



(43) International Publication Date  
30 August 2012 (30.08.2012)

(10) International Publication Number  
**WO 2012/116253 A2**

- (51) **International Patent Classification:** Not classified
- (21) **International Application Number:**  
PCT/US2012/026436
- (22) **International Filing Date:**  
24 February 2012 (24.02.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/446,619 25 February 2011 (25.02.2011) US  
61/529,981 1 September 2011 (01.09.2011) US
- (71) **Applicant (for all designated States except US):** **UNIVERSITY OF GEORGIA RESEARCH FOUNDATION INC.** [US/US]; Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US).
- (72) **Inventor; and**
- (75) **Inventor/Applicant (for US only):** **HE, Biao** [US/US]; 1150 Rowan Oak Circle, Bogart, GA 30622 (US).
- (74) **Agent:** **JOHNSON, Nancy, A.**; Mueting, Raasch & Gebhardt, P.A., P.O. box 581336, Minneapolis, MN 55458-1336 (US).
- (81) **Designated States (unless otherwise indicated, for every kind of national protection available):** AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States (unless otherwise indicated, for every kind of regional protection available):** ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) **Title:** RECOMBINANT MUMPS VIRUS VACCINE

(57) **Abstract:** The present invention provides the complete genomic sequence of the epidemic mumps virus (MuV) strain MuV<sup>Iowa/us/06</sup>. Further, a reverse genetics system was constructed and used to rescue recombinant viral constructs that are attenuated compared to MuV<sup>Iowa/us/06</sup> and JL vaccine viruses. Such constructs include viral constructs lacking the open reading frame (ORF) of the SH gene (rMuVΔSH) and/or incapable of expressing the V protein (rMuVΔV).



WO 2012/116253 A2

## RECOMBINANT MUMPS VIRUS VACCINE

### CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 61/446,619, filed February 25, 2011, and U.S. Provisional Application Serial No. 61/529,981, filed September 1, 2011, each of which is incorporated by reference herein.

### GOVERNMENT FUNDING

This invention was made with government support under Grant No. K02AI065795, awarded by the National Institutes of Health. The Government has certain rights in the invention.

### BACKGROUND

Mumps virus (MuV), a paramyxovirus, causes acute parotitis in humans, characterized by lateral or bilateral swelling of the salivary glands. MuV is also notable as a highly neurotropic and neurovirulent agent causing a number of central nervous system (CNS) manifestations ranging from mild meningitis to severe, and occasionally fatal, encephalitis. Mumps virus infection was the most common cause of viral meningitis and encephalitis until the arrival of mass immunization with mumps virus vaccine. The incidence of mumps and its complications were dramatically reduced following the introduction of measles, mumps, rubella vaccine (MMR) in 1971. MMR vaccine containing the Jeryl Lynn (JL) strain, an attenuated strain of MuV, is highly efficacious and produces few adverse reactions. Currently, mumps virus vaccination is a part of a two dose MMR (mumps, measles, and rubella) vaccine regimen that is administered to children at one and five years of age in the United States.

In recent years, MuV has caused epidemics among highly vaccinated populations. In 2006, the U.S. experienced the largest mumps epidemic in nearly 20 years (Marin et al., 2008, *Vaccine*; 26(29–30):3601–3607). The outbreak originated at a university in Iowa and spread to eleven other states. Over 5000 mumps cases were reported in 2006 compared to an average of

approximately 250 cases/year in the previous decade. In 2009–2010, a mumps outbreak occurred in the State of New York and the State of New Jersey in the US in which 88% of the patients had one-dose of mumps vaccine and 75% of the patients had two doses of vaccine (*MMWR Morb Mortal Wkly Rep*; 59(5):125-129, 2010).

