a fusion (F) protein and a hemaglutinin-neuraminidase (HN) protein. Paramyxovirus variants that further comprise F and HN proteins are useful in immunogenic compositions. In some embodiments, the polynucleotide encoding a variant P protein and the monocistronic polynucleotide encoding the V protein are on separate vectors.

The paramyxovirus can be a parainfluenza virus (PIV). A number of paramyxovirus have a polynucleotide encoding both a P and V protein including HPIV2, murine P1V1 (Sendai virus) HPIV4A, HPIV5, SV41, SV5, mumps virus, and NDV. In an embodiment, the PIV is human PIV (HPIV). Preferably, the HPIV is HPIV type 2 (HPIV2). In an embodiment, the HPIV2 is strain V94, V98, or Greer. In an embodiment, the genome or antigenome sequence is derived from a variant HPIV2 comprising at least 80% or greater nucleotide sequence identity with a HPIV2 reference sequence. In an embodiment, the reference sequence is the genomic sequence of HPIV2 of strain V94 (SEQ ID NO:4), V98 (SEQ ID NO:5), or Greer (SEQ ID NO:6). In an embodiment, the genomic sequence comprises a nucleotide sequence of SEQ ID NO:4.

The polynucleotide encoding the monocistronic V protein can be inserted anywhere into the genome, antigenome, or vector. In some embodiments, the polynucleotide encoding the monocistronic V protein may be inserted at the 3' end. In some embodiments, the gene order, for example, may be 3' V- N-P-M-F-HN-L-5'. In other embodiments, the gene order, for example, may be 3' N-V-P-M-F-NH-L-5'. In some embodiments, the monocistronic polynucleotide encoding a protein is inserted into a restriction site in the genome. Restriction sites may include *Asc I, Bst EII, AgeI* or *Sac II* as shown in Figure 5. In an embodiment, a

recombinant virus encoding a separate P and V protein has an antigenomic cDNA sequence of SEQ ID NO:1.

The variant polynucleotide encoding the P protein can be inserted anywhere into the genome or antigenome. In addition, the variant polynucleotide encoding the P protein and the monocistronic polynucleotide encoding the V protein can be located on separate vectors. In some embodiments, the variant polynucleotide encoding a P protein is inserted into a restriction site in the genome or antigenome. Restriction sites may include *Asc I, Bst EII, AgeI* or *Sac II* as shown in Figure 5.

The variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein, optionally, are separated by a non-coding polynucleotide spacer sequence. In an embodiment, the spacer sequence is upstream of a V coding sequence or open reading frame (ORF) in the polynucleotide encoding the V protein. In an embodiment, the spacer sequence comprises a gene end transcription signal, intergenic transcription signal, and/or gene start transcription signal. In an embodiment, the gene start transcription signal is cis-acting. In an embodiment, the gene start transcription signal includes a first adenosine at position 6n+1. In an embodiment, the spacer sequence comprises a nucleotide sequence of SEQ ID NO:7.

When a polynucleotide encoding a monocistronic V protein is inserted into the viral genome, the inserted polynucleotide is a heterologous sequence. Viral recombinant virus comprising a polynucleotide encoding a V protein may have an altered phenotype. In some embodiments, the phenotype may be attenuating. In some embodiments, the phenotype is temperature sensitive. Additional nucleotide changes may be introduced into a recombinant virus encoding separate P and V genes to introduce other phenotypic changes selected from a change in growth characteristics, attenuation, temperature sensitivity, cold adaptation, plaque size, host range restriction, or a change in immunogenicity.

An attenuating phenotype can be temperature sensitive. In an embodiment, a recombinant virus encoding separate P and V proteins is not attenuated for replication at permissive temperature (for example, about 30-32°C) but is attenuated for replication at restrictive temperatures, such as for example 37°C - 40°C. In an embodiment, replication of the paramyxovirus variants is reduced at about 38°C. In some embodiments, the shut-off temperature of the variants is preferably about 38°C, more preferably about 37°C. Preferably, a recombinant virus encoding separate P and V proteins have a lower shutoff temperature than

control paramyxovirus. In some embodiments, the control is a wild type virus. In other embodiments, the control is other attenuated paramyxoviruses.

Preferably, a recombinant paramyxovirus encoding separate P and V proteins is attenuated *in vivo*. In an embodiment, the recombinant virus encoding separate P and V proteins exhibit reduced replication in the upper and/or lower respiratory tract of a mammal as compared to wild-type HPIV2 or other attenuated paramyxoviruses. In an embodiment, the replication is reduced at least about 10 fold, 100 fold, more preferably about 500 fold, more preferably about 1000 fold, more preferably about 1500 fold, more preferably about 2000 fold, more preferably about 3000 fold, more preferably about 4000 fold, more preferably about 5000 fold, more preferably about 6000 fold as compared to wild-type HPIV2 or other attenuated paramyxoviruses. In an embodiment, the mammal is a golden Syrian hamster (*Mesocricetus auratus*). In another embodiment, the mammal is an African green monkey (*Cercopithecus aethiops*). In some embodiments, the attenuated paramyxovirus is immunogenic and elicits sufficient antibodies to protect against infection.

The monocistronic polynucleotide encoding a V protein can encode a V protein having a sequence of a naturally occurring or variant V protein. The V protein can be from a heterologous paramyxovirus, including but not limited to HPIV4A, HPIV4B, SV5, SV41, mumps, NDV, or Sendai virus. In an embodiment, the nucleotide sequence encoding the V protein comprises at least 80% sequence identity, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99% or greater sequence identity to a nucleotide sequence encoding V protein in a reference sequence. In an embodiment, the reference sequence is HPIV2 strain V94 (SEQ ID NO:4; Table 6), HPIV strain V98 (SEQ ID NO:5 Table 7), HPIV2 strain Greer (SEQ ID NO:6; Table 8)or a polynucleotide encoding a V protein comprising an amino acid sequence of SEQ ID NO:45. In an embodiment, the antigenomic cDNA sequence encoding the V ORF is that of SEQ ID NO:3, shown in Figure 12. In some embodiments, an isolated monocistronic nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80 % sequence identity to a V protein having a sequence of SEQ ID NO:45 is provided.

In an embodiment, the nucleotide sequence encoding V protein comprises a mutation that inhibits the ability of V protein to interrupt production or signaling of interferon in an infected host or host cell. Preferably, the mutation does not substantially impact virus replication in cell culture. In an embodiment, the V protein has reduced activity as compared to V protein encoded by HPIV2 strain V94, HPIV2 strain V98, or HPIV2 strain Greer. Preferably the mutation does not affect P protein expression or activity in cell culture.

One of several approaches can be taken to identify mutations in the V protein that render rHPIV2 P+V attenuated for replication in vivo. The first is random mutagenesis of the V ORF to generate viruses that are restricted for replication in the respiratory tract of experimental animals. For example, alanine mutations can be introduced at each position and those positions important in the function of the protein can be identified. In other embodiments, deletions of at least 2 amino acids can be generated. Recombinant viruses bearing these mutations can be characterized in vitro and in vivo.

Alternatively, sequence alignment with heterologous paramyxovirus V proteins can be used as a guide for targeted mutagenesis. For example, there are two ways to use the sequence alignment as a guide. First, conserved sequences, which are likely required for specific V protein activities, can be directly targeted with conservative amino acid substitutions or small (2 amino acid) deletions. A less conservative approach can be taken and unrelated amino acids can be used for amino acid substitutions, or large portions of the conserved regions (6 or more amino acids) can be deleted. Unrelated amino acids may be selected that require at least two nucleotide changes in the codon as compared to the codon encoding the wild type amino acid at that position. The selection of sites for mutagenesis is not limited to conserved sequences.

Substantial modifications in the biological properties of V protein are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet conformation, helical conformation, or loop structure, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: leucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions entail exchanging a member of one of these classes for another class. Such substituted residues also can be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

Examples of candidate sites for point or deletion mutagenesis in V protein are summarized and highlighted in Figure 9A: (I) similarity to a sequence identified in SV5 that is required for RNA binding; (II) similarity to a sequence identified in SV5 that is required for STAT binding; (III) similarity to a sequence identified in SV5 that is required for STAT degradation; (IV) putative leucine (bold font) rich nuclear export signal sequence; (V) highly conserved sequence that may form part of a zinc finger binding domain; (VI) region includes 5 of 7 conserved cysteine residues in the cysteine rich domain (CRD). Figure 9B shows an amino acid sequence comparison of a highly conserved carboxy-terminal cysteine-rich domain, a critical V protein domain known to bind zinc ions, of 22 members of the Paramyxovirus family. Boxed and bolded sequences are highly conserved. Residues indicated with * may directly interact with one or more zinc ions and are targets for amino acid deletion or substitution mutagenesis. In some embodiments, a variant V protein comprises at least one amino acid mutation of a least one residue corresponding to an amino acid residue in at least one of the domains, more preferably in more than one of the domains.

In an embodiment, the V protein comprises one or more amino acid substitutions or deletions at or between residues corresponding to positions 67, 68, 69, 70, 71, 72, 105, 106, 107, 108, 121, 122, 123, 124, 125, 126, 127, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 167, 168, 169, 170, 171, 172, or any one of amino acids 174-225 of SEQ ID NO:4. Preferably, the nucleotide changes that encode a mutation comprise at least two nucleotide changes compared to the codon encoding the wild type amino acid at that position to increase the stability of the mutation. Preferably, the variant V protein has at least 80% sequence identity to that of a reference sequence.

The monocistronic polynucleotide encoding a V protein includes an mRNA editing site. Preferably the editing site includes a heptaguanosine run. In an embodiment, the editing site comprises a nucleotide sequence of SEQ ID NO:8. In an embodiment, the heptaguanosine run is substituted such that editing of nucleotide sequence encoding V protein is inhibited. Nucleotide deletion, insertion, or substitution mutagenesis can be used to inhibit mRNA editing. For example, in V94 HPIV2, mRNA editing of the nucleotide sequence encoding V protein can be inhibited by substituting the nucleotide corresponding to G9 of the editing site

(SEQ ID NO:8) with A and the nucleotide corresponding to G12 of the editing site (SEQ ID NO:8) with C. Nucleotide deletions or insertions must conform to the "rule of six", as described herein and in WO 04027037.

The variant polynucleotide encoding a P protein includes a nucleotide sequence encoding a P protein. The variant polynucleotide encoding the P protein is altered so that it does not encode a V protein. The amino acid sequence of the P protein may also be a variant sequence. The P protein can be from a heterologous paramyxovirus of the Rubulavirus genus, including but not limited to, SV5, SV41, HPIV4A and HPIV4B. In an embodiment, the nucleotide sequence encoding P protein comprises at least 80% sequence identity, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99% or greater sequence identity to a nucleotide sequence encoding P protein in a reference sequence. In an embodiment, the reference sequence is HPIV2 strain V94 (SEQ ID NO:4; Table 6), HPIV2 strain V98 (SEQ ID NO:5; Table 7), or HPIV2 strain Greer (SEQ ID NO:6; Table 8). In an embodiment, an antigenomic cDNA sequence encoding a P protein has a sequence of SEQ ID NO:2.

The variant polynucleotide encoding a P protein includes a P coding sequence or open reading frame (ORF). Preferably the P ORF includes one or more nucleotide substitution(s) that introduce one or more stop codons in an overlapping V ORF reading frame but does not alter the amino acid sequence of P protein encoded by the P ORF. In an embodiment, third base codon substitutions encoding stop codons in the V ORF reading frame at codon positions 167, 176, 184, and/or 186 are introduced into the P ORF (SEQ ID NO:2). In another embodiment, the P ORF comprises a nucleotide sequence of SEQ ID NO:2. In some embodiments, insertion of one or more stop codons in a V ORF may be preferably utilized when it may be undesirable to make modifications to the mRNA editing site in the coding sequence for the P ORF.

The variant polynucleotide encoding a P protein includes an mRNA editing site. Preferably the editing site includes a heptaguanosine run. In an embodiment, the editing site comprises a nucleotide sequence of SEQ ID NO:8. In an embodiment, the heptaguanosine run

is substituted such that editing of the nucleotide sequence encoding P protein is inhibited. Nucleotide deletion, insertion, or substitution mutagenesis can be used to inhibit mRNA editing. Preferably, the insertions and/or substitutions destroy the heptaguanosine stretch in the editing site but do not alter the reading frame encoding the carboxy-terminal portion of P protein. In HPIV2, for example, 2 guanosines are inserted into the mRNA editing site such that the nucleotide sequence encoding the carboxy-terminal portion of P protein is shifted in frame. In an embodiment, the number of guanosines necessary to shift the reading frame to the nucleotide sequence encoding the carboxy terminal portion of P protein are inserted in the mRNA editing site and 2 or more guanosines in the heptaguanosine run are substituted with A or T. See, for example, Figure 6. In another embodiment, A, T, or a combination thereof corresponding to the number of guanosines necessary to shift the reading frame to the nucleotide sequence encoding the carboxy terminal portion of P protein are inserted into the heptaguanosine run of the mRNA editing site. See, for example, Figure 6. Nucleotide deletions or insertions must conform to the "rule of six", as described herein and in WO 04027037, and maintain the correct P ORF reading frame.

A paramyxovirus or polynucleotide of the invention including a variant polynucleotide encoding a P protein and monocistronic polynucleotide encoding a V protein can be made using known recombinant methods such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, PCR mutagenesis, site-directed mutagenesis (Zoller *et al.*, 1987, *Nucl. Acids Res.*, 10: 6487-6500), cassette mutagenesis (Wells *et al.*, 1985, *Gene*, 34:315), restriction selection mutagenesis (Wells *et al.*, 1986, *Philos. Trans. R. Soc. London SerA*, 317:415), and the like.

B. Combinations of Mutations

A paramyxovirus of the invention, including a partial or complete polyhexameric genome or antigenome having a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein, can include any of the attenuating mutations in the L polymerase and/or 3' leader described herein or other known attenuating mutations. Recombinant viral variants having more than one mutation are likely to have increased stability over those variants having a single mutation or a small number of mutations (eg., less than 3 mutations). The paramyxovirus of the invention can also include any of the attenuating L polymerase mutations described in WO 04/027037. Preferably, the attenuating effect of the mutations in the L polymerase and/or 3' leader are additive and serve to further increase the attenuation of the paramyxovirus of the invention including a variant

polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein. In some embodiments, it is desirable to balance the level of attenuation with the immunogenicity. In preferred embodiments, the variant paramyoviruses have about 100 fold to 5000 fold decrease in viral titer in a mammal. In some embodiments, a decrease of viral replication greater than about 100,000 fold may result in a loss of immunogenicity or an inability to produce the virus on a large scale.

In some embodiments, the variant paramyxoviruses of the invention have at least one temperature sensitive mutation and at least one non-temperature senstitive mutation. In a preferred embodiment, the recombinant variant paramyxoviruses of the invention have at least one temperature sensitive mutation due to insertion of monocistronic polynucleotide encoding a V gene or a mutation in a polynucleotide encoding a L polymerase, wherein the change in the amino acid is due to at least two nucleotide changes to the codon encoding the wild type amino acid. In another embodiment, the recombinant variant paramyxoviruses of the invention comprise a mutation that provides a host range restriction phenotype. In a preferred embodiment, the variant virus having a host range restriction comprises a mutation at position 15 of the 3' terminus of the viral genome.

C. Vectors including heterologous antigens

The paramyxoviruses of the invention are also useful as vectors for expressing heterologous antigens in an immunogenic composition. One or more supernumerary genes encoding one or more heterologous polypeptides can be cloned into and expressed by the paramyxovirus of the invention. For example, an immune response against multiple PIV serotypes or strains can be elicited by engineering protective epitopes of multiple PIV serotypes and strains into a single paramyxovirus. The supernumerary genes can be cloned and expressed in a recombinant virus encoding a separate P and V proteins as described herein, as well as recombinant virus comprising one or more mutations in L polymerase and/or a mutation in the 3' leader region. Insertion of additional heterologous genes may also result in an attenuated phenotype. Preferably, the paramyxovirus comprising a polynucleotide encoding a heterologous gene is attenuated about 100 to 5000 fold or more in a cell or mammal.

In an embodiment, the genome or antigenome includes one or more heterologous genes or genome segments encoding one or more antigenic determinants of a heterologous pathogen. For example, one or more heterologous antigenic determinant(s) from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1

and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses, human metapneumovituses, or influenza viruses can be expressed by the paramyxovirus of the invention. Examples of useful antigenic determinants include, but are not limited to, measles virus HA and F proteins, subgroup A or subgroup B respiratory syncytial virus F, G, SH and M2 proteins, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein, filovirus G protein, bunyavirus G protein, flavivirus pre E, and NS1 proteins, human metapneuomovirus (HMPV) G and F proteins, and alphavirus E protein, and antigenic domains, fragments and epitopes thereof.

In an embodiment, a polynucleotide encoding an open reading frame (ORF) of a measles virus HA gene is incorporated into a HPIV2 vector genome or antigenome to yield a chimeric candidate useful to immunize against measles and/or HPIV2 or another HPIV. In another embodiment, a polynucleotide comprising genes or genome segments encoding one or more heterologous PIV(s) *(e.g.,* HPIV1, HPIV3, and/or HPIV4) HPIV2 N, P, V, F, HN and/or L protein(s) or fragment(s) thereof is incorporated into a HPIV2 vector genome or antigenome. In another embodiment, one or more supernumerary heterologous gene(s) or genome segment(s) selected from HPIVl HN, HPIV2 F, HPlV3 HN, HPIV3 F, measles HA and F, HMPV G and F proteins, and/or RSV subgroup A or B G and F proteins are cloned into a paramyxovirus of the invention.

Some methods of inserting one or more supernumerary genes or transcriptional units into a paramyxovirus viral genome or antigenome are described in WO04/027037, hereby incorporated by reference. Supernumerary heterologous gene(s) or genome segment(s) can be inserted at various sites within the recombinant genome or antigenome, for example at a position 3' to N, between the N/P, P/M, and/or HN/L genes, or at another intergenic junction or non-coding region of a HPIV2 vector genome or antigenome. Preferably, the heterologous or supernumery gene or transcriptional unit is inserted at a restriction site, for example, *AscI, BstEII, AgeI,* or *SacII* as shown in Figure 5C. Any insertions or deletions of the viral genome, preferably, conform to the rule of six.

D. Recombinant Methods, Vectors, and Host Cells

The infectious paramyxoviruses and polynucloetides of the invention are produced by synthetic and recombinant methods. Accordingly, the invention relates to polynucleotides encoding infectious paramyxovirus clones of the invention and host cells including the infectious clone, as well as methods of making such vectors and host cells by recombinant methods.

The paramyxovirus or polynucleotides of the invention may be synthesized or prepared by techniques well known in the art. See, for example, WO 94/027037. Nucleotide sequences for wild type paramyxovirus genomes are known and readily available, for example, on the Internet at GenBank (accessible at www-ncbi-nlm-nihgov/entrez). The nucleotide sequences encoding the paramyxovirus of the invention may be synthesized or amplified using methods known to those of ordinary skill in the art including utilizing DNA polymerases in a cell free environment.

Amino acid substitutions, insertions, and deletions can be made using known recombinant methods such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, PCR mutagenesis, site-directed mutagenesis (Zoller *et al.*, 1987, *Nucl. Acids Res.*, 10: 6487-6500), cassette mutagenesis (Wells *et al.*, 1985, *Gene*, 34:315), restriction selection mutagenesis (Wells *et al.*, 1986, *Philos. Trans. R. Soc. London SerA*, 317:415), and the like. Examples of PCR primers suitable for use in generating the attenuating mutations of the invention are described in Examples 1, 2, and 3

The paramyxovirus of the invention can be produced from virus isolated from biological samples. The polynucleotides and vectors may be produced by standard recombinant methods known in the art, such as polymerase chain reaction (Sambrook, et al., 1989, Molecular Cloning, A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY). Methods of altering or modifying nucleic acid sequences are also known to those of skill in the art.

The paramyxovirus genome may be assembled from polymerase chain reaction cassettes sequentially cloned into a vector including a selectable marker for propagation in a host. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria.

The polynucleotide may be inserted into a replicable vector for cloning using standard recombinant methods. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid

sequence may be inserted into the vector by a variety of procedures. In general, a nucleic acid is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors including one or more of these components employs standard ligation techniques that are known to the skilled artisan.

Examples of suitable replicable vectors include, without limitation, pUC19 or pTM1. The polynucleotide can be operably linked to an appropriate promoter such as, for example, T7 polymerase promoter, cytomegalovirus promoter, cellular polymerase II promoter, or SP1 promoter. The replicable vectors may further include sites for transcription initiation, transcription termination, and a ribosome binding site for translation.

In an embodiment, a paramyxovirus of the invention including a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein are cloned by introducing unique restriction enzyme recognition sequences into paramyxovirus cDNA such that the recognition sequences flank the bicistronic polynucleotide encoding the P/V proteins, digesting the genome with one or more restriction enzymes that cut the genome at the restriction sites flanking the bicistronic polynucleotide encoding P/V proteins, inserting the variant polynucleotide encoding a P protein and /or monocistronic polynucleotide encoding a V protein at the cleaved restriction sites, and religating the genome. Examples of suitable restriction enzyme recognition sequences, include but are not limited to, *NotI, AscI, BstEII, AgeI,* and *Sac II.* In some embodiments, the restrictions sites are introduced into the non-coding regions upstream or downstream of the bicistronic P/V ORFs. In an embodiment, an *AscI* site is upstream of the bicistronic P/V ORFs and *BstEII, AgeI,* and *SacII* sites are downstream of the bicistronic P/V ORFs (Figures 5A and 5B). In an embodiment, the variant polynucleotide encoding a P protein is introduced into the genome using the *AscI* and *BstEII* restriction sites. In an embodiment, the monocistronic polynucleotide encoding a V protein is introduced into the genome using the *AgeI* and *SacII* restrictions sites.

Introduction of a recombinant vector composed of a paramyxovirus genome or polynucleotide encoding a paramyxovirus protein into a host cell, such as for example a bacterial cell or eukaryotic cell, can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, electrical nuclear transport, chemical transduction, electrotransduction, infection, or other methods. Such methods are described in standard laboratory manuals such as Sambrook, et al., 1989,

Molecular Cloning, A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY or Davis et al., 1986, *Basic Methods in Molecular Biology*. Commercial transfection reagents, such as Lipofectamine (Invitrogen, Carlsbad, CA) and FuGENE 6™ (Roche Diagnostics, Indianapolis, IN), are also available. In some embodiments, transfection efficiency of the host cells is about 15% or greater, about 20% or greater, about 30% or greater, about 40% or greater, or about 50% or greater. Suitable host cells include, but are not limited to, HEp-2 cells, FRhL-DBS2 cells, LLC-MK2 cells, MRC-5 cells, and Vero cells.

E. Immunogenic compositions

The invention provides isolated, infectious, recombinant paramyxovirus including one or more attenuating mutations for use in immunogenic compositions, including live attenuated virus vaccines. The paramyxoviruses of the invention are useful in immunogenic compositions for eliciting an immune response in a mammal. Preferably, the attenuated paramyxovirus includes a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein. In an embodiment, the V gene comprises a nucleotide sequence encoding a V protein having a mutation that inhibits the ability of the V protein to interrupt production and /or signaling of interferon in an infected host. In an embodiment, the attenuated virus is HPIV2.