While definitive causes for these recent outbreaks are not known, possible reasons (not mutually exclusive) for these outbreaks include waning immunity, high velocity of infection, and vaccine failure due to emerging of a new mumps virus strain. See, for example, (Crowley and Afzal, 2002, *Commun Dis Public Health*; 5(4):311–313; Lim et al., 2003, *J Med Virol*; 70(2):287-292; Otto et al., 2010, *Euro Surveill*; 15(50); Strohle et al., 1996, *Arch Virol*; 141(3–4):733-741; Utz et al., 2004, *J Med Virol*; 73(1):91-96; and Whelan et al., 2010, *Euro Surveill*; 15(17). The results of a large study to examine the efficacy of the two-dose MMR against mumps virus by CDC indicate that titers of anti-MuV dropped dramatically 12 years after the second dose of MMR (17 years of age), to the level of pre-second dosage inoculation. Furthermore, neutralizing antibody titers are low in adults: out of 101 sera tested, 74 were positive using ELISA and only one had neutralization antibody titer higher than 1:8. This is consistent with the fact that in the 2006 outbreak, the most affected population was 18 to 24 years of age. In the 2010 outbreak, most affected patients were 13 to 14 years of age. Both recent outbreaks occurred in high-density populations (college campus and religious school). High velocity infection (for example, large quantity of infectious virions transmitted from one to another due to close contact) may have overwhelmed the anti-MuV immunity in recent outbreaks.

The current vaccine Jeryl Lynn (JL) is based in MuV genotype A, while recent outbreaks have been caused by genotype G. It is possible that vaccine generated immunity based on strain A is ineffective in preventing infection of strain G, leading to the outbreak. Because of re-emerging of mumps virus outbreaks even in vaccinated populations, mumps virus has been listed as a high priority pathogen by National Institute of Allergy and Infectious Diseases (see "Emerging and Re-emerging Infectious Diseases" on the worldwide web at [niaid.nih.gov/topics/emerging/list.htm](http://niaid.nih.gov/topics/emerging/list.htm)). Currently, live attenuated MuV vaccines are obtained through serial passages in embryonic eggs and cells. This is a time consuming process and a strategy with a poor record of generating safe vaccines.

Thus, there is a need for new and improved mumps vaccines, including the development of vaccines directed at the genotype G and a need for new and improved methods for developing mumps vaccines.

### SUMMARY OF THE INVENTION

The present invention includes an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus, wherein the isolated nucleotide sequence encodes a mumps virus unable to express a small hydrophobic (SH) protein product and/or unable to express a V protein product, and fragments and derivatives thereof.

In some aspects, an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus unable to express a small hydrophobic (SH) protein product includes a deletion of the open reading frame (ORF) encoding the SH protein, a mutation converting a start codon into a stop codon, or a mutation in the region between F protein ORF and the SH protein ORF that disrupts transcription of the SH gene. In some aspects, a deletion of the open reading frame (ORF) encoding the SH protein includes a deletion of 156 nucleotides of the ORF encoding the SH protein.

In some aspects, an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus unable to express a V protein product includes one or more mutations to the V/I/P gene abrogating expression of the V protein. In some aspects, one or more mutations to the V/I/P gene abrogating expression of the V protein include the nucleotide sequence GAGGAGGG at the editing site in the P/V gene.

In some aspects, an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus includes a deletion of the open reading frame (ORF) encoding the SH protein or a mutation converting a start codon into a stop codon and includes one or more mutations to the V/I/P gene abrogating expression of the V protein. In some aspects, the one or more mutations to the V/I/P gene abrogating expression of the V protein include the nucleotide sequence GAGGAGGG at the editing site in the P/V gene.

The present invention also includes an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus as described herein, including one or more further mutations and/or deletions. In some aspects, a further mutation or deletion may include a mutation or deletion effecting phosphorylation of the P protein. In some aspects, a

further mutation or deletion effecting phosphorylation of the P protein may include a mutation or deletion at T147 and/or S307 of the P protein.

The present invention also includes an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus as described herein, further including expression of an I protein product and/or further including mutations in the L protein product.

The present invention also includes an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus as described herein, wherein the mumps genome further encodes a heterologous polypeptide.

In some aspects, an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus belongs to genotype G.

In some aspects, an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus is MuV/IowaUS/2006 (MuV-IA). In some aspects, MuV/IowaUS/2006 (MuV-IA) includes SEQ ID NO:1.

The present invention includes an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of the MUV/IowaUS/2006 (MuV-IA) strain of the mumps virus, and fragments and derivatives thereof. In some aspects, the nucleotide sequence includes SEQ ID NO:1.

The present invention includes a recombinant mumps virus (rMuV) having an isolated nucleotide acid sequence including a cDNA sequence encoding a full length RNA genome of a mumps virus, as described herein, or a fragment or derivative thereof.

The present invention includes a plasmid encoding a measles virus genome (pMuV) including a cDNA sequence encoding a full length RNA genome of a mumps virus, as described herein, or a fragment or derivative thereof.

The present invention includes a viral expression vector including an isolated nucleotide sequence including a cDNA sequence encoding the full length RNA genome of a mumps virus as described herein, or a fragment or derivative thereof.

The present invention includes an infectious viral particle including an isolated nucleotide sequence or plasmid as described herein.

The present invention includes a composition including an isolated nucleotide sequence, plasmid, pMuV, rMuV, or infectious viral particle as described herein. In some embodiments, a

composition further includes a rubella and/or measles antigenic determinant. In some embodiments, the composition is formulated for intranasal, oral, intradermal, or intramuscular administration.

The present invention includes a method of inducing an immune response to mumps virus in a subject, the method including administering an effective amount of an isolated nucleotide sequence plasmid, pMuV, rMuV, viral particle, or composition as described herein, to the subject. In some embodiments, administration includes intranasal, oral, intradermal, or intramuscular administration.

The present invention includes a method of vaccinating a subject against mumps, the method including administering an effective amount of an isolated nucleotide sequence plasmid, pMuV, rMuV, viral particle, or composition as described herein to the subject. In some embodiments, administration includes intranasal, oral, or intramuscular administration.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

## BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C. Analysis of MuV-IA genome. Fig. 1A is an alignment of the SH proteins. SH protein sequences of three strains, Glouc1/UK96 (SEQ ID NO:4), UK01-22 (SEQ ID NO:5) and MuV-IA (SEQ ID NO:6) are shown. MuV-IA was different from Glouc1/UK96\* and UK01-22 for only five nucleotides and two amino acids respectively. The transmembrane domain of mumps virus SH protein, outlined in rectangular box, was predicted using TMHMMserver v.2.0 (CBS; Denmark). Fig. 1B is a sequence comparison of different mumps viruses. The genome sequences of 32 strains of mumps viruses were obtained from NCBI Genbank and aligned with genome sequence of MuV-IA using MEGA4 version 4.0.2. The 32 MuV Strains were (accession numbers are given in the brackets): 87–1004 (AF314560), SIPAR 02 (AF314558), Biken (AF314561), 87–1005 (AF314562), MuV(2001) (AF314559), Urabe 1004-10/2 (FJ375177), Urabe Gw7 (FJ375178), Hoshino (AB470486), Miyahara (1992) (NC\_002200), MuV Miyahara (1992) (2) (AB040874), Y213(AB576764), Dg1062/Korea/98 (32172464), L3/Russia/Vector (AY508995), L-Zagreb master seed (AY685921), L-Zagreb vaccine strain (AY685920), 9218/Zg98 (299766355), Novosbrisk genotype C (50404164), PetroNov genotype H (AY681495), 88–1961 (AF467767), Glouc1/UK96(AF280799), Du/CRO05 (EU370207), SP-A (FJ556896), SP (EU884413), SP(2006) (DQ649478), JL2 (AF3452901), Jeryl Lynn sub strain (FN31985), Enders (GU9800521), Jeryl Lynn major component (AF338106), MuV(2000) (AF201473), JL1 (FJ211586), RIT4385 (FJ211585), RIT4385(2) (FJ211584). Fig. 1C is a sequence comparison between MuV-IA and Jeryl Lynn (JL) strain (major component). The gene and protein sequences of NP, P/V (encoding P protein and V protein), M, F, SH, HN and L of MuV-IA and Jeryl Lynn live vaccine major component were aligned using NCBI BLAST program. Nucleotide identities and amino acid identities were shown above. \*The SH genes were aligned using MEGA 4.0.2.