Recombinant HPIV2 of the invention can be combined with viruses of other PIV serotypes or strains and paramyxoviruses from multiple genera in a composition to elicit an immune response against multiple genera, serotypes, and strains. The immunogenic composition can comprise paramyxoviruses from two or more serotypes. In an embodiment, at least one of the serotypes is HPIV1, HPIV2, HPIV3, or HPIV4. The immunogenic composition can comprise paramyxovirus from two or more strains. In an embodiment, at least one of the strains is an HPIV2 strain, such as for example, V94, V98, or Greer. The immunogenic composition can comprise paramyxovirus from two of more genera. In an embodiment, one genus is Rubulavirus genus.

The paramyxoviruses of the invention are also useful as vectors for expressing heterologous antigens in an immunogenic composition. One or more supernumerary genes encoding one or more heterologous polypeptides can be cloned into and expressed by the paramyxovirus of the invention. For example, an immune response against multiple PIV serotypes or strains can be elicited by engineering protective epitopes of multiple PIV serotypes and strains into a single paramyxovirus. In an embodiment, the genome or antigenome includes

one or more heterologous genes or genome segments encoding one or more antigenic determinants of a heterologous pathogen.

For example, one or more heterologous antigenic determinant(s) from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses, human metapneumovituses, or influenza viruses can be expressed by the paramyxovirus of the invention. Examples of useful antigenic determinants include, but are not limited to, measles virus HA and F proteins, subgroup A or subgroup B respiratory syncytial virus F, G, SH and M2 proteins, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein, filovirus G protein, bunyavirus G protein, flavivirus pre E, and NS1 proteins, human metapneuomovirus (HMPV) G and F proteins, and alphavirus E protein, and antigenic domains, fragments and epitopes thereof.

In an embodiment, a polynucleotide encoding an open reading frame (ORF) of a measles virus HA gene is incorporated into a HPIV2 vector genome or antigenome to yield a chimeric candidate useful to immunize against measles and/or HPIV2 or another HPIV. In another embodiment, a polynucleotide comprising genes or genome segments encoding one or more heterologous PIV(s) *(e.g.,* HPIV1, HPIV3, and/or HPIV4) HPIV2 N, P, V, F, HN and/or L protein(s) or fragment(s) thereof is incorporated into a HPIV2 vector genome or antigenome. In another embodiment, one or more supernumerary heterologous gene(s) or genome segment(s) selected from HPIVl HN, HPIV2 F, HPIV3 HN, HPIV3 F, measles HA and F, HMPV G and F proteins, and/or RSV subgroup A or B G and F proteins are cloned into a paramyxovirus of the invention.

Some methods of inserting one or more supernumerary genes or transcriptional units into a paramyxovirus viral genome or antigenome are described in WO04/027037, hereby incorporated by reference. Supernumerary heterologous gene(s) or genome segment(s) can be inserted at various sites within the recombinant genome or antigenome, for example at a position 3' to N, between the N/P, P/M, and/or HN/L genes, or at another intergenic junction or non-coding region of a HPIV2 vector genome or antigenome.

Candidate viruses for use in an immunogenic composition, such as for example a vaccine, are selected based on their attenuation and immunogenicity. These vaccine selection criteria are determined according to well-known methods. Preferably, candidate viruses have a stable attenuation phenotype, exhibit replication in an immunized host, and effectively elicit production of an immune response in a recipient, preferably a protective immune response. Preferably, the candidate viruses stimulate and expand the immune response, e.g., induce an immune response against different viral strains or subgroups and/or stimulate an immune response mediated by a different immunologic basis (e.g. secretory versus serum immunoglobulins, cellular immunity, and the like).

Recombinant paramyxoviruses of the invention can be tested in well-known and *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity. In *in vitro* assays, the modified virus paramyxovirus of the invention is tested for one or more desired phenotypes, such as, for example, temperature sensitive replication. Paramyxovirus of the invention can also be tested in animal models of PIV infection. A variety of animal models are known. For example, PIV model systems, including rodents and non-human primates, for evaluating attenuation and immunogenic activity of PIV vaccine candidates, are known, and the data obtained therefrom are known to correlate with PIV infection, attenuation, and immunogenicity in humans.

In some embodiments, recombinant variant paramyxoviruses have at least one attenuating mutation with a ts phenotype and at least one mutation with a non-ts phenotype. The recombinant attenuated paramyxoviruses are preferably attenuated about 100 to 5000 fold in a cell or mammal compared to wild type paramyxovirus. In some embodiments, attenuation of greater than 100,000 fold may result in reduced immunogenicity. In some embodiments, it is preferred that the level of viral replication in vitro is sufficient to provide for production of viral vaccine for use on a wide spread scale. In some embodiments, it is preferred that the level of viral replication of attenuated paramyxovirus in vitro is at least 10^6, more preferably at least 10^7, and most preferably at least 10^8 per ml. The attenuating mutation is preferably one that is stable. For example, for mutations in L polymerase, it is preferable that a change in amino acid at a position requires at least two nucleotide changes in the codon as compared to the codon encoding the wild type amino acid at that position. A recombinant paramyxovirus with at least two, three, four or ever more attenuating mutations is likely to be more stable. Insertion of a supernumerary gene whose total length conforms to the rule of six, such as a polynucleotide encoding a separate V gene, can also provide a stable phenotype.

Immunogenicity of a recombinant attenuated paramyxovirus can be assessed in an animal model by determining the number of animals that form antibodies to the paramyxovirus after one immunization and after a second immunization. In some embodiments, a recombinant paramyxovirus has sufficient immunogenicity if about 60 to 80 % of the animals develop antibodies after the first immunization and about 80 to 100% of the animals develop antibodies after the second immunization. The preferred animal for a determination of immunogenicity is African green monkey. Preferably, the immune response protects against infection with a paramyxovirus of the same strain or multiple strains.

The invention also provides for immunogenic compositions comprising isolated polynucleotides or polypeptides of the invention. For example, an immunogenic composition can include a polynucleotide encoding a polypeptide that has at least 80% sequence identity to a V polypeptide having a sequence of SEQ ID NO:45 or a polypeptide having a sequence at least 80% sequence identity to a V polypeptide having an sequence of SEQ ID NO:45. In other embodiments, an immunogenic composition can include a polynucleotide encoding a polypeptide that has at least 80% sequence identity to a L polypeptide having a sequence of SEQ ID NO:17 or a polypeptide having a sequence at least 80% sequence identity to a L polypeptide having an sequence of SEQ ID NO:17. In other embodiments, an immunogenic compositions can include a nucleic acid comprising a polynucleotide of SEQ ID NO:2.

Recombinant paramyxoviruses of the invention are preferably present in the immunogenic composition in an immunogenic effective amount. An immunogenic effective amount is an amount of recombinant paramyxovirus that induces an immune response in an animal. The actual amount of the recombinant paramyxovirus may vary depending on the animal to be immunized, the route of administration and adjuvants. The actual amount of recombinant paramyxovirus necessary to elicit an immune response, and the timing and repetition of administration, can be determined using conventional methods based on the state of health and weight of the host, mode of administration, nature of formulation, etc. Immunogenic dosages can be determined by those of skill in the art. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^6 PFU virus per host. In any event, the formulations should provide a quantity of attenuated recombinant paramyxovirus of the invention sufficient to effectively stimulate or induce an anti-PIV or other anti-pathogenic immune response.

The immune response may be indicated by T and/or B cell responses. Typically, the immune response is detected by the presence of antibodies that specifically bind to a particular

antigen. Methods of detecting antibodies to a particular antigen are known to those of skill in the art and include such assays as ELISA assays, western blot assays, hemagglutination-inhibition assays, and infectivity neutralization assays. Host receiving immunogenic compositions of the invention are preferably monitored for signs and symptoms of upper and lower respiratory tract illness. Preferably, attenuated virus administered intranasally grows in the nasopharynx of recipients at levels about 10-fold or more lower than wild-type virus, or about 10-fold or more lower when compared to levels of incompletely attenuated virus.

In neonates and infants, multiple administrations may be required to elicit sufficient levels of immunity. Administration could begin within the first month of life, and at intervals throughout the first several years of childhood, such as at two months, six months, one year and two years, as necessary to maintain an immune response against native (wild-type) PIV infection. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or immunizations repeated as necessary to maintain desired levels of immune response.

Recombinant paramyxoviruses, polynucleotides, and polypeptides of the invention can be used directly in formulations, or lyophilized, as desired, using well known methods. Lyophilized virus is typically maintained at about 4°C. When ready for use, the lyophilized virus is reconstituted in an appropriate stabilizing solution. Many stabilizing solutions are known. Immunogenic compositions including paramyxovirus of the invention can include a physiologically acceptable carrier and/or adjuvant. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™. Lyophilized preparations are generally combined with a sterile solution prior to administration.

The compositions may include pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, such as for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolarnine oleate, and the like. Acceptable adjuvants include, but are not limited to, Freund's adjuvant (incomplete or complete), MPLTM (3-0-deacylated monophosphoryl lipid A; Corixa, Hamilton IN) and IL-12 (Genetics Institute, Cambridge MA), CpG oligonucleotides, immunostimulating compositions and alum salts.

The immunogenic compositions of the invention can be administered nasally in droplet, aerosol, or nebulizer form, orally, or parentally, including subcutaneous injection, intravenous, intramuscular, intrasternal or infusion techniques, in dosage unit formulations including conventional non-toxic pharmaceutically acceptable carriers, adjuvants or vehicles. Compositions of the invention can be in the form of suspensions or tablets suitable for oral administration or sterile injectable preparations, such as sterile injectable aqueous or oleagenous suspensions.

For administration as injectable solutions or suspensions, the immunogenic compositions of the invention can be formulated according to techniques well-known in the art, using suitable dispersing or wetting and suspending agents, such as sterile oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

Immunization by the nasal route may be more effective compared with intramuscular or subcutaneous injection because the production of local secretory IgA in the upper respiratory tract can protect against PIV infection. For example, PIV specific secretory IgA can show a broader cross-reactivity for variant strains of PIV and thus may offer a greater degree of protection against mutant PIV. In contrast, injectable vaccines are inefficient at inducing mucosal IgA. In particular, nasal administration of the immunogenic compositions of the invention may be more effective in the elderly since, unlike the systemic immune system, mucosal immune responses do not deteriorate with age. Immunogenic compositions of the invention that also stimulate systemic immune responses may protect the lower respiratory tract (lungs) due to transudation of antibodies from the serum. In addition, PIV-specific cytotoxic T cells (CTL) in nasal associated lymphoid tissue can contribute to recovery from infection.

Immunogenic compositions for nasal administration are preferably formulated so that they are similar to nasal secretions in regard to toxicity, pH, and viscosity so that normal ciliary action is maintained. In an embodiment, the immunogenic compositions of the invention are

formulated in an aqueous solution that is isotonic and slightly buffered to maintain a pH of about 5.5 to about 6.5. Antimicrobial preservatives and appropriate stabilizers, if required, are included in the formulation.

F. Methods of use

The invention also provides methods of making and using the recombinant paramyxovirus of the invention. In one embodiment, the invention relates to methods of generating an infectious, self-replicating paramyxovirus as have been described herein. The methods generally include the steps of removing or altering a bicistronic polynucleotide encoding a P and V protein from the viral genome or antigenome of a paramyxovirus and inserting a variant polynucleotide encoding a P protein and/or a monocistronic polynucleotide encoding a V protein. In an alternative embodiment, the variant polynucleotide encoding the P gene in the paramyxovirus can be altered in situ to no longer encode the V protein using the standard methods and the monocistronic polynucleotide encoding the V protein can be inserted into the paramyxovirus genome or antigenome. Preferably, the variant polynucleotide encoding a P protein includes a mutated mRNA editing site such that editing of mRNA encoding P protein is inhibited and the monocistronic polynucleotide encoding a V protein includes a mutated mRNA editing site such that editing of mRNA encoding V protein is inhibited. More preferably, the V protein has at least one mutation that reduces the ability of the V protein to inhibit production and/ or signaling of interferon.

In an embodiment, the removing step includes introducing unique restriction enzyme recognition sequences into the genome or antigenome such that the recognition sequences flank the bicistronic polynucleotide encoding P and V proteins and digesting the genome or antigenome with one or more restriction enzymes that cut the genome or antigenome at the restriction sites flanking the bicistronic polynucleotide. In an embodiment, the inserting step includes inserting the variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein at the cleaved restriction sites and religating the genome or antigenome.

In another embodiment, the invention relates to methods of producing infectious, self-replicating, recombinant paramyxovirus. The methods of the invention include transfecting a population of cells with an expression vector comprising a partial or complete polyhexameric genome or antigenome sequence and one or more supporting vectors including one or more polynucleotides encoding N protein, P protein, and L polymerase, and incubating the

transfected cells under conditions to allow for viral replication. Preferably, the paramyxoviruses have a variant polynucleotide encoding a P protein and/or a monocistronic polynucleotide encoding a V protein.

In another embodiment, the invention relates to methods of eliciting an immune response in a mammal. The methods of the invention include administering an immunogenic composition of the invention. Preferably, the immune response produces antibodies that are protective (e.g. inhibit infection or reduce the severity of infection). In an embodiment, the antibodies are anti-PIV antibodies. In an embodiment, the anti-PIV antibodies are IgA. In some embodiments, the immune response produces antibodies that bind one or more antigenic determinants of a heterologous pathogen encoded by supernumerary genes or genome segments. Examples of heterologous pathogens include, but are not limited to, HPIV1, HPIV3, measles virus, subgroup A or subgroup B respiratory syncytial virus, mumps virus, human papilloma virus, type 1 or type 2 human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rabies virus, Epstein Barr virus, filovirus, bunyavirus, flavivirus, alphavirus, human metapneumovirus, or influenza virus. In an embodiment, the antigenic determinants include measles virus HA, HPIV1 HN, and/or HPIV3 HN.

In another embodiment, the invention relates to methods of inhibiting a paramyxovirus infection including, but not limited to, PIV infection. The methods of the invention include administering an immunogenic composition of the invention comprising an attenuated paramyxovirus of the invention. Preferably the paramyxovirus of the invention includes a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein. In an embodiment, the paramyxovirus is PIV. In an embodiment, the PIV is HPIV2. Preferably the immunogenic composition elicits antibodies that are protective (e.g. inhibit infection or reduce the severity of infection). In an embodiment, the antibodies are anti-PIV antibodies. In an embodiment, the anti-PIV antibodies are IgA.

All publications, patents and patent applications are hereby incorporated by reference. The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

Example 1

Level of attenuation and stability conferred by L protein mutations enhanced by alternative codon substitution

Attenuated HPIV1 and HPIV2 vaccine candidates were previously generated by amino acid substitutions at loci in the L protein (WO04/027037; (McAuliffe et al., 2004)). In this example, the level of attenuation conferred by alternative codon substitutions at mutant loci in the L protein was studied. Codon substitution mutations were designed so that at least two nucleotide changes would be required to regenerate a codon specifying the wild type amino acid at each position, thereby reducing the chance of spontaneous reversion to a codon specifying the wild type amino acid. For example, taking the commonly cited value of 10^{-4} for the mutation rate for a RNA virus, reversion that requires a single nucleotide substitution would occur at a frequency of $\sim 10^{-4}$ whereas reversion requiring two or three substitutions would occur at the greatly reduced frequencies of $\sim 10^{-8}$ and $\sim 10^{-12}$, respectively.

Material and Methods

Preparation of plasmids and generation of mutants

HPIV2 V94 strain antigenomic sense cDNA (SEQ ID NO:4), which is 15,654 nucleotides in length and conforms to the "rule of six" (Calain and Roux, 1993; Kolakofsky et al., 1998; Vulliemoz and Roux, 2001), was modified by PCR mutagenesis and standard molecular cloning techniques to include a *Not*I site (GCGGCCGC (SEQ ID NO:11)) at nucleotide sequence positions 149 to 156 in the HPIV2 genome, which is within the N gene and upstream of the N ORF. The *Not*I restriction site was introduced to aid in subsequent cloning steps and as a site for the subsequent insertion of supernumerary genes. Unless otherwise noted, each of the recombinant mutant HPIV2s described includes this *Not*I site.

The original biologically derived wild-type clinical HPIV2 isolate is designated V94 (SEQ ID NO:4); its recombinant wild-type counterpart lacking the *Not*I site is designated rV94 (SEQ ID NO:12), and the version including the *Not*I site is designated rV94Not (SEQ ID NO:13). Wild-type and recombinant HPIV2 were assembled and recovered as described in WO04/027037 and Skiadopoulos et al., 2003, *J. Virol.*, 77:270-279.

Four L protein mutations conferring *ts* attenuation (*att*) phenotypes were previously identified in several heterologous paramyxoviruses (WO04/027037). These mutations were imported into HPIV2 as follows: F460L, imported from the RSV*cpts*530 L protein, F521L (Juhasz et al., 1999; Whitehead et al., 1999; Skiadopoulos et al., 1999c; Juhasz, Murphy, and

Collins, 1999)); Y948H and L1566I, imported from the HPIV3 *cp*45 L protein, Y942H and L1558I, respectively (Skiadopoulos et al., 1998; Skiadopoulos et al., 1999a)); and S1724I, imported from the bovine PIV3 (BPIV3) L protein, T1711I (Skiadopoulos et al., 2003a).

In the present example, the L protein mutations were designed to involve more than one nucleotide change including two mutations at the codons specifying amino acid position 460 (F460A and F460P) and three at amino acid position 948 (Y948A, Y948G, and Y948L). A codon substitution at position 1724 (S1724I) specifying the mutation originally identified in the BPIV3 L ORF (T1711I) was also generated, although this mutant codon differed from wild type by only a single nucleotide substitution. The codons at positions 1724 and 1725 were both deleted in order to maintain a polyhexameric genome length, i.e. to conform the genome length to the rule of six. Thus, six mutations, including novel mutations at codon positions encoding amino acids at positions 460, 948, 1566, 1724, and 1725, were generated by PCR mutagenesis (Moeller et al., 2001) and standard molecular cloning techniques (Skiadopoulos et al., 2003, *J. Virol.*, 77:270-279; Newman et al., 2004) using mutagenic PCR primers designed to achieve the nucleotide sequence indicated in Table 2.

Table 2 summarizes the substitution mutations introduced into the L ORF of HPIV2. Mutants bearing a mutation in the L protein were designated by the amino acid substitution generated (e.g., rF460A, recombinant V94 with the F-460 to A mutation).

Table 2

Virus	Amino acid		Codon		Number of nucleotide changes to revert to wild type[a]
	Wild type	Mutant	Wild type	Mutant	
rF460L[b]	Phe	Leu	TTT	CTG	2
rF460A[c]		Ala	TTT	GCA	3
rF460P[c]		Pro	TTT	CCA	3
rY948H[b]	Tyr	His	TAC	CAC	1
rY948A[c]		Ala	TAC	GCA	3
rY948L[c]		Leu	TAC	CTA	3
rY948G[c]		Gly	TAC	GGA	3
rL1566I[b]	Leu	Ile	TTG	ATC	1
rS1724I[b, c]	Ser	Ile	TCT	ATT	1
r1724del	Ser-Thr	deletion	TCT-ACT	deletion	6[d]

a. Number of nucleotide changes required to revert the codon to any possible codon specifying the indicated wild type amino acid.
b. Original imported mutations corresponding the RSV L protein F521L, HPIV3 L protein Y942H and L1558I, or BPIV3 L protein T1711I mutations.
c. Novel recombinant HPIV2 mutants.
d. Six nucleotides would need to be inserted to restore the codons encoding Ser and Thr.

Additional codon substitution mutations not shown in Table 2 were introduced into the HPIV2 full-length antigenomic cDNA, but rHPIV2 could not be recovered from these constructs after 1 or 2 attempts, suggesting that these mutations may specify a lethal phenotype. These mutations included Ala-998 to Phe or Cys, Leu-1566 to Ala, Gly, Lys, and Asn, and Tyr-948 to Thr.

rV94Not comprising the indicated L gene mutations (Table 2) was recovered from cDNA using a reverse genetics system that employed a full length HPIV2/rV94Not plasmid and three HPIV2 support plasmids as described in WO04/027037 and below.

A support plasmid encoding the N protein of HPIV2/V94 (pTM-N2) was derived from vRNA using the Thermoscript RT-PCR System (Invitrogen, Inc.) and the Advantage-HF PCR kit (Clontech) using an antigenomic sense oligonucleotide that included an *Afl III* site spanning the N ORF ATG translation initiation codon site and an anti-sense oligo including an *Eco*RI site. The PCR product was digested with *Afl* III and *Eco*RI and cloned into pTMl (Durbin etal., Virology 235: 323-332,1997 ; Durbin et al., Virology 234: 74-83,1997 ;Elroy- Stein et al. , Proc. Natl. Acad. Sci. USA. 86: 6126-30,1989), that was digested with Nco I and EcoRI.

A support plasmid encoding the P protein of HPIV2/V94 (pTM-P2) was generated from two overlapping PCR fragments (Moeller et al., J. Virol. 75: 7612-20, 2001, incorporated herein by reference) and engineered to include a two guanosine nucleotide insertion within the HPIV2 P gene editing site (nt 2481-2487) to generate the complete P ORF (as distinguished thereby from the V ORF) which was subcloned into pTMl as an Nco I to EcoRI fragment.

A support plasmid encoding the L polymerase of HPIV2 (pTM-L2) was made by PCR amplification with a sense oligo including an *Nco I* site spanning the L gene ATG translation initiation codon, and an antisense oligo downstream of a unique *Aat II* site (nt 10342) in the L ORF. The remainder of the L ORF was derived from a subclone used to construct the HPIV2 full-length clone. The PCR product was digested with *Asp718* and *Aat II* and was cloned into a pUC19 plasmid including the HPIV2 nts 10342 to 15654 followed by the unique extragenomic *Rsr II* site. The complete HPIV2/V94 L ORF was then subcloned into a modified pTMl as an *Nco I* to *Rsr II* fragment.

HEp-2 cells (ATCC CCL 23) in 6-well plates (Costar, Coming Inc., Coming, NY) were co-transfected with a cDNA plasmid encoding the mutant HPIV2 of the invention and the three HPIV2 support plasmids (pTM-N2, pTM-P2, pTM-L2), using Lipofectamine-2000 reagent (Invitrogen,Inc.). The HEp-2 cells were simultaneously infected with MVA-T7 as described previously (Durbin etal., Virology 235: 323-332,1997 ; Schmidt et al. , J. Virol. 74:_8922-9,2000). Supernatant was harvested on day three or four post-transfection and was passaged two times on LLC-MK2 (ATCC CCL 7.1) monolayers.

To confirm that viruses were derived from cDNA, rather than representing contamination by biologically derived virus, RT was performed and segments of the viral genome were amplified by PCR. Sequence analysis of the PCR products revealed the presence of the two point mutations that are present in the F and L genes of the recombinant virus, designated rHPIV2/V94, but that are not present in the wild type parental virus. Each rHPIV2/V94 was then cloned by plaque to plaque purification on LLC-MK2 monolayers and

passaged 6 to 8 times on LLC-MK2 cells using standard techniques (Skiadopoulos et al., 1999, Virology, 260:125-35).