Figures 2A-2D. Analysis of rMuV, rMuV-EGFP and rMuV-RL. Fig. 2A shows the growth rate of rMuV. rMuV was obtained by transfecting BSRT-7 cells with pMuV-IA, pCAGGS-NP, pCAGGS-P and pCAGGS-L. Growth rates of rMuV (empty square) and MuV-IA (filled triangle) were compared. Fig. 2B shows viral protein expression levels of rMuV and MuV-IA. Six well plates of Vero cell were infected with mock, rMuV or MuV-IA at a MOI of 0.5. Cell lysates were subjected to immunoblotting with anti-NP, P or V. Fig. 2C shows rescue of rMuV-EGFP. rMuV-EGFP was rescued in a similar manner as described for rMuV. Vero

cell in six well plates were infected with mock or rMuV-EGFP at a MOI of 0.05 and photographed at 2 dpi. Fig. 2D shows rescue of rMuV-RL. rMuV-RL was recovered from cloned DNA as described for rMuV. 24 well plates of Vero cell were infected with mock or rMuV-RL at MOI of 0.1. At 2 dpi, cells were assayed for renilla luciferase activity.

Figures 3A-3D. Generation of a MuV lacking SH (rMuV $\Delta$ SH). Fig. 3A is a schematic of the production of rMuV $\Delta$ SH. The SH ORF (SEQ ID NO:7) was replaced with a 5 amino acid coding sequence containing an Nhe I site (SEQ ID NO:8; restriction site is underlined). Fig. 3B is confirmation of rMuV $\Delta$ SH by RT-PCR. After plaque purification, viral RNA was extracted and was subjected to RT-PCR, in which two primers flanking the SH gene were used to perform a PCR to confirm the deletion. Lane 1 and Lane 6 are 100 bp and 1 kb DNA ladder respectively; Lane 2 is the negative control - PCR without polymerase. Lane 3, 4 and 5 are PCR products from rMuV $\Delta$ SH, rMuV and wtMuV-infected cells respectively. Fig. 3C is confirmation of rMuV $\Delta$ SH by sequencing. PCR products were sequenced (SEQ ID NO:9). The inserted sequence is underlined. Fig. 3D shows expression of SH in MuV-IA, rMuV and rMuV $\Delta$ SH-infected cells. Vero cells were mock infected or infected with MuV-IA, rMuV or rMuV $\Delta$ SH. Cell lysates were subjected to immunoblotting with anti-NP, P or SH.

Figures 4A-4D. Growth rates and viral protein expression of rMuV $\Delta$ SH and rMuV. Figure 4A shows growth rates of rMuV $\Delta$ SH and rMuV. Vero cell were infected with rMuV $\Delta$ SH (filled diamond) and rMuV (empty square) at MOI of 0.01. Supernatants were used for plaque assay. Fig. 4B shows viral protein expression levels in rMuV $\Delta$ SH and rMuV infected cells. Vero cells were mock infected or infected with rMuV $\Delta$ SH or rMuV at a MOI of 0.5. Fig. 4C shows HN expression level in rMuV $\Delta$ SH and rMuV infected cells. Vero cells were mock infected or infected with rMuV $\Delta$ SH or rMuV at a MOI of 0.5. Cells were collected at 24 hpi and expression levels of total HN and NP were examined using flow cytometry. Mean fluorescence intensity (MFI) of HN and NP were calculated. Y-axis represents the relative ratio of MFI of HN normalized by MFI of NP. Fig. 4D shows HN mRNA level in rMuV $\Delta$ SH or rMuV infected Vero cells. Viral RNA was extracted from rMuV $\Delta$ SH or rMuV-infected Vero cells, reverse transcribed with oligodT and used for real time PCR. HN and F mRNA levels were calculated and HN mRNA level was normalized by F mRNA level. Y-axis represents ratio of HN mRNA verses F mRNA.