The HEp-2 and LLC-MK2 cells were maintained in OptiMEM I (LifeTechnologies, Gaithersburg, MD) supplemented with 5% FBS and gentamicin sulfate (50 ug/mL). Recombinant and biologically derived HPIV2s were propagated in LLC-MK2 cells and were quantified by limiting dilution with virus-infected cultures identified by hemadsorption with guinea pig erythrocytes using standard techniques (Hall et al., 1992, Virus Res., 22:173-184).

Replication of recombinant and mutant rHPIV2 in vitro

Replication of recombinant wild type and mutant rHPIV2 at permissive (32°C) and restrictive (37-40°C) temperatures was tested *in vitro*. Recombinant or biologically derived HPIV2 was inoculated in triplicate onto LLC-MK2 cell monolayers in six-well plates at a multiplicity of infection of 0.01, and cultures were incubated at 32°C with and without 5 μg of porcine trypsin/ml added to the culture medium (Skiadopoulos et al., 1999, *Vaccine*, 18:503-510). Medium (0.5 ml) from each well was harvested and replaced with 0.5 ml of fresh medium at 0 h and at 1 to 6 days postinfection. Virus present in the samples was quantified by titration on LLC-MK2 monolayers in 96-well plates that were incubated for 6 days at permissive (32°C) or restrictive (37-40°C) temperatures (Skiadopoulos et al., 1999, *Vaccine*, 18:503-510). Virus grown in the presence of trypsin was titered with trypsin in the medium. Virus was detected by hemadsorption with guinea pig erythrocytes (Skiadopoulos et al., 1999, *Vaccine*, 18:503-510).

Replication of HPIV2 in vitro (Multi-cycle growth curves)

Recombinant or biologically derived HPIV2 was inoculated in triplicate onto LLC-MK2 cell monolayers in 6-well plates at a multiplicity of infection (m.o.i.) of 0.01, and cultures were incubated at 32°C. 0.5 ml of medium from each well was harvested and replaced with 0.5 ml of fresh medium at 0 hr and at 1 to 7 days post-infection. Virus present in the samples was quantified by titration on LLC-MK2 monolayers in 96-well plates that were incubated for 6-7 days at 32°C. Virus was detected by hemadsorption and the titer is reported as $\log_{10}TCID_{50}$/ml (50% tissue culture infectious dose/ml).

Replication of Recombinant HPIV2 L Protein Mutants *In Vivo*

Golden Syrian hamsters (*Mesocricetus auratus*) have been demonstrated to be an appropriate small animal model for evaluating the level of replication of human parainfluenza

viruses. Therefore, the level of replication of wild type and recombinant mutant HPIV2 in the upper and lower respiratory tract of hamsters was examined, as described previously (Skiadopoulos et al., 2003b). Briefly, four week-old Golden Syrian hamsters (Charles River Laboratories, NY) in groups of 6 were inoculated intranasally (IN) with 0.1 ml of L15 medium including $10^{6.0}$ $TCID_{50}$ of HPIV2. On day 4 post-infection, the lungs and nasal turbinates were harvested, and the virus was quantified by serial dilution of tissue homogenates on LLC-MK2 monolayers, as previously described (Newman et al., 2002). The mean virus titer was calculated for each group of hamsters and is expressed as $\log_{10}$ $TCID_{50}$ per gram of tissue.

African green monkeys (*Cercopithecus aethiops*) have been previously demonstrated to be an appropriate non-human primate animal model for HPIV2 replication (Durbin, Elkins, and Murphy, 2000). Monkeys that lacked serum antibodies to HPIV2 (hemagglutination inhibition (HAI) titer of ≤ 1 $\log_2$) were inoculated simultaneously by the IN and intratracheal (IT) routes using a one ml inoculum per site including 10^6 $TCID_{50}$ of virus in L15 medium, as described previously (Durbin, Elkins, and Murphy, 2000). Nasopharyngeal (NP) swab samples were collected on days 1-10, and tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10 post-infection. Virus present in NP and TL specimens was quantified by titration on LLC-MK2 cell monolayers, and the mean peak virus titer obtained was expressed as $\log_{10}$ $TCID_{50}$/ml. The monkeys were challenged IN and IT with one ml per site including 10^6 $TCID_{50}$ of the biologically-derived V94 HPIV2 strain 28 or 31 days after the first infection, and NP and TL samples were collected on days 2, 4, 6, 8 and 10 post-challenge. HPIV2 present in the samples was quantified as described above. Serum samples were collected to determine the serum antibody titer before immunization (on day 0), post-immunization (on day 28 or 31), and 28 days post-challenge (day 56 or 59 post first infection).

Results and Discussion

The L protein mutants shown in Table 2 were found to grow to high titer at 32°C on LLC-MK2 cell monolayers ($\geq 10^{6.8}$ $\log_{10}$ $TCID_{50}$/ml), indicating the mutations in the L protein are not attenuating for replication at permissive temperature (32°C) *in vitro* (Table 3). The shut–off temperature of a rHPIV2 mutant is defined as the lowest temperature at which the reduction in virus titer compared to its titer at 32°C was 100-fold greater than the difference of HPIV2/V94 between the same two temperatures.

Mutant recombinants rF460A, rF460P, rY948A, rY948G, rY948L, rS1724I, and rdel1724 were designated as having a *ts* phenotype (Table 3).

Table 3

Virus [a]	Mean titer at 32°C	Mean log_{10} reduction in virus titer at the indicated temperature (°C)[b]			
		37	38	39	40
V94	7.2	0.0	0.3	0.4	0.5
rV94Not	7.5	-0.2	-0.1	0.3	0.5
rV94	7.8	nd[c]	-0.1	0.8	0.7
rV94 N/A/B	7.3	nd	-0.4	0.4	0.7
r F460L	7.5	0.0	-0.3	0.6	1.1
rF460A	7.0	0.7	1.1	2.1	5.0
rF460P	6.8	1.3	1.1	2.2	5.1
rY948H	8.1	0.2	0.5	1.0	1.8
rY948A	7.2	0.1	0.6	3.0	5.7
rY948G	7.4	-0.1	0.5	0.9	3.1
rY948L	7.1	1.1	1.9	5.5	5.7
rL1566I	7.6	0.4	0.6	0.8	0.9
rS1724I	7.6	-0.3	0.5	2.9	5.8
rdel1724	7.3	1.0	1.5	4.0	6.2
rV94 P+V	7.3	nd	2.8	4.8	6.0
rV94 RSV-F	5.7	nd	0.3	0.6	1.0

[a] See Table 2. V94 is the original HPIV2 clinical isolate; rV94 is its recombinant counterpart; rV94Not is rV94 including the pre-N ORF *Not*I site. rV94 N/A/B is rV94Not including additional unique restriction sites flanking the P/V ORFs.

[b] Values are the mean of at least two experiments. Values in bold type indicate temperatures at which the mean log_{10} reduction versus 32°C was $\geq$2.0 log_{10} compared to that of wild type rHPIV2. The lowest temperature in bold is the shut-off temperature. Viruses in bold have a *ts* phenotype.

[c] nd = Not determined.

As shown in Figure 1 and Table 4, recombinant HPIV2 (rF460A, rF460P, rY948A, rY948G and rY948L) comprising an alternative codon substitution were more attenuated than the rV94Not parent virus. rF460A and rF460P were more attenuated in both the upper and lower respiratory tract than F460L which bears the original imported mutation. Similarly, rY948A, rY948G, and rY948L were more attenuated in both the upper and lower respiratory tract than rY948H, which bears the original imported mutation. Thus, the codon substitution mutants exhibited increased attenuation, a desirable property for a vaccine virus.

Both the newly derived rS1724I and the previously derived rdel1724 (WO04/027037) recombinants were more attenuated in the upper and lower respiratory tract of hamsters than the rV94Not parent virus. These results indicated that amino acid 1724 in the HPIV2 L protein is a susceptible site for yielding an attenuation phenotype. Importantly, the rdel1724, which is highly attenuated in hamsters, includes a two amino acid deletion and thus would require the insertion of six specific nucleotides to revert to wild type, whereas rS1724I includes a single nucleotide substitution and would be much more susceptible to reversion. Therefore, the deletion mutation is a preferred mutation for inclusion in a HPIV2 vaccine, because it specifies an *att* phenotype that should be stable *in vivo*.

Table 4

Virus[a]	No. of animals	Mean virus titer (log_{10} $TCID_{50}$/g ± S.E.[c])	
		Nasal Turbinates	Lungs
V94	6	4.9 ± 0.1	5.9 ± 0.4
rV94Not	18[b]	5.3 ± 0.1	5.3 ± 0.2
rV94	6	5.0 ± 0.2	5.5 ± 0.4
rV94 N/A/B	6	5.3 ± 0.2	5.1 v 0.2
r F460L	6	5.0 ± 0.1	3.1 ± 0.3
rF460A	6	1.7 ± 0.2	≤1.5 ± 0.0
rF460P	6	1.6 ± 0.1	≤1.5 ± 0.0
rY948H	6	5.6 ± 0.1	4.5 ± 0.4
rY948A	6	3.9 ± 0.2	2.2 ± 0.2
rY948G	6	3.5 ± 0.3	2.2 ± 0.3
rY948L	6	1.7 ± 0.1	≤1.5 ± 0.0
rL1566I	6	4.6 ± 0.4	3.1 ± 0.5
rS1724I	6	3.5 ± 0.3	2.2 ± 0.2
rdel1724	6	2.4 ± 0.2	≤1.5 ± 0.0
rV94 P+V	6	2.4 ± 0.2	3.1 ± 0.1
rV94 RSV-F	6	4.1 ± 0.2	2.1 ± 0.4

[a] Hamsters in groups of 6 were inoculated IN with 10^6 $TCID_{50}$ of the indicated virus. Nasal turbinates and lung tissues were harvested on day 4. Virus present in the tissues was quantified by serial dilution on LLC-MK2 monolayers at 32°C.

[b] Total number of animals from three independent experiments.

[c] Data shown were compiled from multiple experiments. The mean virus titer per gram of tissue for each group of animals receiving the same inoculum is shown. S.E., standard error. A

statistical analysis of the level of reduction of replication of mutant virus versus wild-type viruses is presented in Figure 1.

The Y948L mutation attenuated HPIV2 replication to the greatest extent in the respiratory tract of hamsters (4000 and 6000-fold reduction of replication in the upper and lower respiratory tract, respectively, compared to rV94Not). We therefore examined the ability of the Y948L mutant to replicate in the respiratory tract of non-human primates.

As shown in Figure 2 and Table 5, the novel HPIV2 L protein Y948L mutation conferred an additional 160-fold reduction in the mean peak level of virus replication in the lower respiratory tract of African green monkeys compared to its rV94Not parent, as well as a significant reduction in the mean sum of daily virus shed in the lower respiratory tract. Furthermore, infection with rY948L protected animals from challenge with the biologically derived V94 (Table 5). These results indicate the rY948L mutant is appropriately attenuated and efficacious in non-human primates. In addition, the Y948L codon substitution would require 3 nucleotide changes to revert to the wild type Tyr codon thereby rendering this mutation genetically more stable. Thus, the use of alternative codon substitutions at positions known to confer an attenuation phenotype in a heterologous virus is an effective means of enhancing the level of HPIV2 attenuation for non-human primates.

The HPIV2 mutant bearing a deletion of amino acids 1724-1725 was also highly attenuated and efficacious in African green monkeys (Figure 2 and Table 5). rV94 del1724 has a greater level of stability than rV94 Y948L, since rV94 del1724 requires insertion of 6 nucleotides to revert to the wild type.

Table 5

Immunizing virus[a]	Group size[b]	Mean peak titer of immunizing virus ($log_{10}TCID_{50}$/ml ± S.E.)[c]		Mean of sum of daily virus titers of immunizing virus ($log_{10}TCID_{50}$/ml ± S.E.)[d]		Post-immunization serum HPIV2	Mean peak challenge HPIV2 titer[f] (log_{10} $TCID_{50}$/ml ± S.E)		Post-challenge serum HPIV2
		NP	TL	NP	TL	HAI antibody titer (recip. log_2 ± S.E)[e]	NP	TL	HAI antibody titer (recip. log_2± S.E)[e]
V94	8	2.6 ± 0.3	4.8 ± 0.3	11.7 ± 1.0	14.5 ± 1.3	1.3 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	3.1 ± 0.6
V98	4	2.6 ± 0.6	4.6 ± 0.5	11.5 ± 2.5	14.5 ± 2.7	6.3 ± 0.3[j]	≤0.5 ± 0.0	0.6 ± 0.1	7.3 ± 0.3[j]
Greer	4	2.7 ± 0.6	3.7 ± 0.5	11.8 ± 2.6	12.9 ± 2.1	6.0 ± 0.4[j]	1.4 ± 0.1	1.1 ± 0.4	8.8 ± 0.5[j]
RV94 Not	7	2.3 ± 0.5	3.2 ± 0.2[A]	8.8 ± 1.2	9.0 ± 0.7[A]	2.0 ± 0.6	0.6 ± 0.1	≤0.5 ± 0.0	3.7 ± 0.4
RV94	6	3.1 ± 0.4	2.7 ± 0.5[A]	13.6 ± 1.4	5.9 ± 0.8[A]	3.7 ± 1.0	0.6 ± 0.1	1.1 ± 0.3	5.0 ± 0.4
RV94 del1724	8	2.0 ± 0.4	2.0 ± 0.1[A]	10.8 ± 1.9	6.2 ± 0.3[A]	1.5 ± 0.2[h]	0.6 ± 0.1	1.1 ± 0.2	3.1 ± 0.6[i]
RV94 Y948L	4	2.9 ± 0.4	1.0 ± 0.5[A,B]	13.5 ± 2.2	3.0 ± 0.5[A,B]	2.8 ± 0.8	≤0.5 ± 0.0	0.8 ± 0.3	4.8 ± 0.9
RV94 P+V	4	2.2 ± 0.3	2.4 ± 0.6[A]	11.1 ± 0.7	6.2 ± 0.7[A]	1.0 ± 0.0	≤0.5 ± 0.0	≤0.5 ± 0.0	3.8 ± 0.8

a. Animals were inoculated IN and IT on day 0 with 10^6 $TCID_{50}$ per site of the indicated virus.
b. Includes data collected from similarly infected and sampled African green monkeys from three studies.

c. Nasopharyngeal (NP) swab samples were collected on days 1 to 10 post-infection. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8 and 10 post-infection. The mean of the peak virus titer for each animal in its group irrespective of sampling day is shown. S.E. = standard error. The limit of detection of virus titer was 10 $TCID_{50}$/ml. Values indicated by A differ significantly from the group that received V94 ($P < 0.05$). Values indicated by B differ significantly from the group that received rV94Not.

d. The sum of the viral titers obtained for each animal on all sampling days (days 1-10 for NP and days 2, 4, 6, 8 and 10 for TL) was calculated and the mean (log_{10})± S.E. for each group is shown. The lower limit of detection is 5.0 $log_{10}TCID_{50}$/ml for NP swab and 2.5 $log_{10}TCID_{50}$/ml for TL samples. Values indicated with A differed significantly from the group that received V94. Values indicated with B differed significantly from the group that received rV94Not.

e. Sera were collected 28 to 31 days post-challenge and were titered in the same HPIV2 hemagglutination inhibition assay.

f. Animals were challenged IN and IT on day 28 or 31 post-first immunization with 10^6 $TCID_{50}$ per site of the V94 strain of HPIV2. NP swab and TL samples were collected on days 2, 4, 6, and 8 post-infection. Virus titrations were performed on LLC-MK2 cells at 32°C. Mean of the peak virus titers ± S.E. for each animal in its group irrespective of sampling day is shown. The limit of detection of virus titer was 10 $TCID_{50}$/ml. Compare to mean peak virus titer values obtained in HPIV2 naïve monkeys that received V94.

g. Post-challenge antibody titers were obtained 28 days post-challenge and were titered in the same HPIV2 hemagglutination inhibition assay.

h. Quantity of serum not sufficient from 2 AGMs; mean was calculated from 6 animals

i. Quantity of serum not sufficient from 1 AGM; mean was calculated from 7 animals.

j. HAI data from previous study, samples not tested at the same time as other samples.

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Analysis of the replicative properties of these mutants in vivo and in animal models demonstrated that the F460L and L1566I mutations each conferred approximately a 250 fold reduction of replication in the lower respiratory track of hamsters, and the 1724-1725 deletion mutation conferred a 4 to 25 fold reduction in replication in the respiratory tract of African Green monkeys. Interestingly, while the Y942H mutation conferred ts and att phenotypes in HPIV3 and HPIV1, the analogous mutant did not attenuate HPIV2. This shows that the importation strategy frequently, but not always, results in an *att* phenotype in a recipient virus.

Example 2

Identification of a Putative Non-*ts*, Host-Range *att* Mutation in the 3' Leader Sequence of HPIV2/V94.

In African green monkeys, we noted a significant and unexpected difference between the level of replication of the rV94 bearing the pre-N ORF *Not*I restriction site (rV94Not) versus the biologically derived V94 in the lower respiratory tract (Table 5 and Figure 2). With the exception of the introduced pre-N ORF *Not*I restriction site and a translationally silent nucleotide substitution in the F ORF, the recombinant V94 sequence was identical to that determined for the biologically derived virus (Skiadopoulos et al., 2003b). To establish whether the introduced *Not*I site was responsible for the attenuation phenotype, we compared the level of replication of biologically derived V94 to that of a recombinant V94 (rV94) that does not includes the pre-N ORF *Not*I site. We found that rV94 was also significantly attenuated in the lower respiratory tract of African green monkeys (Figure 2 and Table 5). These results indicated that another factor was responsible for the difference in the observed level of replication of recombinant and biologically derived V94 in the lower respiratory tract of African green monkeys and that the *Not*I site likely did not specify an *att* phenotype.

Materials and Methods

Passaging and Sequencing

The uncloned, biologically derived HPIV2 V94 clinical isolate administered to the monkeys in Example 1 was sequentially passaged nine times on Vero cell monolayers and then passaged once on LLC-MK2 cell monolayers to generate a virus preparation (V94(a)).

The virus preparation used to determine HPIV2/V94 consensus sequence, however, was prepared from V94 that was first passaged nine times on Vero cells and then passaged multiple

times on LLC-MK2 cells (V94(b)). Therefore, the possibility existed that V94(a) and V94(b) were not genetically identical. We determined the sequence of V94(a) and compared it to the V94(b) consensus sequence (Figure 3A). Sequence was determined by sequencing overlapping RT-PCR cDNA fragments spanning the entire genome as described previously (WO04/027037; Skiadopoulos et al., 2003, *J. Virol.*, 77:270-279). Sequence electropherograms from uncloned RT-PCR products derived from primary isolates of virus recovered from the lower repsiratory tracts of four African green monkeys infected with V94(a) are shown in Figure 3b. V94(a) passaged in LLC-MK2 cells is shown as a control. The electorpherograms were generated by ABI Prism Sequencing Analysis software (version 3.0) (Perkin-Elmer, UK) on a Perkin–Elmer ABI 3100 sequencer with a Big Dye sequencing kit (Perkin–Elmer Applied Biosystems, Warrington, UK).

Sequencing of viral isolates of tracheal lavage of African Green monkeys

Tracheal lavage samples collected from four African green monkeys that were infected with V94(a) (including a mixed population of T or C at nucleotide position 15) were amplified by a single passage on LLC-MK2 monolayers. As a control, the original V94(a) inoculum was also passaged once on LLC-MK2 cells. Primary isolates were similarly prepared from tracheal lavage samples from animals that were administered rV94 (including exclusively C at position 15). These primary isolates were harvested after incubation at 32°C for six days and vRNA was prepared and used in 3' RACE (Rapid Amplification of cDNA Ends) reactions to generate RT-PCR products spanning the 3' end of the viral genome. The PCR products were sequenced in the vicinity of nucleotide position 15.

Results and Discussion

The sequences of V94 (a) and V94 (b) were found to be completely identical except for a single position in the 3' leader. While the sequence at nucleotide position 15 of V94(b) is a cytosine (C, antigenomic cDNA sense), the sequence determined for the V94(a) preparation administered to the monkeys was a mixture of cytosine and thymidine (T). This difference in sequence is likely a spontaneous change that arose during passage of V94 in Vero cells and was selected for by multiple passage on LLC-MK2 cells. The sequences of the recombinant versions of V94 (rV94 and rV94Not) had a C at position 15. Because this is the only sequence difference observed between the biologically derived V94 (V94(a)) and the recombinant V94 (V94(b)), we conclude that a C at position 15 has an attenuating effect on replication of the virus in the lower respiratory tract of African green monkeys.

Interestingly, there was no difference between the level of replication of the biologically derived V94 and the rV94 in the respiratory tract of hamsters. Moreover, the growth characteristics of the biologically derived and recombinant viruses *in vitro* were also identical (Table 3), indicating that this nucleotide substitution specifies a host-range *att* phenotype, i.e., attenuated in African green monkeys but is a wild type phenotype in hamsters. The results in nonhuman primates are likely predictive of results in humans. Therefore, T15C constitutes another novel attenuating mutation available for producing a live-attenuated HPIV2 vaccine.

In the sequence electropherogram of the V94(a) preparation used to inoculate the monkeys, the relative signal strengths of the C and T nucleotides at position 15 were approximately the same (not shown), suggesting that the preparation included populations of viruses bearing C or T that were nearly equivalent in abundance. Based on this observation, we suggest that the population of T-including virus might account for the higher level of V94 replication observed in the monkeys that received V94 (a). If this was indeed the case, then the virus that replicated in the lungs of these animals should predominately have a T at position 15.

To test this hypothesis, tracheal lavage samples collected from four African green monkeys that were infected with V94(a) (including a mixed population of T or C at nucleotide position 15) were amplified by a single passage on LLC-MK2 monolayers. As shown in Figure 3B, the original V94(a) inoculum passaged once on LLC-MK2 cells maintained a mixed population (C+T) at the position-15 locus. Significantly, each of the isolates obtained from animals that had been administered the same V94(a) inoculum now had a predominantly homogeneous population (T15) at the same locus. This indicates that the virus population

including a T at position 15 is the predominant replicating population in the respiratory tract of the monkeys.

Primary isolates were similarly prepared from tracheal lavage samples from animals that were administered rV94 (including exclusively C at position 15). Sequence analysis of RT-PCR products prepared from vRNA purified from these isolates verified that the input virus sequence at this position (C-15) was unchanged (data not shown), indicating that it is genetically stable following *in vivo* replication. Thus, a C at position 15 of the V94 genome is associated with attenuation in the lower respiratory tract of African green monkeys, but not in hamsters. Since rV94 is not restricted for replication at 39°C or 40°C (Table 3), this change does not confer a *ts* phenotype.

Because T15C confers a non-*ts*, host-range *att* phenotype, it is a useful component to include in a live-attenuated HPIV2 vaccine. Figure 2 indicated that the attenuation specified by the Y948L mutation is additive with the 3' leader T15C mutation, indicating that *ts* and non-*ts* *att* mutations are compatible and additive. These mutations provide a method to incrementally adjust the level of attenuation of an HPIV2 vaccine virus.