Figures 5A-5C. Induction of cell death by rMuVΔSH. Fig. 5A shows cytopathic effects of rMuVΔSH or rMuV in tissue culture cell lines. Vero cells, MDBK cells or HeLa cells were mock infected or infected with rMuVΔSH or rMuV at MOI of 0.01 and photographed at 1 dpi. Fig. 5B shows cytopathic effects of rMuVΔSH infection in L929 cells. L929 cells were mock infected, or infected with rMuVΔSH, or rMuV at a MOI of 3 and were photographed at 1 dpi and 2 dpi. Fig. 5C shows rMuVΔSH infection induced apoptosis in L929 cells. L929 cells were infected as in Fig. 5B. At 1 dpi, both floating cells and attached cells were collected, fixed, permeabilized and used for TUNEL assay.

Figures 6A-6D. The role of TNF- $\alpha$  in rMuVΔSH-mediated cell death. Fig. 6A shows rMuVΔSH infection activated P65 in L929 cells. L929 cells on glass cover slips were infected with mock, rMuVΔSH or rMuV at a MOI of 10. At 1 dpi, L929 cells on the cover slips were stained with anti-P65. Fig. 6B shows rMuVΔSH infection in L929 cells induced TNF- $\alpha$  production. L929 cells were mock infected or infected with rMuVΔSH, or rMuV at a MOI of 5. TNF- $\alpha$  in the media was measured using ELISA. Fig. 6C shows treatment with anti-TNF- $\alpha$  antibody reduced CPE in rMuVΔSH infected L929 cells. L929 cells were mock infected or infected with rMuVΔSH or rMuV at a MOI of 5. Cells were cultured in media containing TNF- $\alpha$  neutralizing antibody or control antibody and were photographed at 1 dpi. Fig. 6D shows TNF- $\alpha$  antibody treatment inhibited apoptosis in rMuVΔSH infected L929 cells. L929 cells in 6 well plates were infected and treated as in Fig. 6C. At one day and two days post infection, cells were collected and used for TUNEL assay.

Figures 7A and 7B. MuV-IA SH inhibited TNF- $\alpha$  activation of NF- $\kappa$ B. L929 cells were transfected with a reporter plasmid (pKB-TATA-FL) and pCAGGS-MuV SH, pCAGGS-PIV5 SH or pCAGGSMuV-NP (Fig. 7A). At one day post-transfection, cells were treated with TNF- $\alpha$  at 10 ng/ml for four hours and then assayed for fire fly luciferase activity. Similarly, the effect of the sequence of the SH ORF was examined using a plasmid encoding the SH ORF sequence without expressing the SH polypeptide due to in-frame stop codon insertion downstream of the start codon of the SH ORF (Fig. 7B).

Figure 8. Neurotoxicity of MuVΔSH in vivo. Newborn rats were infected intracerebrally with rMuV or rMuVΔSH. The animals were sacrificed at 30 days post infection. The brains of the animals were sectioned, stained and neurotoxicity scores were calculated based on relative hydrocephalus score as described in the Material and Methods.

Figures 9A-9D. Generation of a  $\text{MuV}^{\text{Iowa/US/06}}$  lacking V protein ( $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ ). Fig. 9A is a schematic of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ . The GGGGGG (nucleotides 1-6 of SEQ ID NO:14) editing site in the P/V gene of  $\text{MuV}^{\text{Iowa/US/06}}$  was changed to GAGGAGGG (nucleotides 1-8 of SEQ ID NO:15) to eliminate expression of the V protein. To maintain the genome length of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  to be a multiple of six, four basepairs (bp) were added to the P/V gene 3' UTR (SEQ ID NO:16). Fig. 9B shows confirmation of the rescue of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ . Viral RNAs extracted from  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ - and  $\text{rMuV}^{\text{Iowa/US/06}}$ -infected cells were reverse transcribed into cDNA, followed by reverse transcription (RT)-PCR using two primers flanking the P/V gene. Lane 1 is 100-bp DNA ladder; lane 2 is negative control (PCR without polymerase); lanes 3 and 4 are PCR products from  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ - and  $\text{rMuV}^{\text{Iowa/US/06}}$ -infected cells, respectively. Fig. 9C is confirmation of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ . The PCR products shown in Fig. 9B were sequenced (SEQ ID NO:17). Fig. 9D shows expression of the V protein in  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ - and  $\text{rMuV}^{\text{Iowa/US/06}}$ -infected cells. Vero cells were mock infected or infected with  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  or  $\text{rMuV}^{\text{Iowa/US/06}}$ . Cell lysates were immunoblotted using anti-NP, -P, or -V.