Example 3

Recombinant HPIV2 Including Separate Polynucleotides encoding P or V Protein

The bicistronic P/V gene of HPIV2 encodes the nucleocapsid-associated P phosphoprotein (395 amino acids) and the accessory V protein (225 amino acids) from two overlapping reading frames (Ohgimoto et al., 1990). Since the V protein is not absolutely required for virus replication (Kato et al., 1997; Fukuhara et al., 2002; He et al., 2002; Sun et al., 2004), it is an attractive target for the introduction of mutations that do not interfere with the replication of a vaccine virus in cell culture but attenuate the virus for replication in a human host. The overlapping nature of the P and V ORFs, however, greatly restricts the number and types of mutations that can be introduced into the V protein without also affecting the P protein (and vice versa). To overcome this obstacle we have undertaken a novel approach and modified the HPIV2 V94 strain antigenomic cDNA such that the P and V proteins are encoded by a separate polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein, respectively. This will allow the introduction of a variety of substitution and deletion mutations in the V protein without compromising the integrity of the P protein.

The alternative reading frames of the P/V gene are accessed by an unusual cotranscriptional editing of the P/V gene mRNA (Ohgimoto et al., 1990), a feature unique to most members of the *Paramyxovirinae* subfamily of Paramyxoviruses (Kawano et al., 1993; Chanock, Murphy, and Collins, 2001). The HPIV2 P protein is translated from a P/V gene mRNA, which has had two additional guanosine residues, inserted into the heptaguanosine stretch (underlined) (TTTAAGAGGGGGGG, V94 nucleotide 2474-2487; SEQ ID NO:8) of the mRNA editing site. This editing site, which shares common features with the editing sites of other paramyxoviruses, includes the uninterrupted heptaguanosine tract (Jacques and Kolakofsky, 1991; Chanock, Murphy, and Collins, 2001) (Figures 4A and B). During mRNA synthesis, the viral polymerase is believed to insert one or more guanosine residues by stuttering at the heptaguanosine tract. In the case of HPIV2, two guanosine residues are inserted. The inserted residues shift the translational reading frame at codon 164 and the translation machinery subsequently accesses an alternative ORF specifying the carboxy-terminal half of the P protein (aa 165-395). The shorter V protein is generated from an unedited P/V gene mRNA. Thus the P and V proteins have a common amino-terminal sequence (aa 1-164) and unique carboxy-termini (Figure 4C).

Paramyxovirus V proteins have a range of functions from activities affecting virus morphogenesis to counteracting host cell innate immune response (Parisien et al., 2001; Andrejeva et al., 2002a; Parisien, Lau, and Horvath, 2002). Analogous to Rubulavirus V proteins, putative HPIV2 V protein activities include RNA binding (Lin, Paterson, and Lamb, 1997), viral nucleoprotein (N) binding (Precious et al., 1995; Randall and Bermingham, 1996; Watanabe et al., 1996a), subcellular localization activities (Watanabe et al., 1996b; Rodriguez, Cruz, and Horvath, 2004), E2- or E3-like ubiquitin ligase activity (Ulane and Horvath, 2002), induction of ubiquitination, STAT binding and degradation (Andrejeva et al., 2002a; Kozuka et al., 2003), binding to the damage specific DNA binding protein (DDBP) (Lin et al., 1998; Andrejeva et al., 2002b), blocking apoptosis (Sun et al., 2004), and zinc ion binding via a highly conserved cysteine rich carboxy-terminal domain (Liston and Briedis, 1994; Paterson et al., 1995; Huang et al., 2000). The active sites for many of these functions are thought to localize to separate domains of the V protein, while some activities likely overlap.

Since the V protein is not absolutely required for virus replication (Kato et al., 1997; Fukuhara et al., 2002; He et al., 2002; Sun et al., 2004), it is an attractive target for the introduction of mutations that do not interfere with the replication of a vaccine virus in cell culture but attenuate the virus for replication in a human host. Paramyxoviruses that express a V

protein with a carboxy-terminal deletion were found to be defective for replication *in vivo* and sometimes *in vitro*, including in Vero cells which do not express antiviral interferons α and β (Kato et al., 1997; Delenda et al., 1997; Durbin et al., 1999; Kawano et al., 2001; He et al., 2002; Park et al., 2003). Thus, it is important to determine the protein domains of the HPIV2 V protein that are responsible for the growth promoting function *in vitro* and for the anti-innate immune response activities *in vivo*. However, the overlapping nature of the P and V ORFs greatly restricts the number and types of mutations that can be introduced into the V protein without also affecting the P protein (and vice versa).

Materials and Methods

Generation and recovery of recombinant HPIV2 including a genetic rearrangement of the P/V gene. To facilitate subsequent cloning steps, unique endonuclease restriction recognition sequences [*AscI* (nt 1986-1993); *Bst*EII (nt 3188-3194); *Age*I (nt 3192-3197); *Sac*II (nt 3221-3226)] were introduced into rV94Not cDNA (Figure 5A). The restriction sites were introduced by nucleotide substitution PCR mutagenesis in the non-coding regions upstream (*AscI*) or downstream (*Bst*EII, *Age*I, and *Sac*II) of the P/V ORFs using mutagenic PCR primers designed to achieve the sequence indicated in Figure 5B or 5C. The restrictions sites were translationally silent because they occurred outside of the ORFs. Recombinant V94 comprising the introduced restrictions sites (termed rV94N/A/B) was recovered as described in Example 1.

The naturally occurring P/V ORF in rV94N/A/B was replaced by a modified P ORF (SEQ ID NO:2) encoding only the P protein. In the P gene unit, the editing site was modified by the insertion of two nucleotides, which serve to access the appropriate P reading frame specifying the carboxy-terminal half of the P protein, and the translationally-silent substitution of 3 guanosines to prevent editing of the mRNA encoding P protein. Four additional nucleotides (TGAC; SEQ ID NO:14) were inserted in the non-coding region flanking the P ORF (Figure 5C) so that the genome length of the final construct conformed to the "rule of six" (Calain and Roux, 1993; Kolakofsky et al., 1998; Vulliemoz and Roux, 2001). The editing sites were modified by PCR mutagenesis (Moeller et al., 2001) using mutagenic PCR primers designed to achieve the sequences indicated in Figure 6. Silent third base codon substitutions in the editing site are bolded and underlined. The two nucleotides (TG) inserted to access the 3'-terminal half of the P ORF are indicated by arrows. Parainfluenza viruses generated from cDNAs that do not conform to the rule of six accumulate spontaneous nucleotide deletion or

insertion mutations at positions that cannot be predicted (Skiadopoulos et al., 2003b), and therefore cannot be reliably used for live-vaccine production.

To further ensure that a V protein could not be generated from the polynucleotide encoding P protein, silent third base codon substitution mutations were introduced into the P ORF downstream of the ablated editing site by PCR (Moeller et al., 2001) using mutagenic primers designed to achieve the nucleotide sequence indicated in Figure 6. These nucleotide substitutions encoded stop codons in the overlapping V reading frame. Thus, in the unlikely event that the translation apparatus shifts translation back into the V reading frame, translation termination codons would be encountered at codon positions 167, 176, 184 and 186 of the V ORF remnant. The modified P ORF was subcloned into rV94N/A/B cDNA between the *AscI* and *Bst*EII sites, as indicated in Figure 5C, using standard methods.

A modified V ORF designed to encode only the V protein (SEQ ID NO:3). The P and V genes were separated by a non-coding polynucleotide spacer sequence. The spacer sequence was located upstream of the modified V ORF (Figure 5C). The spacer sequence included an *AgeI* site and upstream gene end (GE), intergenic (IG), and gene start (GS) transcription signals that served to terminate P gene transcription and initiate V gene transcription, respectively.

The editing site in the V gene unit was engineered so that the heptaguanosine run in the mRNA editing site was mutated by the substitution of 2 guanosines to either adenosine or cytosine (Figure 6) using PCR mutagenesis and standard molecular cloning techniques (Moeller et al., 2001; Skiadopolous et al., 2003, *J. Virol.*, 77:270-279). This mutation eliminated RNA editing so that the gene unit encoded only the V protein, and was silent at the level of translation. Four additional nucleotides (TGAC) were inserted in the non-coding region flanking the V ORF (Figure 5C) so that the genome length of the final construct conformed to the "rule of six" (Calain and Roux, 1993; Kolakofsky et al., 1998; Vulliemoz and Roux, 2001).

It is believed that the phasing of the first adenosine in the transcription gene start sequence, with respect to its position in the polyhexameric genome, may be important for the appropriate production of the viral mRNA of all members of the *Paramyxovirinae* subfamily (Kolakofsky et al., 1998). Therefore, the cis-acting transcription gene start sequence of the inserted polynucleotide encoding V protein was designed so that the phasing of the first adenosine (antigenomic cDNA sequence) in the signal sequence was in a 6n + 1 position (Figure 5C), similar to that of the naturally occurring P/V gene start cis-acting transcription control regions.

The modified V ORF was subcloned into pFLC V94 using the *Age*I and *Sac*II restrictions sites, as indicated in Figure 5c, using standard methods. The transcription control sequences upstream of the P ORF and downstream of the V ORF were not affected by the cis-acting transcription gene start sequence in the V gene unit and the phasing of the polynucleotide encoding P protein in rV94 P+V remained unaltered.

The final full-length antigenomic cDNA plasmid (pFLC V94 P+V) was sequenced using standard methods. HPIV2 P+V cDNA (SEQ ID NO:1) was 16,350 nucleotides in length and is shown in figure 10. The supernumerary polynucleotide encoding V protein increased the number of encoded mRNAs to 7, which is one mRNA more than that of wild type HPIV2.

Recovery and sequencing of a recombinant V94 P+V. Recombinant HPIV2 expressing the P and V proteins from separated polynucleotide (rV94 P+V) was recovered in HEp-2 cells using the full-length antigenomic HPIV2 plasmid pFLC V94 P+V and the HPIV2 N, P and L support plasmids in the reverse genetics system described in Example 1. Virus recovered from transfected HEp-2 cells was passaged onto Vero cell monolayer culture or onto LLC-MK2 cells. The recovered virus, rV94 P+V, grew to high titer in LLC-MK2 (1.3×10^8 $\log_{10}$ $TCID_{50}$/ml) and in Vero cell culture (5×10^8 $\log_{10}$ $TCID_{50}$/ml).

The identity of the recovered virus was confirmed by RT-PCR amplification from purified viral RNA (vRNA) of a subgenomic fragment including a polynucleotide encoding P or V protein (Figure 7A). Oligonucleotide primers approximately 25-30 nucleotides in length, in both sense and antisense orientation, spanning the HPIV2 genome were used as sequencing primers. A PCR product was not obtained from similar RT-PCR reactions that did not have the RT enzyme added to them (data not shown), indicating that the RT-PCR product was generated from an RNA template rather than from contaminating plasmid DNA. The size of the PCR product obtained from rV94 P+V was compared to that of the biologically derived V94 virus and to a PCR product obtained with the pFLC V94 P+V cDNA.

Western Blot of Protein Extracts

To demonstrate that the V protein was produced by rV94 P+V, total cellular protein was purified from Vero cells infected with rV94 P+V or wild type V94. Uninfected Vero cells and Vero cells infected with HPIV3 were used as controls. Forty-eight hours after infection Vero cell monolayers were harvested and washed once with PBS. Cell pellets were disrupted with 2X sample buffer (100mM Tris-Cl [pH 6.8], 4% sodium dodecylsulfate, 20% glycerol, 0.2% bromophenol blue, 200 mM dithiothreitol) and centrifuged through Qiashredders (Qiagen). Five µl of each sample was electrophoresed through sodium dodecylsulfate –4 to 20%

polyacrylamide gels (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were incubated with rabbit polyclonal antisera raised against a V protein specific peptide representing amino acids 186 to 201 of HPIV2 V protein (SEQ ID NO:45). Bound antibodies were visualized by incubation with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G antibodies and chemilluminescence (Pierce). Western blot analysis of protein extracts from the infected cells confirmed that V protein was produced by rV94 P+V (Figure 7B).

Replication of rV94 P+V *in vitro* at permissive and restrictive temperatures.

The kinetics of replication of rV94 P+V was determined by inoculation of HPIV2 onto LLC-MK2 monolayers on 6-well plates at a multiplicity of infection (m.o.i.) of 0.01. rV94Not and the *ts* and *att* recombinant rdel1724 were used as control viruses and the cultures were incubated at 32°C. 0.5 ml of medium from each well was harvested and replaced with 0.5 ml of fresh OptiMEM medium at 0 hr and at 24 hour intervals for up to 7 days post-infection. Virus present in the daily harvest was quantified by titration on LLC-MK2 monolayers in 96-well plates incubated at 32°C.

Recombinant HPIV2 expressing RSV F protein (rV94 RSV-F)

Recombinant HPIV2 expressing RSV F protein were constructed as described in WO04/027037. Briefly, a polynucleotide expressing RSV subtype A fusion (F) protein ORF as a supernumerary HPIV2 gene unit was cloned into V94Not cDNA upstream of the N coding sequence and under the control of the cis-acting HPIV2 transcription signals. Recombinant V94Not vector viruses expressing RSV F protein were recovered using a reverse genetics system that employed a full length HPIV2/rV94Not plasmid and three HPIV2 support plasmids as described in WO04/027037 and Example 1.

Results and Discussion

A modified HPIV2 V94 strain antigenomic cDNA comprising separate polynucleotides encoding P and V proteins was prepared and designated rV94 P+V. The identity of the recombinant V94 P+V virus was confirmed by RT-PCR amplification from purified viral RNA (vRNA) of a subgenomic fragment including the P and V genes (Figure 7A). As shown in Figure 7A, the RT-PCR product obtained from rV94 P+V vRNA is approximately 700 bp larger than that obtained from wild type V94 and is the same size as that obtained from the pFLC V94 P+V cDNA.

The genomic sequence of rV94 P+V was determined by sequencing of the RT-PCR

products spanning the entire genome generated from purified vRNA, with the exception of nucleotides 16287 to 16350 from the 5' end which were not sequenced. The genetic structure of the monocistronic polynucleotides was confirmed by sequence analysis. Several spontaneous point mutations were also identified. These nucleotide substitutions were not encoded in the pFLC P+V cDNA and thus arose during propagation of the virus in cell culture. Coding substitutions identified included mutations Phe25Leu and Leu27Pro in the V protein, and Asn1120His and Asp1892Asn in the L protein. Western blot analysis of protein extracts from the infected cells confirmed that V protein was produced by rV94 P+V (Figure 7B).

The growth kinetics of rV94 P+V in Vero cells was similar to that of rdel1724 (Figure 8), which grew at a moderately slower rate than rV94Not. However, the peak virus titers of rV94 P+V and rdel1724, which are an important consideration for the manufacture of live-vaccine viruses, were comparable to that of rV94Not.

The ability of rV94 P+V to replicate at higher temperatures (37-40°C) was also examined. rV94 P+V was found to be highly *ts* with a shut-off temperature of 38°C (Table 3). In contrast, rV94 and rV94 N/A/B did not exhibit significant shut off even at 40°C. Replication of a previously described recombinant HPIV2 expressing the RSV fusion F protein from a supernumerary gene unit inserted upstream of the HPIV2 N ORF (rV94 RSV-F; WO 04/027037) was found to not be restricted at 39 or 40°C (Table 3). This data indicated an increase in the number of inserted supernumerary gene units or an increase in genome length was not responsible for the *ts* phenotype of rV94 P+V. Recombinant HPIV3 with gene unit insertions has been shown to exhibit a moderate level of temperature sensitivity (Skiadopoulos et al., 2000; Skiadopoulos et al., 2002). The basis for this phenomenon, however, is not known.

Replication of rV94 P+V in hamsters was examined as described in Example 1. rV94 P+V was approximately 800-fold restricted in replication in the upper respiratory tract and approximately 100-fold restricted in lower respiratory tract of hamsters (Figure 1 and Table 3) compared to parent viruses rV94Not and rV94 N/A/B. Similarly, recombinant HPIV2 expressing RSV F protein (rV94 RSV-F) was attenuated for replication in the upper and lower respiratory tract of hamsters. The basis for the high level of attenuation conferred by these supernumerary gene unit insertions is not known. The level of attenuation, however, is more pronounced than that observed in other recombinant paramyxoviruses including additional gene units (e.g., HPIV3 and HPIV1) (Skiadopoulos et al., 2000; Skiadopoulos et al., 2002).

Replication of rV94 P+V in African green monkeys was examined as described in Example 1. As shown in Figure 4 and Table 4, rV94 P+V was approximately 250-fold

restricted for replication in the lower respiratory tract of African green monkeys compared to biologically derived V94. rV94 P+V was approximately 6-fold restricted for replication in the upper respiratory tract of African green monkeys compared to parent rV94Not virus. This difference, however, is not statistically significant. It remains to be determined whether attenuation in monkeys conferred by the T15C substitution in the 3' leader is additive to attenuation conferred by the P/V gene rearrangement. Attenuation of rV94 P+V in hamsters was independent of the T15C mutation as both rV94Not and rV94 P+V possessed the mutation and rV94 P+V was significantly more restricted in replication than rV94Not. The independent contribution of the P+V rearrangement to attenuation for African green monkeys will be determined by constructing and characterizing V94 P+V lacking the T15 C mutation.

Although the rV94 P+V vaccine candidate is highly attenuated for replication in monkeys, it conferred a high level of protection against the wild type HPIV2 challenge virus (Table 5). Thus, the P/V ORF rearrangement and supernumerary polynucleotide insertion are useful as a method of conferring an attenuation phenotype to HPIV2. Importantly, the attenuating effect of the gene rearrangement, similar to that of the codon substitutions and deletion mutations described in Examples 1 and 2, should be genetically and phenotypically stable following replication *in vivo*.

The ability to recover recombinant HPIV2 encoding P and V proteins from separate genes will be valuable in examining the domain structure of the V protein and for introducing attenuating V protein mutations. Candidate sites for mutations in V protein are summarized in Figure 9. These include mutations in the highly conserved cysteine-rich carboxy-terminal domain, a critical domain of the V protein shown to bind zinc ions and to play a major role in several V protein activities in other paramyxoviruses. Mutations in the N-terminal half of V protein directly affect RNA binding or STAT binding and degradation may be used to attenuate HPIV2. Useful mutations will minimally impact virus replication *in vitro*, disable the ability of the virus to block interferon response resulting in virus that are more immunogenic, and/or attenuate virus replication in a human host. Mutations at the indicated sites include, but are not limited to, amino acid substitutions or deletion of the indicated residues.

Table 6

ACCAAGGGGAGAATCAGATGGCATCGTTATATGACGAATTGCAAAAAGATTACGTAGGTCCGGA

ACCACTAGATTCCGGTGCCGGTAACGATCTCAGTTTTATACTATCTGATCATTCTTTATCTCTA

CTAAGGATATTTCTAATCTAAGGTTCAAAATGTCAAGTGTCTTAAAGACATTTGAAAGATTTAC

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Table 7

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Table 8

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AGAAAGAGCAAGAGGCAACCATAGGAGAGAATGGAGTATTGCATGGGTCGGAGATCAGGTCAAA
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CATGCGGATCCTGCCCCAGCATCTGCGGAGAATGTGAAGGAGATCATTGAGCTCTTAAAGGGAC
TTGATCTTCGCCTTCAGACTGTAGAAGGGAAAGTAGATAAAATTCTTGCAACTTCTGCAACTAT
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GTTTACACAATGTTCCAGTAGTAATTGCCGGTCCAACTAGTGGAGGCTTCACAGCCGAAGGCAG
TGATATGATTTCAATGGATGAACTAGCTAGACCTACACTCTCATCAACAAAAAGGATCACACGA
AAGCCTGAATCCAAGAAAGATTTAACAGGCATAAAACTAACTTTGATGCAGCTTGCAAATGACT
GCATCTCGCGTCCAGATACCAAGACTGAGTTCGTGACTAAGATTCAGGCAGCAACCACAGAATC
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AAGAGACACTCAACATGCATCCAATCAAGATCCAGACTCCATCCATCCAAAAACACGCCCACAA
TTGTCAACACCAAGAAACAACCACAGCCGAACCATGCTCAACCAAAAGACCCAAACAACACCTC
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TCCAAACCCCAAACACAGCAGCAGACATGCCTATAATATCATTACCAGCAGATCCAACTTCACC
CAGTCAATCCCTTACTCCGTTTCCAATACAACTTGACACCAAAGATGGCAAGGCAGGGAAACTC
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TGACCTAGCTGCAGGGTCCATAACAGCATGCATGATGACGCTAGGTCCTGGTCCAAATATTCAG
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GGATTTGAGTATGTATACATACCAACTTTTGTCTCCATTACATACTCACCACGAAATCTGAATT
ACCAAGTTGCCAGACCTATCCTTAAGTTCAGATCACGCTTTGTGTATAGCATTCATTTGGAATT
AATCCTGAGATTGCTATGCAAATCTGACTCCCCCTTGATGAAATCCTACAATGCAGACAGAACA
GGTCGGGGATGCCTCGCATCAGTCTGGATCCTTGTATGTAACATTCTGAAAAACAAAAGCATCA
AGCAACAAGGCAGAGAATCATATTTCATAGCTAAGTGCATGAGCATGCAGCTGCAGGTGTCCAT
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ACATTTGAGAGGTCCAAATTCAGCCCATTCAAAAAATATGCATCAATTCCAAACTTGGAAGCCA
TCAACTGAATGCTCCAGCATCTGAGAATAGAACCACAATCAAGTCATACTACTAGTCACTATAC
AATAATCAACAATTTTAGTCAACTGATTACCAAGATGTTATCATAGGTCCGAACTGATCAATCT
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CCTGCATCCAATGATAGTATGCATCTTTGTTATGTACACTGGAATTGTAGGTTCAGATGCCATT
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CTGATGGTGGTGCTAGCTTTATTGTTGTAAAATTGCTACCTAATCTTCCCCCAAGCAATGGAAC
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CCAGATTCATTCATTAATTATAGCAACTACTGTTCTTAAGAGGGGTGGGATATTAATTTATAAA
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ACTTGCCGCAGATTTTAGAACTATCGGTTTCAGTGCAGCTCTAGTAACTGCTACTACTCTTCAC
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GTTCGGAGACAGTTGATATACCTCTAATTAAAGACATTCAGATAGAAGATATTAATGATTTTGA
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GTTTTATTTAAGAAAAACATATTGATTTTCCCCTTGGT

Table 9

MSSVLKTFERFTIQQELQEQSEDTPIPLETIRPTIRVFVINNNDPIVRSRLLFFNLRIIMSNTA
REGHRAGALLSLLSLPSAAMSNHIKLAMHSPEASIDRVEITGFENNSFRVIPDARSTMSRGEVL
AFEALAEDIPDTLNHQTPFVNNDVEDDIFDETEKFLDVCYSVLMQAWIVTCKCMTAPDQPPVSV
AKRMAKYQQQGRINARYVLQPEAQRLIQNAIRKSMVVRHFMTYELQLSQSRSLLANRYYAMVGD
IGKYIEHSGMGGFFLTLKYGLGTRWPTLALAAFSGELQKLKALMLHYQSLGPMAKYMALLESPK
LMDFVPSEYPLVYSYAMGIGTVLDTNMRNYAYGRSYLNPQYFQLGVETARKQQGAVDNRTAEDL
GMTAADKADLTATISKLSLSQLPRGRQPISDPFAGANDRETGGQATDTPVYNFNPINNRRYDNY
DSDSEDRIDNDQDQAIRENRGEPGQPNNQTSENQQRLNLPVPQRTSGMSSEEFQHSMNQYIRAM
HEQYRGSQDDDANDATDGNDISLELVGDFDS

Table 10

MAEEPTYTTEQVDELIHAGLGTVDFFLSRPIDAQSSLGKGSIPPGVTAVLTNAAEAKSKPVAAG
PVKPRRKKVISNTTPYTIADNIPPEKLPINTPIPNPLLPLARPHGKMTDIDIVTGNITEGSYKG
VELAKLGKQTLLTRFTSNEPVSSAGSAQDPNFKRGGELIEKEQEATIGENGVLHGSEIRSKSSS
GVIPGVPQSRLQLASSPAHVDPAPASAENVKEIIELLKGLDLRLQTVEGKVDKILATSATIINL
KNEMTSLKASVATVEGMITTIKIMDPSTPTNVPVEEIRKSLHNVPVVIAGPTSGGFTAEGSDMI
SMDELARPTLSSTKKITRKPESKKDLTGIKLTLMQLANDCISRPDTKTEFVTKIQAATTESQLN
EIKRSIIRSAI

Table 11

MAASSEILLPEVHLNSPIVKHKLIYYLLLGHFPHDLDISEISPLHNNDWDQIAREESNLAERLG
VAKSELIKRVPAFRATRWRSHAAVLIWPSCIPFLVKFLPHSKLQPIEQWYKLINASCNTISDSI
DRCMENISIKLTGKNNLFSRSRGTAGAGKNSKITLNDIQSIWESNKWQPNVSLWLTIKYQMRQL
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CTISHYLSPLGPRIDRLFSIVDELAQLLGDTVYKIIASLESLVYGCLQLKDPVVELTGSFHSFI
TQEIIDILIGSKALDKDESITVTTQLLDIFSNLSPDLIAEMLCLMRLWGHPTLTAAQAAGKVRE
SMCAGKLLDFPTIMKTLAFFHTILINGYRRKKNGMWPPLILPKNASKSLIEFQHDNAEISYEYT
LKHWKEISLIEFRKCFDFDPGEELSIFMKDKAISAPKSDWMSVFRRSLIKQRHQRHHIPMPNPF
NRRLLLNFLEDDSFDPVAELQYVTSGEYLRDDTFCASYSLKEKEIKPDGRIFAKLTNRMRSCQV
IAEAILANHAGTLMKENGVVLNQLSLTKSLLTMSQIGIISEKAKRYTRDNISSQGFHTIKTDSK
NKKKSKIASSYLTDPDDTFELSACFITTDLAKYCLQWRYQTIIHFARTLNRMYGVPHLFEWIHL
RLIRSTLYVGDPFNPPATTDAFDLDKVLNGDIFIVSPKGGIEGLCQKMWTMISISVIILSSAES
KTRVMSMVQGDNQAIAVTTRVPRSLPSVQKKELAYAASKLFFERLRANNYGLGHQLKAQETIIS
STFFIYSKRVFYQGRILTQALKNASKLCLTADVLGECTQASCSNSATTIMRLTENGVEKDTCYK
LNIYQSIRQLTYDLIFPQYSIPGETISEIFLQHPRLISRIVLLPSQLGGLNYLACSRLFNRNIG
DPLGTAVADLKRLIKCGALESWILYNLLARKPGKGSWATLAADPYSLNQEYLYPPTTILKRHTQ
NTLMEICRNPMLKGVFTDNAKEEENLLAKFLLDRDIVLPRVAHIIIDQSSIGRKKQIQGFFDTT
RTIMRRSFEIKPLSTKKTLSVIEYNTNYLSYNYPVILNPLPIPGYLNYITDQTCSIDISRSLRK
LSWSSLLNGRTLEGLETPDPIEVVNGSLIVGTGDCDFCMQGDDKFTWFFLPMGIIIDGNPETNP
PIRVPYIGSRTEERRVASMAYIKGATHSLKAALRGAGVYIWAFGDTVVNWNDALDIANTRVKIS
LEQLQTLTPLPTSANITHRLDDGATTLKFTPASSYAFSSYTHISNDQQYLEIDQRVVDSNIIYQ
QLMITGLGIIETYHNPPIRTSTQEITLHLHTSSSCCVRSVDGCLICESNGEVPQITVPYTNSFV
YDPDPLADYEIAHLDYLSYQAKIGSTDYYSLTDKIDLLAHLTAKQMINSIIGLDETVSIVNDAV
ILSDYTNNWISECSYTKIDLVFKLMAWNFLLELAFQMYYLRISSWTNIFDYTYMTLRRIPGTAL
NNIAATISHPKLLRRAMNLDIITPIHAPYLASLDYVKLSIDAIQWGVKQVLADLSNGIDLEILI
LSEDSMEISDRAMNLIARKLTLLALVKGENYTFPKIKGMPPEEKCLVLTEYLAMCYQNTHHLDP
DLQKYLYNLTNPKLTAFPSNNFYLTRKILNQIRESDEGQYIITSYYESFEQLETDIILHSTLTA
PYDNSETLTKFDLSLDIFPHPESLEKYPLPVDHDSQSAISTLIPGPPSHHVLRPLGVSSTAWYK
GISYCRYLETQKIQTGDHLYLAEGSGASMSLLELLFPGDTVYYNSLFSSGENPPQRNYAPLPTQ
FVQSVPYKLWQADLADDSNLIKDFVPLWNGNGAVTDLSTKDAVAFIIHKVGAEKASLVHIDLES
TANINQQTLSRSQIHSLIIATTVLKRGGILVYKTSWLPFSRFSQLASLLWCFFDRIHLIRSSYS
DPHSHEVYLVCRLAADFRTIGFSAALVTATTLHNDGFTTIHPDVVCSYWQHHLENVGRVEKVID

EILDGLATNFFAGDNGLILRCGGTPSSRKWLEIDQLASFDSVQDALVTLITIHLKEIIEVQSSH
TEDYTSLLFTPYNIGAAGKVRTIIKLILERSLMYTVRNWLVLPSSIRDSVRQDLELGSFRLMSI
LSEQTFLKKTPTKKYLLDQLTRTYISTFFNSHSVLPLHRPYQKQIWKALGSVIYCSETVDIPLI
RDIQIEDINDFEDIERGIDGEEL

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What is claimed:

1. A recombinant, infectious, self replicating paramyxovirus of the *Paramyxovirinae* subfamily comprising a partial or complete polyhexameric genome or antigenome comprising a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein.

2. The recombinant paramyxovirus of claim 1, further comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L).

3. The recombinant paramyxovirus of claim 3, where the paramyxovirus is a human parainfluenza virus (PIV)

4. The recombinant paramxyovirus of claim 3, wherein the HPIV is HPIV2.

5. The recombinant paramxyovirus of claims 1-4, wherein the recombinant virus has an attenuated phenotype.

6. The recombinant paramyxovirus of claim 1, wherein the variant polynucleotide encoding a P protein and the monocistronic polynucleotide encoding a V protein are separated by a non-coding polynucleotide spacer sequence comprising a gene end transcription signal, intergenic transcription signal, and gene start transcription signal.

7. The recombinant paramyxovirus of claim 6, wherein the spacer sequence is upstream of a V ORF.

8. The recombinant paramyxovirus of claim 6, wherein the gene start transcription signal is cis-acting.

9. The recombinant paramyxovirus of claim 8, wherein the gene start transcription signal comprises a first adenosine at position 6n+1.

10. The recombinant paramyxovirus of claim 1, wherein the monocistronic polynucleotide encoding a V protein comprises a nucleotide sequence encoding a variant V protein.

11. The recombinant paramyxovirus of claim 10, wherein the nucleotide sequence comprises a mutation that inhibits the ability of the V protein to interrupt production of interferon in an infected host or signaling through its receptor.

12. The recombinant paramyxovirus of claim 5, wherein the recombinant virus has an attenuated phenotype.

13. The recombinant paramyxovirus of claim 11, wherein the V protein comprises one or more amino acid substitutions at or between amino acid residues corresponding to a position 67, 68, 69, 70, 71,72, 105, 106, 107,108, 121, 122, 123,124, 125, 126,127, 130,131,132,133,134,135,136,137,138,139,140, 167, 168,169,170,171,172, or any one of the amino acids of 174-225 of SEQ ID NO:3.

14. The recombinant paramyxovirus of claim 1, wherein the monocistronic polynucleotide encoding a V gene is located at the 3' terminus of the genome.

15. The recombinant paramyxovirus of claim 11, wherein the mutation is amino acid substitution or deletion.

16. The recombinant paramyxovirus of claim 10, wherein the V protein comprises an amino acid sequence having at least 80% sequence identity with SEQ ID NO:3.

17. The recombinant paramyxovirus of claim 1, wherein the antigenome comprises a nucleotide sequence having at least 80% sequence identity with SEQ ID NO:1.

18. The recombinant paramyxovirus of claim 17, wherein the antigenome comprises a nucleotide sequence of SEQ ID NO:1.

19. The recombinant paramyxovirus of any of claims 1-18, wherein the paramyxovirus has at least one additional mutation that is an attenuating mutation.

20. The recombinant paramyxovirus of claim 19, wherein said at least one additional attenuating mutation is stabilized by at least two changes in the codon specifying the mutation.

21. The recombinant paramyxovirus of claim 19 or 20, wherein the attenuating phenotype is attenuated *in vitro* at about 37°C or greater, as compared to wild type.

22. The recombinant paramyxovirus of any of claims 19 to 21, where replication is attenuated in a mammal.

23. The recombinant paramyxovirus of claim 22, wherein the mammal is a hamster, monkey, or human.

24. The recombinant paramyxovirus of claim 1, wherein the variant polynucleotide encoding a P protein comprises a mRNA editing site having a heptaguanosine run.

25. The recombinant paramyxovirus of claim 24, wherein the mRNA editing site comprises a nucleotide sequence of SEQ ID NO:8.

26. The recombinant paramyxovirus of claim 24, wherein the heptaguanosine run is substituted such that editing of P gene mRNA is inhibited.

27. The recombinant paramyxovirus of claim 26, wherein the substitution is G9A, G12A, and G15T of SEQ ID NO:8.

28. The recombinant paramyxovirus of claim 24, wherein one or more nucleotides are inserted into the mRNA editing site such that the nucleotide sequence encoding a carboxy-terminal portion of P protein is in frame.

29. The recombinant paramyxovirus of claim 28, wherein two nucleotides are inserted after nucleotide position 2487 in SEQ ID NO:1.

30. The recombinant paramyxovirus of claim 1, wherein the variant polynucleotide encoding a P protein comprises a P ORF having one or more nucleotide substitutions wherein

the substitution(s) introduces one or more stop codons in an overlapping V ORF reading frame but does not alter an amino acid sequence of P protein encoded by the variant polynucleotide encoding a P protein.

31. The recombinant paramyxovirus of claim 30, wherein the P ORF comprises a nucleotide sequence of SEQ ID NO:2.

32. The recombinant paramyxovirus of claim 30, wherein the P protein comprises an amino acid sequence of SEQ ID NO:15.

33. The recombinant paramyxovirus of claim 1, wherein the monocistronic polynucleotide encoding a V protein comprises a mRNA editing site having a heptaguanosine run.

34. The recombinant paramyxovirus of claim 33, wherein the mRNA editing site comprises a nucleotide sequence of SEQ ID NO:8.

35. The recombinant paramyxovirus of claim 33, wherein the heptaguanosine run is substituted such that editing of the polynucleotide encoding the V protein mRNA is inhibited.

36. The recombinant paramyxovirus of claim 35, wherein the substitution is G9A and G12C of SEQ ID NO:8.

37. The recombinant paramyxovirus of claim 2, wherein the N protein comprises an amino acid sequence having at least 80% sequence identity with SEQ ID NO:16.

38. The recombinant paramyxovirus of claim 2, wherein the P protein comprises an amino acid sequence having at least 80% sequence identity with SEQ ID NO:15.

39. The recombinant paramyxovirus of claim 2, wherein the L protein comprises an amino acid sequence having at least 80% sequence identity with SEQ ID NO:17.

40. The recombinant paramyxovirus of claim 2, wherein the genome or antigenome comprises one or more attenuating mutations.

41. The recombinant paramyxovirus of claim 40, wherein at least one of the attenuating mutations is temperature sensitive (*ts*).

42. The recombinant paramyxovirus of claim 41, wherein the attenuating mutation comprises an amino acid substitution or deletion at one or more amino acid residues corresponding to position 460, 948, 1566, 1724, or 1725 of an L protein having an amino acid sequence of SEQ ID NO:17.

43. The recombinant paramyxovirus of claim 42, wherein the substitution comprises F460L, F460A, or F460P.

44. The recombinant paramyxovirus of claim 42, wherein the substitution comprises Y948A, Y948L, or Y948G.

45. The recombinant paramyxovirus of claim 42, wherein the substitution comprises L1566I.

46. The recombinant paramyxovirus of claim 42, wherein the substitution comprises S1724I.

47. The recombinant paramyxovirus of claim 42, wherein amino acid residues at positions 1724 and 1725 are deleted.

48. The recombinant paramyxovirus of claim 47, wherein S1724 and T1725 are deleted.

49. The recombinant paramyxovirus of claim 40, wherein the attenuating mutation comprises a nucleic acid substitution at a position corresponding to position 15 of a 3' leader sequence having an nucleic acid sequence of SEQ ID NO:18.

50. The recombinant paramyxovirus of claim 49, wherein the substitution comprises T15C.

51. The recombinant paramyxovirus of claim 1, wherein the genome or antigenome comprises one or more supernumerary heterologous polynucleotides or genome segments encoding one or more antigenic determinants of a heterologous pathogen.

52. The recombinant paramyxovirus of claim 51, wherein the heterologous pathogen comprises HPIV1, HPIV3, measles virus, subgroup A or subgroup B respiratory syncytial virus, mumps virus, human papilloma virus, type 1 or type 2 human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rabies virus, Epstein Barr virus, filovirus, bunyavirus, flavivirus, alphavirus, human metapneumovirus, or influenza virus.

53. The recombinant paramyxovirus of claim 51, wherein the antigenic determinant comprises measles HA.

54. The recombinant paramyxovirus of claim 51, wherein the antigenic determinant comprises HPIV1 HN or F.

55. The recombinant paramyxovirus of any of claims 51-54, wherein the paramyxovirus has an attenuated phenotype.

56. A method for producing an infectious, self-replicating, recombinant paramyxovirus comprising:

(a) coexpressing in a cell:

(i) an expression vector comprising a partial or complete polyhexameric paramyxovirus genome or antigenome comprising a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein of any of claims 1-55, and

(ii) one or more vectors comprising one or more polynucleotides encoding N protein, P protein, and L polymerase; and

(b) incubating the cell under conditions to allow for viral replication.

58. The method of claim 56, wherein the transfected cells are incubated at about 32°C.

59. The method of claim 56, wherein the transfected cells are HEp-2 cells, Vero cells, or LLC-MK2 cells.

60. The method of claim 56, where the paramyxovirus is parainfluenza virus (PIV)

61. The method of claim 60, wherein the PIV is human parainfluenza virus (HPIV).

62. The method of claim 61, wherein the HPIV is HPIV2.

63. The method of claim 56, wherein the antigenome comprises a nucleotide sequence having at least 80% sequence identity with SEQ ID NO:1.

64. The method of claim 63, wherein the antigenome comprises a nucleotide sequence of SEQ ID NO:1.

65 The method of claim 56, wherein the variant polynucleotide encoding a P protein comprises a nucleotide sequence of SEQ ID NO:2.

66. The method of claim 56, wherein the monocistronic polynucleotide encoding a V protein comprises a nucleotide sequence of SEQ ID NO:3.

67. A recombinant infectious variant human parainfluenza virus type 2 (HPIV2) comprising an amino acid substitution at one or more amino acid residues corresponding to positions 460 or 948 of L protein (SEQ ID NO:17), wherein:

(a) position 460 is substituted with A or P;

(b) position 948 is substituted with A, L, or G; or

(c) position 1724 is substituted with I.

68. The recombinant HPIV2 of claim 67, wherein the variant HPIV2 has a temperature sensitive phenotype.

69. A recombinant and infectious variant human parainfluenza virus type 2 (HPIV2) comprising an attenuating mutation, wherein the mutation comprises a nucleic acid substitution at a position corresponding to position 15 of 3' leader (SEQ ID NO:18).

70. The recombinant HPIV2 of claim 69, wherein the variant HPIV2 does not have a temperature sensitive phenotype.

71. The recombinant HPIV2 of claim 69, wherein the variant HPIV2 has a host range restriction phenotype.

72. The recombinant HPIV2 of claim 70, wherein the variant HPIV2 replicates in hamsters, but not in African green monkeys.

73. The recombinant HPIV2 of claim 70, wherein the substitution comprises T15C.

74. The recombinant HPIV2 of claim 67, further comprising an attenuating mutation in a 3' leader, wherein the mutation comprises a nucleic acid substitution at a position corresponding to position 15 of the 3' leader (SEQ ID NO:18).

75. The recombinant HPIV2 of claim 74, wherein the attenuating mutation in the 3' leader is not temperature sensitive.

76. The recombinant HPIV2 of claim 74, wherein the variant HPIV2 has a host range restriction.

77. The recombinant HPIV2 of claim 76, wherein the variant HPIV2 replicates in hamsters and not in African green monkeys.

78. The recombinant HPIV2 of claim 73, wherein the substitution comprises T15C.

79. A method for producing an infectious, self-replicating, recombinant paramyxovirus comprising:

(a) coexpressing in a cell:

(i) an expression vector comprising a partial or complete polyhexameric genome or antigenome encoding the HPIV2 of claim 67 or 69, and

(ii) one or more polynucleotides encoding N protein, P protein, and L polymerase; and

(b) incubating the cell under conditions to allow for viral replication.

80. The method of claim 79, wherein the transfected cells are incubated at about 32°C.

81. The method of claim 79, wherein the transfected cells are HEp-2 cells, Vero cells, or LLC-MK2 cells.

82. A composition comprising an immunogenic effective amount of a paramyxovirus of any one of claims 1-78 and a physiologically acceptable carrier.

83. The composition of claim 82, wherein the composition further comprises an adjuvant.

84. The composition of claim 82, wherein the composition comprises paramyxoviruses from two or more serotypes.

85. The composition of claim 84, wherein at least one of the serotypes is HPIV1, HPIV2, HPIV3, or HPIV4.

86. The composition of claim 82, wherein the composition comprises paramyxovirus from two or more strains.

87. The composition of claim 86, wherein the paramyxovirus are HPIV2.

88. The composition of claim 87, wherein at least one strain is V94, V98, or Greer.

89. The composition of claim 82, wherein the composition comprises paramyxovirus from two of more genera.

90. The composition of claim 89, wherein at least one genus is Rubulavirus genus.

91. The composition of claim 90, wherein the composition comprises HPIV2.

92. A method of eliciting an immune response in a mammal, comprising administering a composition of any of claims 82-91.

93. The method of claim 92, wherein the immune response produces anti-PIV antibodies.

94. The method of claim 93, wherein the anti-PIV antibodies are protective.

95. The method of claim 94, wherein the antibodies are IgA.

96. The method of claim 92, wherein the immune response produces antibodies that bind one or more antigenic determinants of a heterologous pathogen encoded by the supernumerary genes or genome segments.

97. The method of claim 96, wherein the heterologous pathogen comprises HPIV1, HPIV3, measles virus, subgroup A or subgroup B respiratory syncytial virus, mumps virus, human papilloma virus, type 1 or type 2 human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rabies virus, Epstein Barr virus, filovirus, bunyavirus, flavivirus, alphavirus, human metapneumovirus, or influenza virus.

98. The method of claim 96, wherein the antigenic determinant comprises measles HA.

99. The method of claim 96, wherein the antigenic determinant comprises HPIV1 HN or F.

100. The method of claim 96, wherein the antigenic determinant comprises HPIV3 HN or F.

101. The method of any one of claims 96-100, wherein the antibodies are protective.

102. A method of inhibiting parainfluenza virus (PIV) infection, comprising administering a composition of claim 82-91.

103. The method of claim 100, wherein the PIV is HPIV2.

104 The method of claim 100 or 101, wherein the composition elicits anti-PIV antibodies.

105 The method of claim 104, wherein the anti-PIV antibodies are protective.

106. The method of claim 104, wherein the antibodies are IgA.

107. A method of generating an infectious, self-replicating paramyxovirus comprising:

(a) removing a bicistronic polynucleotide encoding P and V protein from viral genome or antigenome of the paramyxovirus; and

(b) inserting a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein, wherein the variant polynucleotide encoding a P protein comprises a mutated mRNA editing site such that editing of mRNA encoding P protein is inhibited and the monocistronic polynucleotide encoding a V protein comprises a mutated mRNA editing unit such that editing of mRNA encoding V protein is inhibited.

108. The method of claim 107, wherein the variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein are separated by a non-coding polynucleotide spacer sequence comprising a gene end transcription signal, intergenic transcription signal, and gene start transcription signal

109. The method of claim 107, wherein removing comprises:

(a) introducing unique restriction enzyme recognition sequences into the genome or antigenome such that the recognition sequences flank the bicistronic polynucleotide, and

(b) digesting the genome with one or more restriction enzymes that cut the genome at the restriction sites flanking the bicistronic polynucleotide.

110. The method of claim 109, wherein inserting comprises:

(a) inserting the variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein at the cleaved restriction sites, and

(b) religating the genome or antigenome.

111. The method of claim 107, wherein the paramyxovirus is attenuated.

112. A recombinant, infectious, self replicating paramyxovirus of the *Paramyxovirinae* subfamily comprising at least one temperature sensitive mutation and at least one non-temperature sensitive mutation, wherein at least one non-temperature sensitive mutation comprises a nucleic acid substitution at a position corresponding to position 15 of 3' leader (SEQ ID NO:18).

113. The recombinant paramyxovirus of claim 112, wherein the temperature sensitive mutation comprises an amino acid substitution or deletion at one or more amino acid residues corresponding to position 460, 948, 1566, 1724, or 1725 of an L protein having an amino acid sequence of SEQ ID NO:17.

114. The recombinant paramyxovirus of claim 112, wherein the substitution comprises F460L, F460A, or F460P.

115. The recombinant paramyxovirus of claim 112, wherein the substitution comprises Y948H, Y948A, Y948L, or Y948G.

116. The recombinant paramyxovirus of claim 112, wherein the substitution comprises L1566I.

117. The recombinant paramyxovirus of claim 112, wherein the substitution comprises S1724I.

118. The recombinant paramyxovirus of claim 112, wherein amino acid residues at positions 1724 and 1725 are deleted.

119. The recombinant paramyxovirus of claim 118, wherein S1724 and T1725 are deleted.

120. The recombinant paramyxovirus of claims 112-119, wherein the paramyxovirus comprises a partial or complete polyhexameric genome or antigenome having a variant polynucleotide encoding a P protein and a monocistronic polynucleotide encoding a V protein

121. The recombinant paramyxovirus of claim 120, wherein the monocistronic polynucleotide encoding a V protein comprises a nucleotide sequence encoding a variant V protein.

122. The recombinant paramyxovirus of claim 121, wherein the nucleotide sequence comprises a mutation that inhibits the ability of the V protein to interrupt production of interferon in an infected host or signaling through its receptor.

123. The recombinant paramyxovirus of claim 122, wherein the V protein comprises one or more amino acid substitutions at amino acid residues corresponding to a position 67, 68, 69, 70, 71,72, 105, 106, 107,108, 121, 122, 123,124, 125, 126,127, 130,131,132,133,134,135,136,137,138,139,140, 167, 168,169,170,171,172, or any one of the amino acids of 174-225 of SEQ ID NO:3.

124. The recombinant paramyxovirus of claim 122 or 123, wherein the mutation is amino acid substitution or deletion.

125. The recombinant paramyxovirus of any one of claims 120-124, wherein the polynucleotide encoding a V protein comprises a nucleotide sequence having at least 80% sequence identity with SEQ ID NO:3.

126. The recombinant paramyxovirus of claims 112-125, wherein the antigenome comprises a nucleotide sequence having at least 80% sequence identity with SEQ ID NO:1.

127. A vector comprising a partial polyhexameric paramyxovirus genome or antigenome comprising a variant polynucleotide encoding a P protein.

128. A vector comprising a partial polyhexameric paramyxovirus genome or antigenome comprising a monocistronic polynucleotide encoding a V protein.

129. An isolated nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO: 1.

130. An isolated nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO:2.

131. An isolated nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO:3.

132. An isolated nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a V polypeptide having a sequence of SEQ ID NO:45.

133. An isolated nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a L polypeptide having a sequence of SEQ ID NO:17.

134. A vector comprising the polynucleotide of any of claims 128-132.

135. A method of producing a paramxyovirus polypeptide comprising culturing a host cell comprising a polynucleotide of any of claims 128-132 under conditions suitable for expression of the polynucleotide.

136. An isolated V polypeptide comprising at least one mutation at an amino acid residue corresponding to a position 67, 68, 69, 70, 71, 72, 105, 106, 107, 108, 121, 122, 123, 124, 125, 126, 127, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 167, 168, 169, 170, 171, 172, or mixtures thereof, of a polypeptide having a sequence of SEQ ID NO:45.

137. An isolated L polypeptide comprising at least one mutation at an amino acid residue corresponding to a position 460, 948, 1566, 1724 of an L protein having an amino acid sequence of SEQ ID NO:17.

138. The isolated L polypeptide of claim 136, comprising one or more of the following mutations: position 460 is substituted with A or P; position 948 is substituted with A, L or G; or position 1724 is substituted with I.

139. A vector comprising a polynucleotide encoding a variant polynucleotide encoding a P protein.

140. A vector comprising a polynucleotide encoding a monocistronic polynucleotide encoding a V protein.

141. A vector comprising a nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO: 1.

142. A vector comprising a nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO:2.

143. A vector comprising a nucleic acid comprising a polynucleotide having a sequence of SEQ ID NO:3.

144. A vector comprising a nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a V polypeptide having a sequence of SEQ ID NO:45.

145. A vector comprising a nucleic acid comprising a polynucleotide encoding a polypeptide having at least 80% sequence identity to a L polypeptide having a sequence of SEQ ID NO:17.

146. A vector comprising one or more nucleic acids comprising a polynucleotide encoding a N polypeptide, a polypeptide having at least 80% sequence identity to a L polypeptide having a sequence of SEQ ID NO:17, or a P polypeptide or mixtures thereof..

147. A vector of claim 146 , further comprising a nucleic acid comprising a polynucleotide encoding a M protein.

148. A vector of claim 147 , further comprising a nucleic acid comprising a polynucleotide encoding a HN protein.

149. A vector of claim 148 , further comprising a nucleic acid comprising a polynucleotide encoding a F protein.

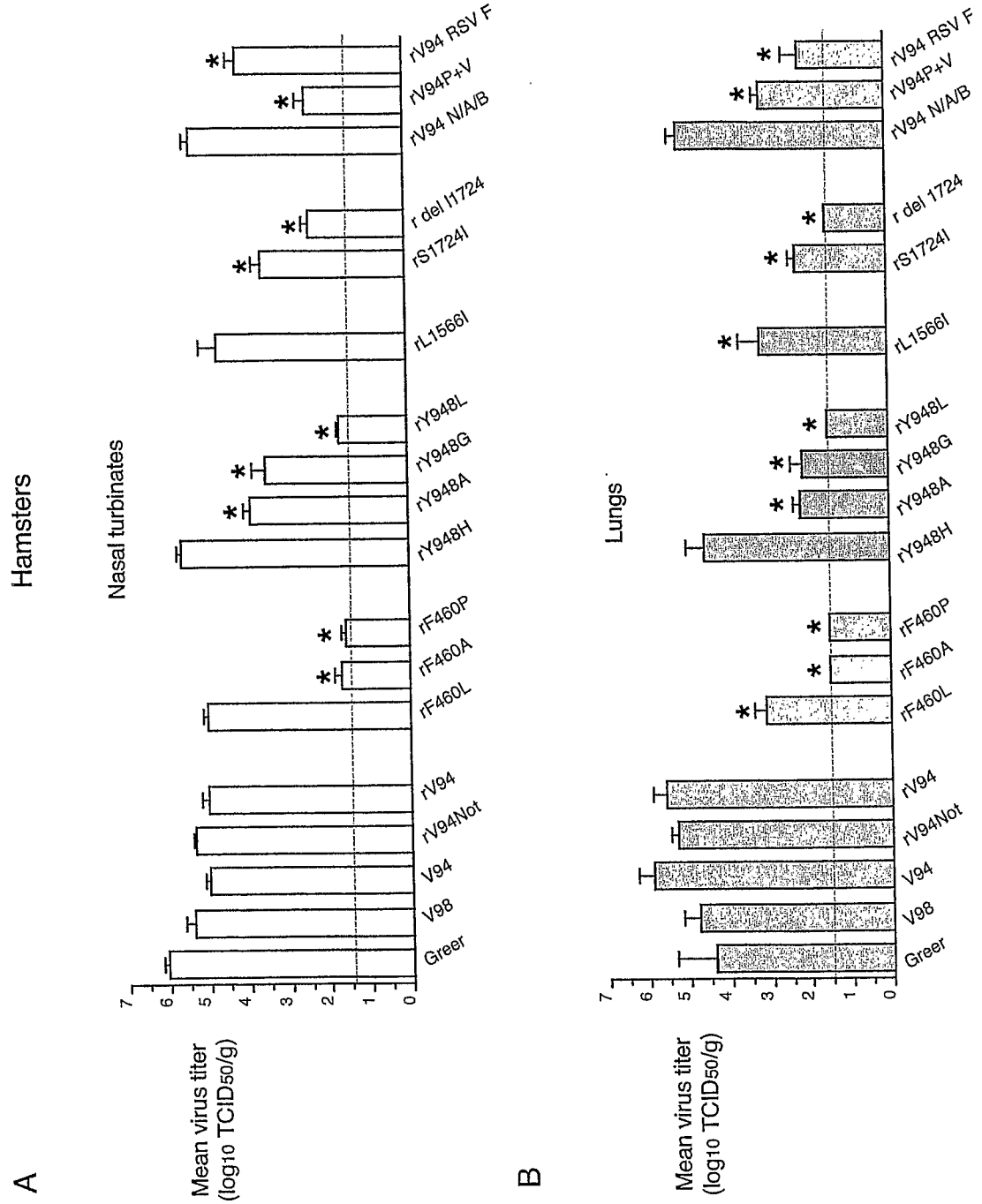

A
Hamsters
Nasal turbinates
Mean virus titer
(log10 TCID50/g)
0
1
2
3
4
5
6
7
Greer
V98
V94
rV94Not
rV94
rF460L
rF460A
rF460P
rY948H
rY948A
rY948G
rY948L
rL1566I
rS1724I
r del I1724
rV94 N/A/B
rV94P+V
rV94 RSV F
B
Lungs
Mean virus titer
(log10 TCID50/g)
0
1
2
3
4
5
6
7
Greer
V98
V94
rV94Not
rV94
rF460L
rF460A
rF460P
rY948H
rY948A
rY948G
rY948L
rL1566I
rS1724I
r del 1724
rV94 N/A/B
rV94P+V
rV94 RSV F

FIGURE 1

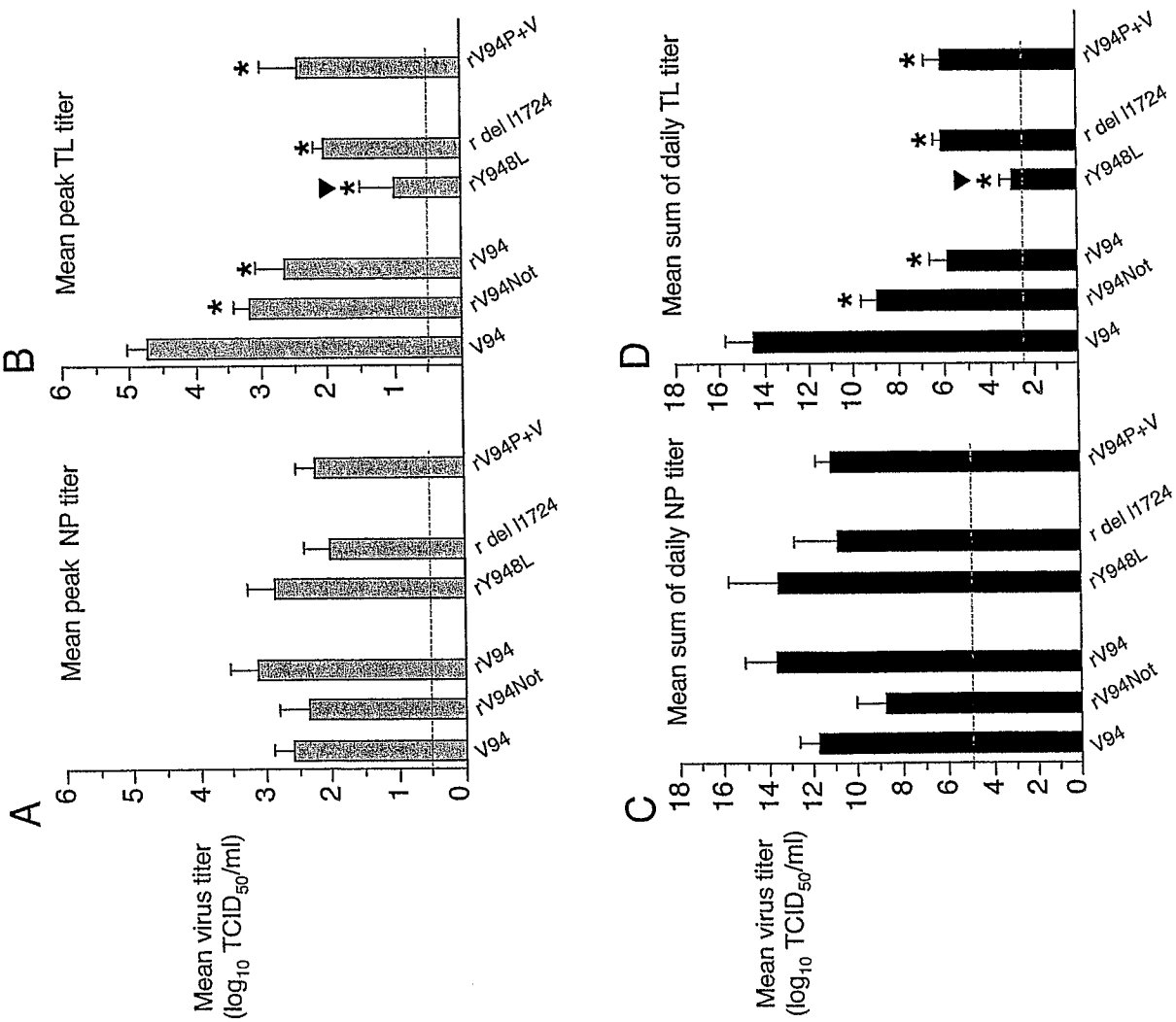

A
Mean peak NP titer
Mean virus titer
(log10 TCID50/ml)
B
Mean peak TL titer
C
Mean sum of daily NP titer
D
Mean sum of daily TL titer
V94
rV94Not
rV94
rY948L
r del I1724
rV94P+V

FIGURE 2 A-D

A

	1 15 30
V98	ACCAAGGGGA GAAT**T**AGATG GCATCGTTAT
V94(a)	ACCAAGGGGA GAAT**T/C**AGATG GCATCGTTAT
V94(b)	ACCAAGGGGA GAAT**C**AGATG GCATCGTTAT
rV94	ACCAAGGGGA GAAT**C**AGATG GCATCGTTAT

B

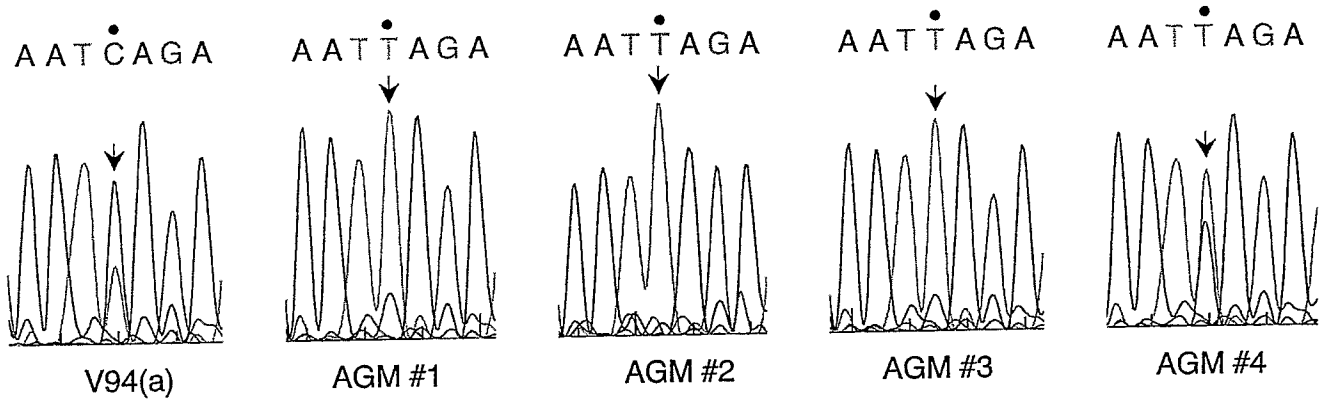


FIGURE 3

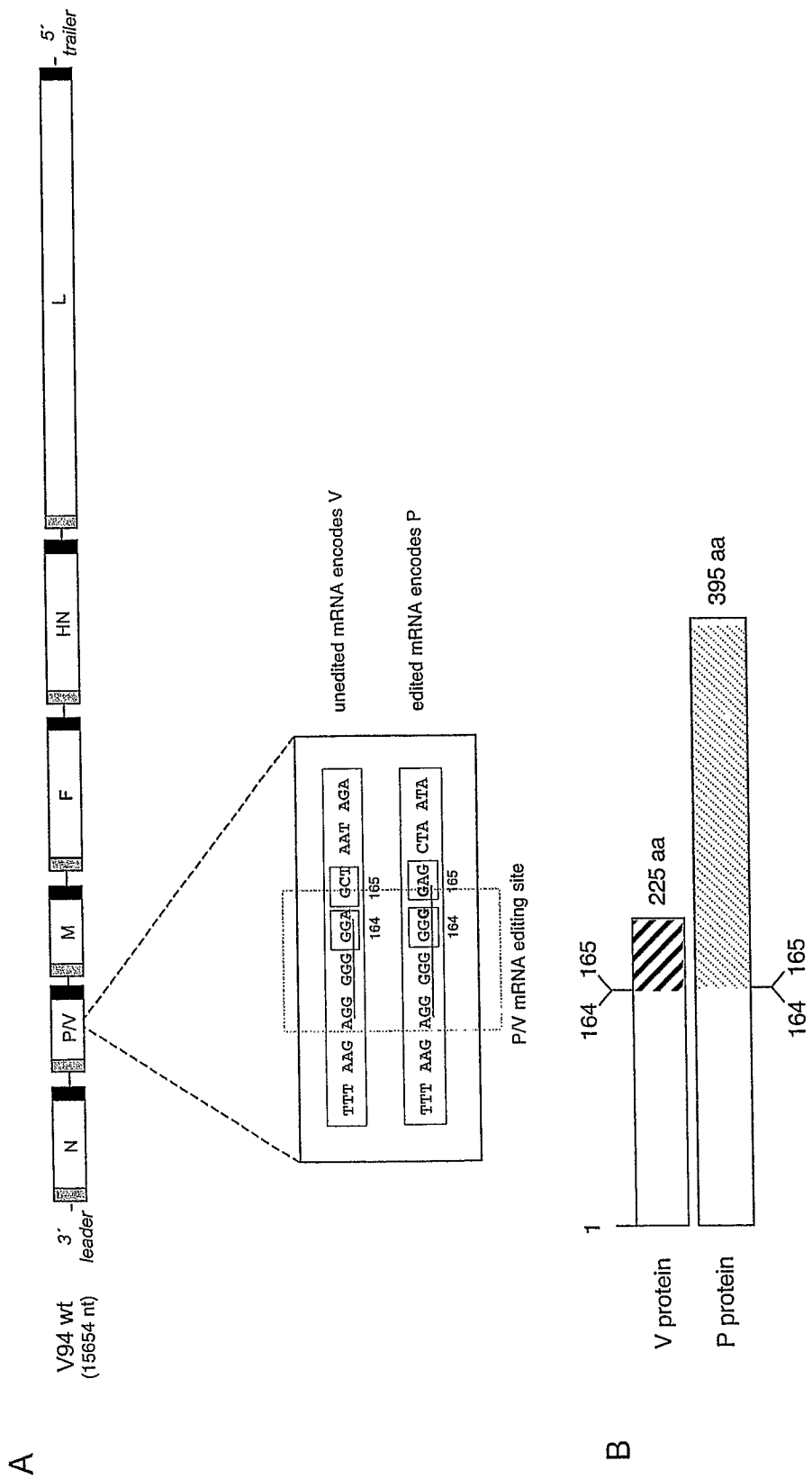
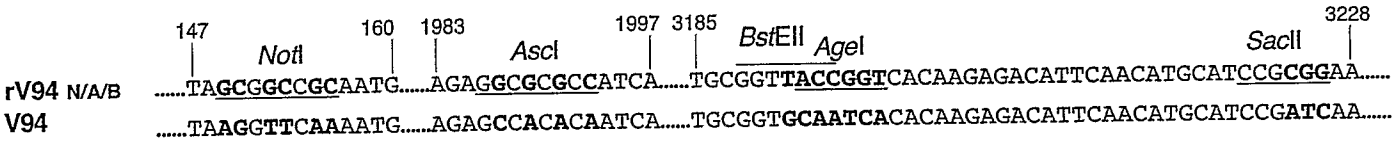

A
V94 wt
(15654 nt)
3′
leader
N
P/V
M
F
HN
L
5′
trailer
unedited mRNA encodes V
edited mRNA encodes P
TTT AAG AGG GGG GGA GCT AAT AGA
TTT AAG AGG GGG GAG CTA ATA
164
165
P/V mRNA editing site
B
1
164
165
225 aa
395 aa
V protein
P protein

FIGURE 4

A



B

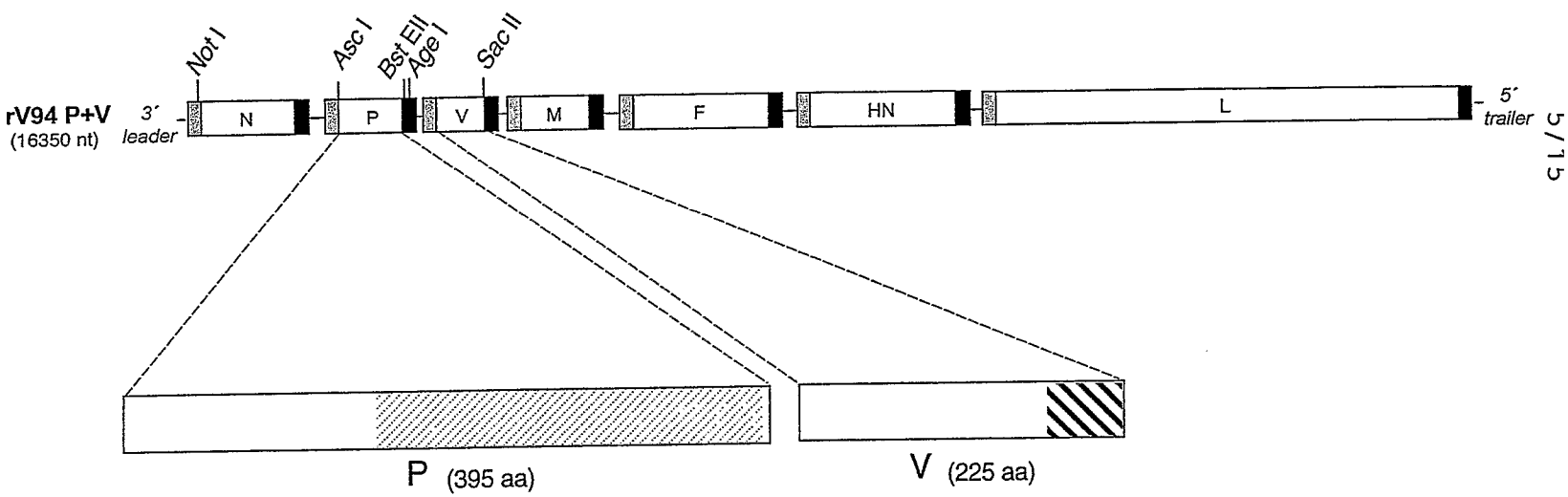


FIGURE 5 A, B

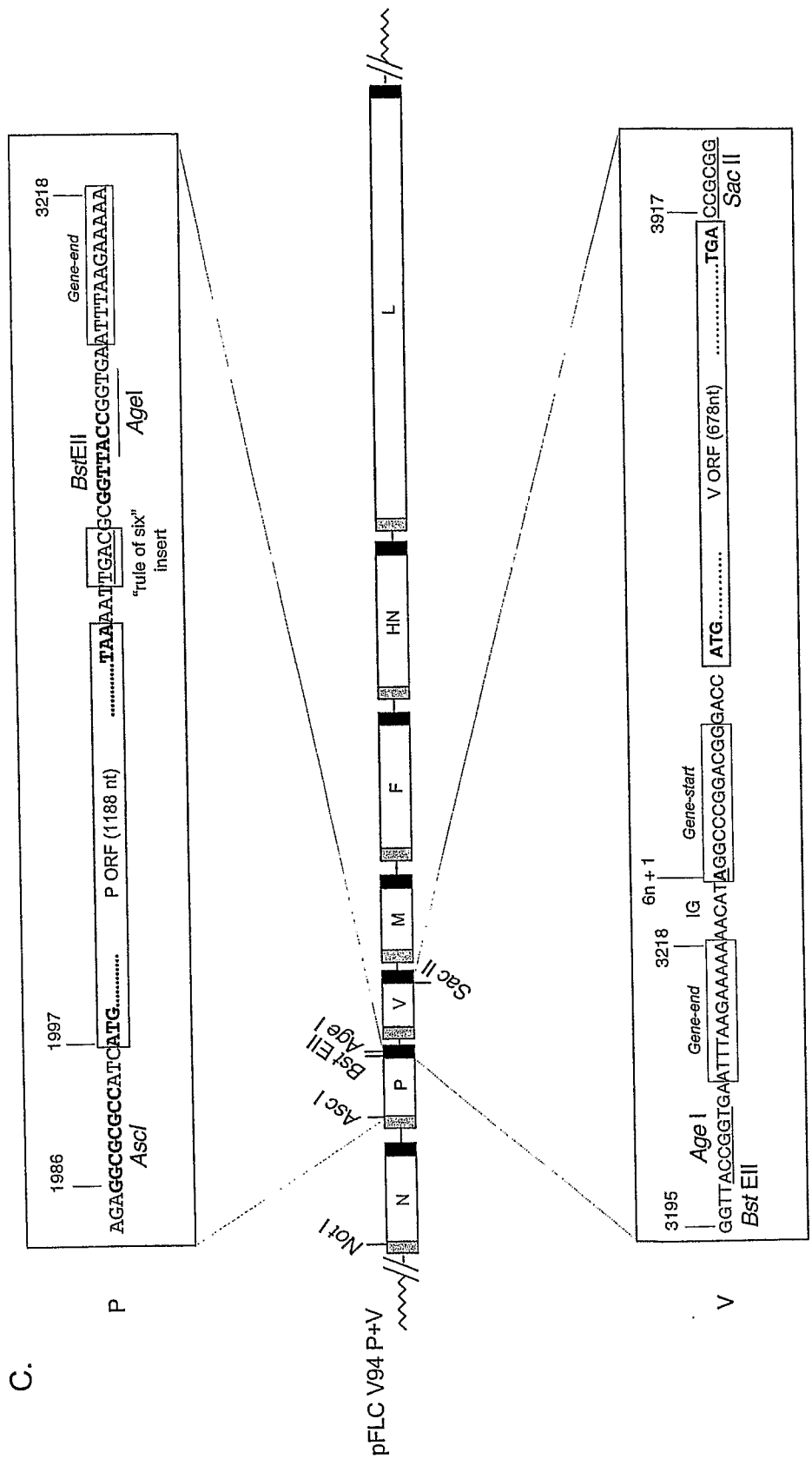

C.
P
1986
AscI
AGAGGCGCGCCATCATG.....
1997
P ORF (1188 nt)
.....TAAAATTGACGCGGTTACCGGTGAATTTAAGAAAAA
"rule of six" insert
BstEII
AgeI
Gene-end
3218
pFLC V94 P+V
N
P
M
F
HN
L
V
NotI
AscI
BstEII
AgeI
SacII
V
3195
Age I
GGTTACCGGTGAATTTAAGAAAAAAACATAGGCCCGGACGGGACC
Bst EII
3218
Gene-end
IG
6n + 1
Gene-start
ATG.....
V ORF (678nt)
.....TGA
3917
CCGCGG
Sac II

FIGURE 5C

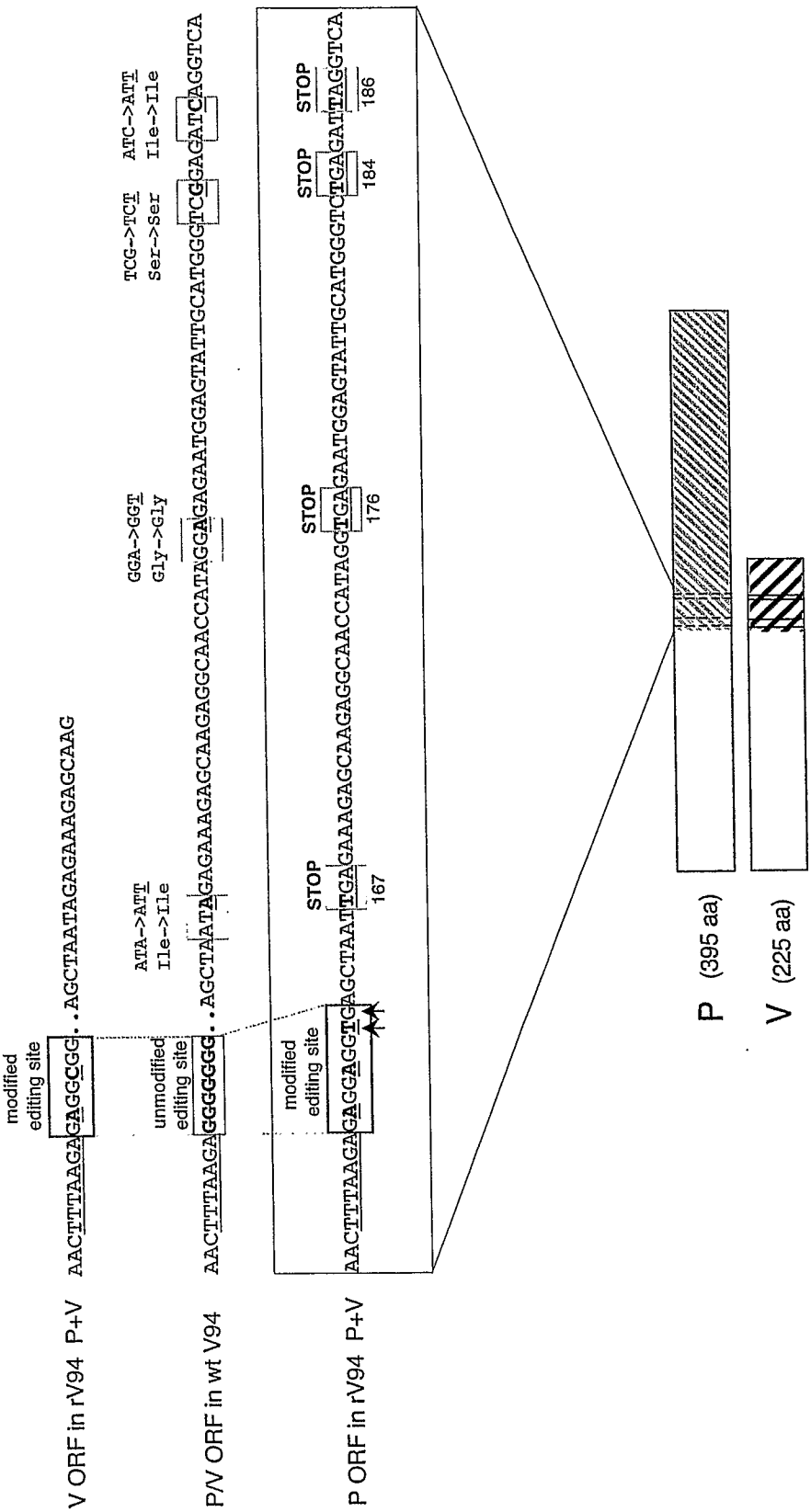
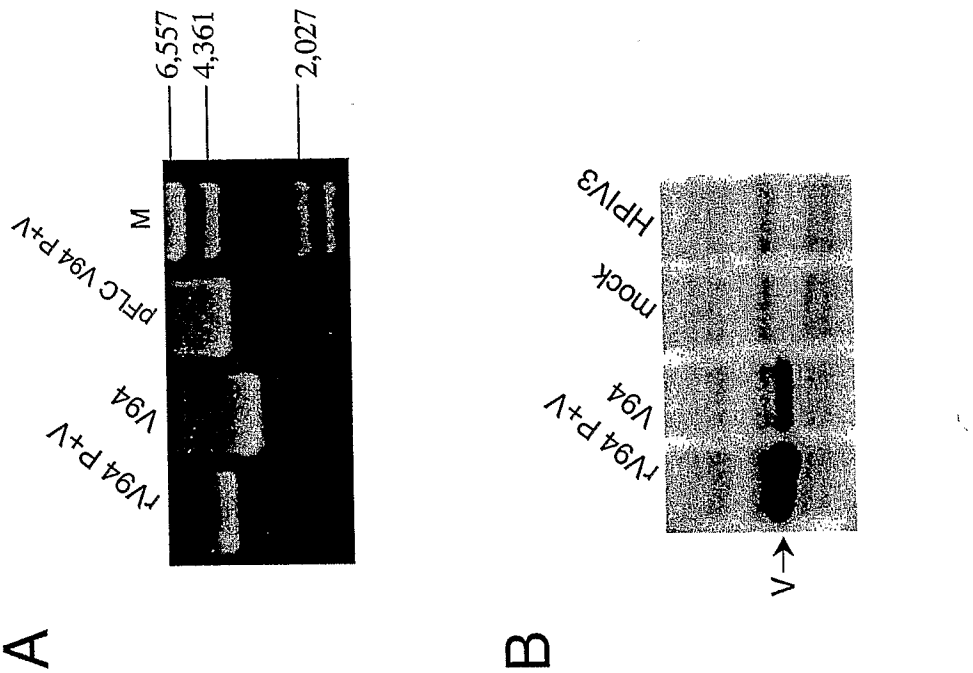

V ORF in rV94 P+V
AACTTTAAGAGAGGCGG..AGCTAATAGAGAAAGAGCAAG
modified editing site
P/V ORF in wt V94
AACTTTAAGAGGGGGGG..AGCTAATAGAGAAAGAGCAAGAGGCAACCATAGGAGAATGGAGTATTGCATGGGTCGGATCAGGTCA
unmodified editing site
ATA->ATT
Ile->Ile
GGA->GGT
Gly->Gly
TCG->TCT
Ser->Ser
ATC->ATT
Ile->Ile
P ORF in rV94 P+V
AACTTTAAGAGAGGTGAGCTAATTGAGAAAGAGCAAGAGGCAACCATAGGTGAGAATGGAGTATTGCATGGGTCTGAGATTAGGTCA
modified editing site
STOP
167
STOP
176
STOP
184
STOP
186
P (395 aa)
V (225 aa)

FIGURE 6

FIGURE 7


A
6,557
4,361
2,027
M
PFLC V9a P+V
V9a
rV9a P+V
B
HPIV3
mock
V9a
rV9a P+V
V

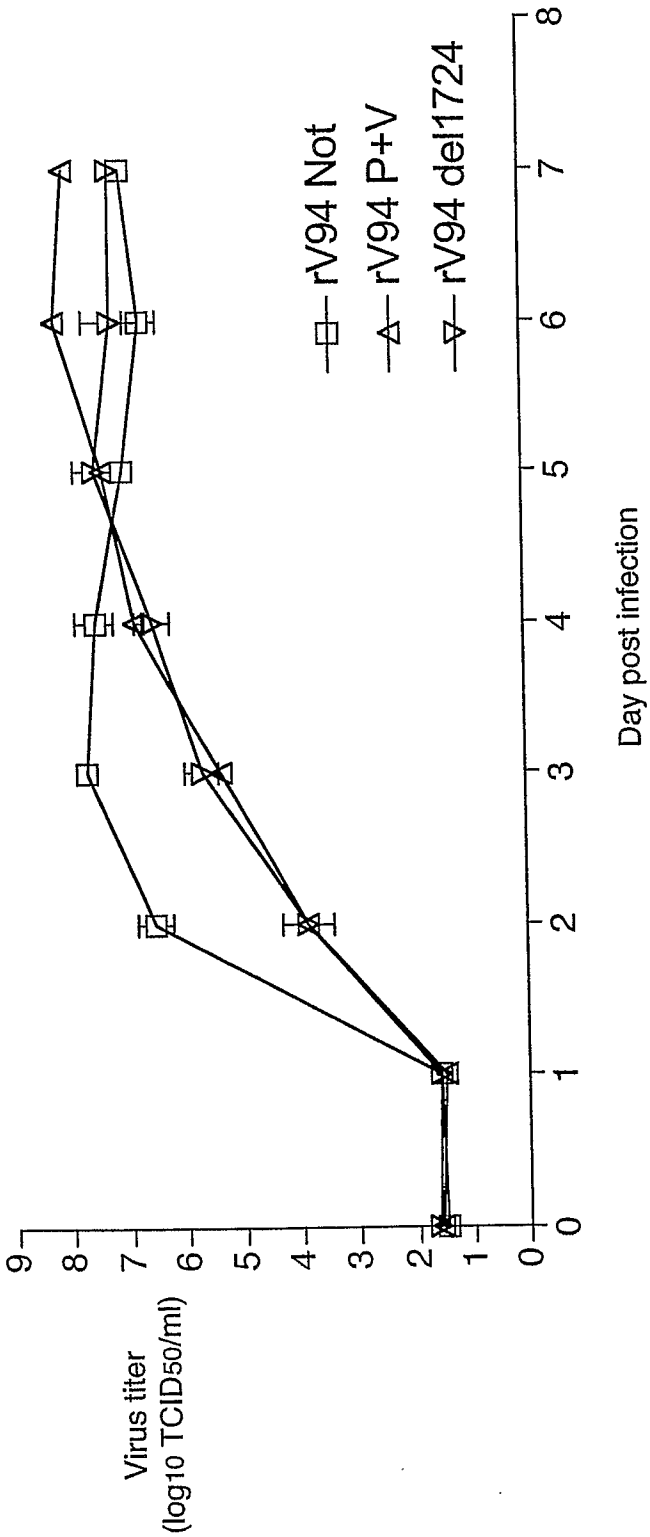

rV94 Not
rV94 P+V
rV94 del1724
Virus titer
(log10 TCID50/ml)
Day post infection
0
1
2
3
4
5
6
7
8
9

FIGURE 8

A

```
1                   20                  40                  60
MAEEPTYTTEQVDELIHAGL GTVDFFLSRPIDAQSSLGKG SIPPGVTAVLTNAAEAKSKP

          I         80                  100       II        120
VAAGPVKPRRKKVISNTTPY TIADNIPPEKLPINTPIPNP LLPLARPHGKMTDIDIVTGN

   III      IV   140                 160     *       V   180
ITEGSYKGVELAKLGKQTLL TRFTSNEPVSSAGSAQDPNF KRGGANRERARGNHRREWSI

                  200           VI    220   225
AWVGDQVKVFEWCNPRCAPV TASARKFTCTCGSCPSICGE CEGDH
```

B

```
                  *                 *  *                 *  *  *  *  *
HPIV2/V94       174 HRREWSIAWVGDQVKVFEWCNPRCAPVTASARKFTCTCGSCPSICGECEGD 224 AF533010
SV41            174 HRREWSIAWVGDEVKVYEWCNPTCAPVTATDRKFSCTCGTCPDRCGECEGD 224 P363153
SV5             171 HRREYSIGWVGDEVKVTEWCNPSCSPITAAARRFECTCHQCPVTCSECERD 221 P11207
Mumps virus     170 HRREWSLSWVQGEVRVFEWCNPICSPITAAARFHSCKCGNCPAKCDQCERD 220 AAL83741
PIV4A           178 HRREYSISWVNGRTTISEWCNPCCAPVKSTASVEKCTCGRCPKICELCIRD 228 P21739
PIV4B           178 HRREHSISWVNGRTTISEWCNPCCAPVKSIASVEKCTCGRCPKICELCIRD 228 P21740
NDV             176 HRREHSISWTMGGVTTISWCNPSCSPIRAEPRQYSCTCGSCPATCRLCASD 226 JQ2393
Tioman virus    164 HRREIAISWATGTPRVTEWCNPICHPISQFTYRGTCRCGCCPDVCSLCERD 214 NP_665866
Menangle virus  166 HRREIAIDWIGGRPRVTEWCNPICHPISQSTFRGSCRCGNCPGICSLCERD 216 AAK62279
Porcine rubulavirus 181 HRREYSIGWVCGTVRVLEWCNPACSPISMEPRYYQCTCGTCPAKCPQCAGD 231 AAL09693
CDV             232 HRREVSLTWNGDSCWIDKWCNPICTQVNWGIIRAKCVCGECPPTCSECKDD 282 AAQ96302
PDV             232 HRREVSLTWNDDRCWIDKWCNPICTQVNWGVIRAKCICGECPPVCDDCKDD 282 P35941
Rinderpest virus 232 HRREIDLIWNDGRVFIDRWCNPTCSKVTVGTVRAKCICGECPRVCEQCITD 282 Q03340
Measles virus   232 HRREISLIWNGDRVFIDRWCNPMCSKVTLGTIRARCTCGECPRVCEQCRTD 282 Q9IC37
Salem virus     252 HRREYSIIWDSEGIQIESWCNPVCSKVRSTPRREKCRCGKCPARCSECGDD 302 AAF63742
Fer-de-lance virus 170 HRREISTSTIDGIFEVWEFCNPMCSRITPDFKPKICVCGECPRYCPRCKEQ 220 NP_899657
BPIV3Ka         348 HRREHSIYRKGDYIITESWCNPICSKIRPIPRQESCVCGECPKQCRYCIKD 398 NC_002161
Sendai virus    318 HRREHIIYERDGYIVNESWCNPVCSRIRVISRRELCVCKACPKICKLCRDD 368 BAB20021
Mossman virus   244 HRREYNFVWTDSGFRVEAWCNPICSRIRNLPRREKCRCGWCPKECPECALG 294 NP_958050
Tupaia paramyxovirus 230 HRREYSMVWSNDGVFIESWCNPMCARIRPLPIREICVCGRCPLKCSKCLLD 280 NP_054692
Hendra virus    406 HRREVSICWDGRRAWVEEWCNPVCSRITPQPRKQECYCGECPTECSQCCHE 456 NP_047108
Nipah virus     408 HRREISICWDGKRAWVEEWCNPACSRITPLPRRQECQCGECPTECFHCG   456 AAM13407
```

FIGURE 9

1 ACCAAGGGGA GAATCAGATG [illegible]
101 TATACTATCT GATCATTCTT TATCTCTACT AAGGATATTT CTAATCTAAG GTTCAAAATG TCAAGTGTCT TAAAGACATT TGAAAGATTT ACTATACAAC 200
201 AGGAGCTTCA GGAGCAATCT GAAGACACTC CAATACCTCT TGAAACAATC AGACCTACAA TCAGAGTATT TGTCATCAAT AATAATGATC CTATTGTAAG 300
301 ATCTAGACTT TTATTCTTTA ATCTACGAAT TATTATGAGT AACACTGCAA GAGAGGGACA TAGAGCTGGT GCTCTCCTCA GTCTTTTATC ACTACCTTCT 400
401 GCAGCTATGA GTAATCACAT CAAACTAGCC ATGCATTCAC CAGAAGCCAG CATAGATAGA GTAGAAATAA CAGGGTTTGA GAATAATTCA TTCCGAGTTA 500
501 TTCCAGATGC TCGATCAACT ATGTCCAGAG GAGAAGTGCT GGCCTTCGAA GCATTAGCTG AGGACATTCC TGATACCCTT AATCACCAAA CTCCATTTGT 600
601 AAATAATGAT GTGGAAGATG ACATATTTGA TGAAACAGAG AAATTCTTGG ATGTTTGCTA TAGTGTACTT ATGCAGGCAT GGATAGTAAC ATGCAAGTGC 700
701 ATGACTGCTC CTGATCAACC ACCAGTATCA GTAGCAAAGC GGATGGCTAA ATATCAACAA CAAGGGAGAA TCAATGCTAG ATATGTACTA CAACCTGAAG 800
801 CACAAAGACT AATTCAGAAT GCCATCCGCA AGTCAATGGT AGTAAGGCAT TTCATGACCT ATGAGCTTCA ACTTTCACAA TCAAGATCTT TGCTAGCGAA 900
901 CCGTTATTAT GCCATGGTGG GAGACATTGG CAAGTATATT GAACACAGCG GAATGGGAGG GTTTTTCTTA ACACTTAAAT ATGGACTTGG AACAAGATGG 1000
1001 CCTACATTGG CTCTTGCAGC ATTCTCTGGG GAACTCCAGA AATTAAAGGC TCTCATGCTA CATTATCAGA GTCTAGGACC CATGGCCAAG TACATGGCTC 1100
1101 TATTAGAATC ACCAAAGCTG ATGGATTTTG TCCCATCTGA ATATCCATTA GTTTATAGCT ATGCAATGGG TATTGGAACT GTCCTTGATA CAAACATGAG 1200
1201 AAACTATGCA TATGGTAGAT CATATCTAAA TCCACAATAT TTTCAGCTAG GGGTAGAAAC AGCAAGGAAA CAGCAAGGAG CTGTTGACAA CAGGACAGCA 1300
1301 GAGGACCTCG GCATGACTGC TGCAGATAAA GCAGACCTCA CTGCAACCAT ATCAAAGCTA TCTTTATCCC AATTACCTAG GGGTAGACAA CCAATATCCG 1400
1401 ACCCATTTGC TGGAGCAAAT GACAGAGAAA CAGGAGGACA AGCAACTGAT ACACCTGTGT ATAACTTCAA TCCAATCAAT AATCGGAGGT ATGACAACTA 1500
1501 TGACAGTGAT AGTGAGGACA GAATTGACAA CGATCAAGAT CAGGCTATCA GAGAGAACAG AGGAGAACCT GGACAACCAA ACAACCAGAC AAGCGAAAAC 1600
1601 CAGCAGAGAC TCAATCTCCC TGTACCGCAA AGAACATCAG GTATGAGTAG TGAAGAGTTC CAACATTCAA TGAATCAGTA CATCCGTGCT ATGCATGAGC 1700
1701 AATACAGAGG CTCCCAGGAT GATGATGCCA ATGATGCCAC AGATGGGAAT GACATTTCAC TTGAGCTAGT TGGAGATTTT GATTCCTAAC TCTCACTTTC 1800
1801 ACATAACCAG ACATACACAT CCACACCACC CAGAGACATA GCTACCATCC ACACACTCAC CCAGACAAAT CAAACTAGAT TCAAATCATT CGGAAACAAT 1900
1901 TCTCCTAGAA TTTAAGAAAA AAACATAGGC CCGGACGGGT TAGAGATCCG GTGCTCGTCT GTGGCCAGAC AACCTCCACA CCAGAGGCGC GCCATCATGG 2000
2001 CCGAGGAACC AACATACACC ACTGAGCAAG TTGATGAATT AATCCATGCT GGACTAGGAA CAGTAGATTT CTTCCTATCT AGACCCATAG ATGCTCAGTC 2100
2101 TTCTTTAGGT AAAGGCAGCA TCCCACCAGG TGTCACGGCT GTTCTAACCA ATGCAGCAGA GGCAAAATCC AAACCAGTTG CTGCTGGTCC AGTAAAACCC 2200
2201 AGACGGAAGA AAGTGATCAG CAATACCACT CCATACACTA TTGCAGACAA CATCCCACCT GAGAAGCTAC CGATCAACAC TCCAATACCC AATCCATTAC 2300
2301 TTCCACTGGC ACGCCCTCAC GGAAAGATGA CAGACATTGA CATTGTCACT GGGAACATTA CAGAAGGATC ATACAAAGGT GTGGAGCTTG CCAAATTAGG 2400
2401 GAAGCAAACA CTACTCACAA GGTTCACCTC GAATGAGCCA GTCTCCTCAG CTGGATCCGC CCAAGACCCC AACTTTAAGA GAGGAGGTGA GCTAATAGAG 2500
2501 AAAGAGCAAG AGGCAACCAT AGGAGAGAAT GGAGTATTGC ATGGGTCGGA GATCAGGTCA AAGTCTTCGA GTGGTGTAAT CCCAGGTGTG CCCCAGTCAC 2600
2601 GGCTTCAGCT CGCAAGTTCA CCTGCACATG TGGATCCTGC CCCAGCATCT GCGGAGAATG TGAAGGAGAT CATTGAGCTC TTAAAAGGGC TTGATCTTCG 2700
2701 CCTTCAGACT GTAGAAGGGA AAGTAGATAA AATTCTTGCA ACCTCTGCAA CTATAATCAA TCTTAAAAAT GAAATGACTA GTCTTAAGGC GAGCGTTGCA 2800
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2901 TAGTAATTGC TGGTCCGACT AGTGGAGGCT TCACAGCCGA AGGCAGTGAC ATGATTTCAA TGGATGAACT AGCTAGGCCT ACACTCTCAT CAACAAAAAA 3000
3001 GATCACACGA AAGCCTGAAT CCAAGAAAGA TTTAACAGGC ATAAAACTAA CCCTGATGCA GCTTGCAAAT GACTGCATCT CGCGTCCAGA TACCAAGACT 3100
3101 GAGTTTGTGA CTAAGATTCA AGCAGCAACC ACAGAATCAC AGCTCAACGA AATCAAACGG TCAATAATAC GCTCTGCAAT ATAAAATTGA CGCGGTTACC 3200
3201 GGTGAATTTA AGAAAAAAAC ATAGGCCCGG ACGGGACCAT GGCCGAGGAA CCAACATACA CCACTGAGCA AGTTGATGAA TTAATCCATG CTGGACTAGG 3300
3301 AACAGTAGAT TTCTTCCTAT CTAGACCCAT AGATGCTCAG TCTTCTTTAG GTAAAGGCAG CATCCCACCA GGTGTCACGG CTGTTCTAAC CAATGCAGCA 3400
3401 GAGGCAAAAT CCAAACCAGT TGCTGCTGGT CCAGTAAAAC CCAGACGGAA GAAAGTGATC AGCAATACCA CTCCATACAC TATTGCAGAC AACATCCCAC 3500
3501 CTGAGAAGCT ACCGATCAAC ACTCCAATAC CCAATCCATT ACTTCCACTG GCACGCCCTC ACGGAAAGAT GACAGACATT GACATTGTCA CTGGGAACAT 3600
3601 TACAGAAGGA TCATACAAAG GTGTGGAGCT TGCCAAATTA GGGAAGCAAA CACTACTCAC AAGGTTCACC TCGAATGAGC CAGTCTCCTC AGCTGGATCC 3700
3701 GCCCAAGACC CCAACTTTAA GAGAGGCGGA GCTAATAGAG AAAGAGCAAG AGGCAACCAT AGGAGAGAAT GGAGTATTGC ATGGGTCGGA GATCAGGTCA 3800
3801 AAGTCTTCGA GTGGTGTAAT CCCAGGTGTG CCCCAGTCAC GGCTTCAGCT CGCAAGTTCA CCTGCACATG TGGATCCTGC CCCAGCATCT GCGGAGAATG 3900
3901 TGAAGGAGAT CATTGACCGC GGAAGATCCA AACTCCTTCC ATCCGAAAAC ACACTCACCA CTGTCAACAC CAAGAAACAA CTACAGCCGA ACCATGCTCA 4000
4001 ACCAAAAGAC CCAAACAACA TCTCAAATCG ACAGAAGGCT AGACATGATA AATTTAATAA AAAATTAAAA GAAGTTAAGT AAAATTTAAA GAACACAATA 4100
4101 GAGAAAACCT AGGTCCGAAA GCTTGCCTTT CAGACAGATC CCAAAATCAT AGTTCAAACT TCAAACACAG CAGCAGACAT GCCTATAATA TCATTACCAG 4200
4201 CAGATCCAAC TTCACCCAGT CAATCCCTTA CTCCGTTTCC AATACAACTT GATACCAAAG ATGGCAAGGC AGGGAAACTC CTTAAACAGA TTAGAATTAG 4300
4301 GTATCTAAAT GAACCTAACT CTCGTCATAC ACCAATAACT TTCATCAATA CGTATGGATT TGTTTATGCT CGAGACACTT CAGGAGGCAT TCACAGCGAG 4400
4401 ATCAGCAGTG ACCTAGCTGC AGGGTCCATA ACGGCATGCA TGATGACACT AGGTCCTGGT CCAAATATTC AGAATGCAAA TCTAGTGCTA AGATCCCTGA 4500
4501 ATGAATTCTA CGTAAAAGTC AAGAAGACAT CAAGCCAGAG GGAGGAAGCA GTGTTTGAAT TAGTTAACAT TCCAACCTTA TTGAGAGAAC ATGCTCTTTG 4600
4601 CAAACGCAAA ACGTTAGTAT GCTCTGCAGA AAAATTCCTC AAGAACCCAT CAAAGCTACA AGCTGGATTT GAATATGTAT ACATCCCAAC TTTTGTCTCC 4700
4701 ATTACATACT CACCACGAAA TCTGAATTAC CAAGTTGCCA GACCTATCCT TAAGTTCAGA TCACGCTTTG TGTATAGCAT TCATTTGGAA TTAATCCTGA 4800
4801 GATTGCTATG CAAATCTGAC TCCCCTTTGA TGAAATCTTA TAATGCAGAT CGAACAGGTC GAGGATGCCT CGCATCAGTC TGGATCCACG TATGTAACAT 4900
4901 TCTGAAAAAC AAAAGCATCA AGCAACAAGG CAGAGAATCA TATTTCATAG CTAAGTGCAT GAGTATGCAG CTGCAGGTGT CCATTGCAGA TCTTTGGGGA 5000
5001 CCAACAATCA TAATTAAATC ATTGGGTCAC ATCCCCAAGA CTGCACTTCC TTTTTTCAGC AAAGACGGGA TTGCCTGTCA TCCACTACAA GATGTTTCCC 5100
5101 CTACTCTGAC AAAATCACTG TGGTCAGTGG GATGTGAGAT AGAATCTGCC AAGTTGATAC TTCAAGAATC TGATATTAAT GAGCTAATGG GCCACCAGGA 5200
5201 CTTGATTACT GATAAGATTG CCATTAGATC AGGTCAACGG ACATTTGAGA GGTCCAAATT CAGCCCATTC AAAAAATACG CATCAATTCC AAACTTAGAA 5300

FIGURE 10A

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5401 AGATATTATC ATAGGTCCGA ACTGATCAAT CTAACAAAAA AACTAAACAT TCAATAATAA ATCAAAGTTC AGGCCAAATT ATCCAGCCAT GCATCAGCTG 5500
5501 CATCCAATGA TAGTATGCAT TTTTGTTATG TACACTGGAA TTGTAGGTTC AGATGCCATT GCTGGAGATC AACTCCTCAA TGTAGGGGTC ATTCAATCAA 5600
5601 AGATAAGATC ACTCATGTAC TACACTGATG GTGGCGCTAG CTTTATTGTT GTAAAATTAC TACCCAATCT TCCCCCAAGC AATGGAACAT GCAACATCAC 5700
5701 CAGTCTAGAT GCATATAATG TTACCCTATT TAAGTTGCTA ACACCCCTGA TTGAGAACCT GAGCAAAATT TCTGCTGTTA CAGATACCAA ACCCCGCCGA 5800
5801 GAACGATTTG CAGGAGTCGT TATTGGGCTT GCTGCACTAG GAGTAGCTAC AGCTGCACAA ATAACCGCAG AGTAAAAGCC AATGCAAATG CAGTTCAAGC 5900
5901 CTGCTGCGAT AAACAATCTT GCATCTTCAA TTCAATCCAC CAACAAGGCA GTATCCGATG TGATAACTGC ATCAAGAACA ATTGCAACCG CAGTTCAAGC 6000
6001 GATTCAGGAT CACATCAATG GAGCCATTGT CAACGGGATA ACATCTGCAT CATGCCGTGC CCATGATGCA CTAATTGGGT CAATATTAAA TTTGTATCTC 6100
6101 ACTGAGCTTA CTACAATATT TCATAATCAA ATAACAAACC CTGCGCTGAC ACCACTTTCC ATCCAAGCTT TAAGAATCCT CCTCGGTAGC ACCTTGCCAA 6200
6201 TTGTCATTGA ATCCAAACTC AACACAAAAC TCAACACAGC AGAGCTGCTC AGTTCCGGAC TGTTAACTGG TCAAATAATT TCCATTTCCC CAATGTACAT 6300
6301 GCAAATGCTA ATTCAAATCA ATGTTCCGAC ATTTATAATG CAACCCGGTG CGAAGGTAAT TGATCTAATT GCTATCTCTG CAAACCATAA ATTACAAGAA 6400
6401 GTAGTTGTAC AAGTTCCTAA TAGAATTCTA GAATATGCAA ATGAACTACA AAACTACCCA GCCAATGATT GTGTCGTGAC ACCAAACTCT GTATTTTGTA 6500
6501 GATACAATGA GGGTTCCCCG ATCCCTGAAT CACAATATCA ATGCTTAAGG GGGAATCTTA ATTCTTGCAC TTTTACCCCT ATTATCGGGA ACTTTCTCAA 6600
6601 GCCATTCGCA TTTGCCAATG GTGTGCTCTA TGCCAACTGC AAATCTTTGC TATGTAAGTC TGCCGACCCT CCCCATGTTG TGTCTCAAGA TGACAACCAA 6700
6701 GGCATCAGCA TAATTGATAT TAAGAGGTGC TCTGAGATGA TGCTTGACAC TTTTTCATTT AGGATCACAT CTACATTCAA TGCTACATAC GTGACAGACT 6800
6801 TCTCAATGAT TAATGCAAAT ATTGTACATC TAAGTCCTCT AGACTTGTCA AATCAAATCA ATTCAATAAA CAAATCTCTT AAAAGTGCTG AGGATTGGAT 6900
6901 TGCAGATAGC AACTTCTTCG CTAATCAAGC CAGAACAGCC AAGACACTTT ATTCACTAAG TGCAATCGCA TTAATACTAT CCACAGGGAG AATCCTGCCG 7000
7001 GTGGGATTGC TGATTGCCTA CATCATCAAG CTGGTTTCTC AAATCCATCA ATTCAGAGCA CTAGCTGCTA CAACAATGTT CAGTGATTAC AATCCTGCGG 7100
7101 TCTTTTCCAA GAACAATCAT GCAAACATAT ATGGGATATC TATCATAAGT CCATATATGT TGCCGATTGA CCTTTAAGAG CCAACCTCCA 7200
7201 ATGATTATCC GTTAAATTCA GATATTAAGC AATATTAAGC CTCCAGATAC CAATGAATAT GAATATATCT CTTAGAAAAC TTGATTATTA 7300
7301 TGTGATAACA TAGTACAATT TAAGAAAAAA CCTAAAATAA GCACGAACCC TTAAGGTGTC GTAACGTCTC GTGACGCCGG GTTCAGTTCA AACATCGACC 7400
7401 CCTGACCCAA TTCAATACCC ATTTTCATAA AGGAACACAG TATAATTTAA TCATAAAAGA CCTCAAAATC TGATACAGCT TAATCCACTC AACATATAAT 7500
7501 TATAAGACTA ATAATAATGG AAGATTACAG CAATCTATCT CTTAAATCAA TTCCTAAAAG GACATGTAGA ATCATTTCCC GAACTGCCAC AATTCTTGGC 7600
7601 ATATGCACAT TAATTGTGCT ATGTTCAAGT ATTCTTCATG AGATAATTCA TCTTGATGTT AATAGTAATT CCTCTTAAAA TTGACAGTAT 7700
7701 TTATTCAGCC TATCATAGAA TCATTAAAAT CATTGATTGC CTTTGGCCAC CAGATTCTAT ATAATGTTGC AATAGTAATT CCTCTTAAAA TTGACAGTAT 7800
7801 CGAAACTGTA ATACTCTCTG CTTTAAAAGA TATGCACACC GGGAGTATGT CCAATGCCAA CTGCACGCCA GGAAATCTGC TTCTGCATGA TGCAGCATAC 7900
7901 ATCAATGGAA TAAACAAATT CCTTGTACTT GAATCATACA ATGGGACGCC TAAATATGGA CCTCTCCTAA ATATACCCAG CTTTATCCCC TCAGCAACAT 8000
8001 CTCCCCATGG GTGTACTAGA ATACCATCAT TTTCACTCAT CAAGACCCAT TGGTGTTACA CTCACAATGT AATGCTTGGA GATTGTCTTG ATTTCACGGC 8100
8101 ATCTAACCAG TATTTATCAA TGGGGATAAT ACAACAATCT GCTGCAGGGT TTCCAATTTT CAGGACTATG AAAACCATTT ACCTAAGTGA TGGAATCAAT 8200
8201 CGCAAAAGCT GTTCAGTCAC TGCTATACCA GGAGGTTGTG TCTTGTATTG CTATGTAGCT ACAAGGTCTG AAAAAGAAGA TTATGCCACG ACTGATCTAG 8300
8301 CTGAACTGAG ACTTGCTTTC TATTATTATA ATGATACCTT TATTGAAAGA GTCATATCTC TTCCAAATAC AACAGGGCAG TGGGCCACAA TCAACCCTGC 8400
8401 AGTCGGAAGC GGGATCTATC ATCTAGGCTT TATCTTATTT CCTGTATATG GTGGTCTCAT AAATGGGACT ACTTCTTACA ATGAGCAGTC CTCACGCTAT 8500
8501 TTTATCCCAA AACATCCCAA CATAACTTGT GCCGGTAACT CCAGCAAACA GGCTGCAATA GCACGGAGTT CCTATGTCAT CCGTTATCAC TCAAACAGGT 8600
8601 TAATTCAGAG TGCTGTTCTT ATTTGTCCAT TGTCTGACAT GCATACAGAA GAGTGTAATC TAGTTATGTT TAACAATTCC GAAGTCATGA TGGGTGCAGA 8700
8701 AGGTAGGCTC TATGTTATTG GTAATAATTT CAACGCAGTT CCTCTTGGTG GTCTGCATCG GTCTGCATGC CATGCAATGC AACAAGTTTT TGCCCTGCTA 8800
8801 AAAGGAATTC CTCCGATCAT TGAGGCTCAA TGGGTACCGT CCTATCAAGT TCCTCGTCCT GGAGTCATGC CATGCAATGC AACAAGTTTT TGCCCTGCTA 8900
8901 ATTGCATCAC AGGGGTGTAC GCAGATGTGT GGCCGCTTAA TGATCCAGAA CTCATGTCAC GTAATGCTCT GAACCCCAAC TATCGATTTG CTGGAGCCTT 9000
9001 TCTCAAAAAT GAGTCCAACC GAACTAATCC CACATTCTAC ACTGCATCGG CTAACTCCCT CTTAAATACT ACCGGATTCA ACACACCAA TCACAAAGCA 9100
9101 GCATATACAT CTTCAACCTG CTTTAAAAAC ACTGGAACCC AAAAAATTTA TTGTTTAATA ATAATTGAAA TGGCTCATC TCTTTTAGGG GAGTTCCAAA 9200
9201 TAATACCATT TTTAAGGGAA CTAATGCTTT AATCCTATTG AATGAAGACT CCAGATTCAA GAATAATTGG AAGCCTCTTT ATTTTATGCC ATAGTTATAC 9300
9301 GTTTTGGCTG TATTAGAATG CTATAGCATT CTGCTGTTTT TCCCATATGG AAAAATCCTT CAACACCAAC TTAGGTTCAA TTTTCTCATC ATTTACTGTT 9400
9401 GTAATTCAAT CTTACTAAAG TTATTCTGAT ATTTAAGAAA AAATAATCTT TATATAATGT AACAATACTA CTAAGATTAT AATATAGGCC AGAATGGCGG 9500
9501 CCTCTTCTGA GATACTCCTT CCTGAAGTCC ATTTGAACTC ACCAATAGTC AAACACAAAC TCATATACTA CTTATTACTA GGGCACTTCC CGCATGATCT 9600
9601 TGACATTTCT GAAATAAGCC CCCTTCACAA TAATGATTGG GATCAGATTG CCAGAGAAGA ATCCAATCTT GCTGAACGAC TCGGAGTAGC TAAATCTGAA 9700
9701 TTAATTAAAC GTGTGCCCGC ATTTAGAGCA ACCAGATGGC GTAGTCATGC AGCCGTCCTT ATATGGCCTT CTTGTATACC ATTCCTTGTT AAATTCCTAC 9800
9801 CCCATTCTAA GCTTCAACCA ATAGAACAAT GGTACAAGTT GATCAATGCT TCATGCAATA CTATATCTGA CTCAATTGAT AGATGTATGG AGAATATTTC 9900
9901 TATTAAGCTT ACTGGGAAAA ACAATCTATT CTCTCGATCC AGAGGAACTG CAGGCGCAGG TAAAAACAGT AAAATCACCC TCAATGATAT CAGCCAACTG 10000
10001 TGGGAATCAA ACAAATGGCA GCCTAATGTA TCTTTATGGC TTACAATTAA ATACCAAATG CGACAACTTA TAATGCATCA AAGTTCTCGT AATGTTTTAA TGTATTTTAC 10100
10101 ATTTAGTTCA CATTGTTGAC ACACGATCTG GTCTAATAGT TATCACACCC GAACTTGTTA TTTGCTTTGA TCGGTTGAAT AATGTTTTAA TGTATTTTAC 10200
10201 ATTTGAGATG ACTTTAATGG TAAGTGACAT GTTTGAGGGA CGGATGAATG TTGCCGCGCT CTGCACTATT AGTCATTACT TATCACCACT AGGGCCAAGG 10300
10301 ATAGATAGAT TGTTTTCTAT TGTAGATGAA TTAGCACAAC TATTGGGTGA CACTGTATAT AAAATTATTG CATCTCTTGA ATCTTTAGTA TATGGGTGTC 10400
10401 TACAACTTAA AGATCCAGTG GTTGAATTAA CAGGATCTAT TCATTCCTTT ATTACGCAAG AGATTATAGA TATCCTAATT GGGTCAAAAG CCCTTGATAA 10500
10501 GGATGAATCA ATAACTGTCA CTACACAATT GCTAGATATA TTTTCCAACC TTTCTCCAGA TTTAATCGCT GAGATGTTGT GTCTCATGAG ACTTTGGGGT 10600
10601 CATCCCACTC TTACTGCTGC GCAAGCTGCA GGTAAAGTGA GAGAATCTAT GTGTGCAGGT AAGTTACTTG ATTTCCCTAC AATAATGAAA ACTCTTGCTT 10700
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FIGURE 10B

FIGURE 10C

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   1 ATGGCCGAGG AACCAACATA CACCACTGAG CAAGTTGATG AATTAATCCA TGCTGGACTA GGAACAGTAG ATTTCTTCCT ATCTAGACCC ATAGATGCTC 100
 101 AGTCTTCTTT AGGTAAAGGC AGCATCCCAC CAGGTGTCAC GGCTGTTCTA ACCAATGCAG CAGAGGCAAA ATCCAAACCA GTTGCTGCTG GTCCAGTAAA 200
 201 ACCCAGACGG AAGAAAGTGA TCAGCAATAC CACTCCATAC ACTATTGCAG ACAACATCCC ACCTGAGAAG CTACCGATCA ACACTCCAAT ACCCAATCCA 300
 301 TTACTTCCAC TGGCACGCCC TCACGGAAAG ATGACAGACA TTGACATTGT CACTGGGAAC ATTACAGAAG GATCATACAA AGGTGTGGAG CTTGCCAAAT 400
 401 TAGGGAAGCA AACACTACTC ACAAGGTTCA CCTCGAATGA GCCAGTCTCC TCAGCTGGAT CCGCCCAAGA CCCCAACTTT AAGAGAGGAG GTGAGCTAAT 500
 501 AGAGAAAGAG CAAGAGGCAA CCATAGGAGA GAATGGAGTA TTGCATGGGT CGGAGATCAG GTCAAAGTCT TCGAGTGGTG TAATCCCAGG TGTGCCCCAG 600
 601 TCACGGCTTC AGCTCGCAAG TTCACCTGCA CATGTGGATC CTGCCCCAGC ATCTGCGGAG AATGTGAAGG AGATCATTGA GCTCTTAAAA GGGCTTGATC 700
 701 TTCGCCTTCA GACTGTAGAA GGGAAAGTAG ATAAAATTCT TGCAACCTCT GCAACTATAA TCAATCTTAA AAATGAAATG ACTAGTCTTA AGGCGAGCGT 800
 801 TGCAACTGTG GAAGGTATGA TAACAACAAT TAAAATCATG GATCCCAGTA CACCAACCAA TGTCCCTGTA GAGGAGATCA GAAAGAGTTT ACACAATGTT 900
 901 CCAGTAGTAA TTGCTGGTCC GACTAGTGGA GGCTTCACAG CCGAAGGCAG TGACATGATT TCAATGGATG AACTAGCTAG GCCTACACTC TCATCAACAA 1000
1001 AAAAGATCAC ACGAAAGCCT GAATCCAAGA AAGATTTAAC AGGCATAAAA CTAACCCTGA TGCAGCTTGC AAATGACTGC ATCTCGCGTC CAGATACCAA 1100
1101 GACTGAGTTT GTGACTAAGA TTCAAGCAGC AACCACAGAA TCACAGCTCA ACGAAATCAA ACGGTCAATA ATACGCTCTG CAATATAA 1188
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FIGURE 11

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   1 ATGGCCGAGG AACCAACATA CACCACTGAG CAAGTTGATG AATTAATCCA TGCTGGACTA GGAACAGTAG ATTTCTTCCT ATCTAGACCC ATAGATGCTC 100
 101 AGTCTTCTTT AGGTAAAGGC AGCATCCCAC CAGGTGTCAC GGCTGTTCTA ACCAATGCAG CAGAGGCAAA ATCCAAACCA GTTGCTGCTG GTCCAGTAAA 200
 201 ACCCAGACGG AAGAAGTGA TCAGCAATAC CACTCCATAC ACTATTGCAG ACAACATCCC ACCTGAGAAG CTACCGATCA ACACTCCAAT ACCCAATCCA 300
 301 TTACTTCCAC TGGCACGCCC TCACGGAAAG ATGACAGACA TTGACATTGT CACTGGGAAC ATTACAGAAG GATCATACAA AGGTGTGGAG CTTGCCAAAT 400
 401 TAGGGAAGCA AACACTACTC ACAAGGTTCA CCTCGAATGA GCCAGTCTCC TCAGCTGGAT CCGCCCAAGA CCCCAACTTT AAGAGAGGCG GAGCTAATAG 500
 501 AGAAAGAGCA AGAGGCAACC ATAGGAGAGA ATGGAGTATT GCATGGGTCG GAGATCAGGT CAAAGTCTTC GAGTGGTGTA ATCCCAGGTG TGCCCCAGTC 600
 601 ACGGCTTCAG CTCGCAAGTT CACCTGCACA TGTGGATCCT GCCCCAGCAT CTGCGGAGAA TGTGAAGGAG ATCATTGA                        678
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FIGURE 12