Figures 10A and 10B. Whole-genome sequencing of rescued  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ . Fig. 10A is a summary of changes found in rescued  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ . The leftmost panel shows the names of individual  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  strains from eight successful virus rescues. Fig. 10B is a schematic of the changes that occurred in the NP GE and P/V GS regions. Changes that occurred during virus rescue of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  are indicated as bold, italic letters. The NP GE and P/V GS sequence in the plasmid is shown (SEQ ID NO:18). The NP GE and P/V GS mutated sequences are shown for the following virus strains: PX2-SP-48 (SEQ ID NO:19), PX2-sp-51 (SEQ ID NO:20), PX2-sp-61 (SEQ ID NO:21), PX2-sp-81 (SEQ ID NO:22), PX2-sp-91 (SEQ ID NO:23), PX2-sp-101 (SEQ ID NO:24), and PX2-sp-106 (SEQ ID NO:25). The "TT" in the middle of the sequence alignment is the gene junction sequence between the NP and P/V genes in  $\text{MuV}^{\text{Iowa/US/06}}$ .

Figures 11A-11E. Growth rates and viral protein expression of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$ ,  $\text{rMuV}^{\text{Iowa/US/06}}$ , and  $\text{MuV}^{\text{Iowa/US/06}}$ . Fig. 11A shows growth rates of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  and  $\text{rMuV}^{\text{Iowa/US/06}}$  in Vero cells. Vero cells were mock infected or infected with  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  or  $\text{rMuV}^{\text{Iowa/US/06}}$  at an MOI of 0.01. Supernatants were collected for plaque assay. Fig. 11B shows plaques of  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  and  $\text{rMuV}^{\text{Iowa/US/06}}$  in Vero cells.  $\text{rMuV}^{\text{Iowa/US/06}}\Delta\text{V}$  or  $\text{rMuV}^{\text{Iowa/US/06}}$  was plated onto Vero cells. The plaques were stained with Giemsa at 6 dpi. Fig.

11C shows viral protein expression of rMuV<sup>Iowa/US/06</sup>ΔV and rMuV<sup>Iowa/US/06</sup> in Vero cells. Vero cells were infected as in Fig. 11A. Viral protein levels were examined by immunoblotting with anti-NP and P. β-Actin was used as a loading control. Fig. 11D presents growth rates of rMuV<sup>Iowa/US/06</sup>ΔV and rMuV<sup>Iowa/US/06</sup> in HeLa cells. HeLa cells were infected as Fig. 11A. Fig. 11E presents growth rates of rMuV<sup>Iowa/US/06</sup>ΔV and rMuV<sup>Iowa/US/06</sup> in 293T cells. 293T cells were mock infected or infected with rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.5. Supernatants were collected for plaque assay.

Figures 12A-12D. Ratios of NP and P in rMuV<sup>Iowa/US/06</sup>ΔV-infected cells. Fig. 12A shows NP and P expression levels in rMuV<sup>Iowa/US/06</sup>ΔV- and rMuV<sup>Iowa/US/06</sup>-infected Vero cells during early time points postinfection. Vero cells were mock infected or infected with rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.5. Vero cells were collected and examined for NP and P expression using flow cytometry. Ratios of mean fluorescence intensity (MFI) of P over NP are shown. P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV at 12 and 16 hpi were calculated using Student's t test and are less than 0.05. Fig. 12B shows NP and P expression levels in rMuV<sup>Iowa/US/06</sup>ΔV- and rMuV<sup>Iowa/US/06</sup>-infected Vero cells during late time points postinfection. Ratios of MFI of P over NP at multiple time points postinfection were examined as in Fig. 12A. P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV at 24 and 48 hpi are less than 0.05. Fig. 12C shows NP and P expression ratios of rMuV<sup>Iowa/US/06</sup>ΔV (P GS). Ratios of MFI of P over NP in rMuV<sup>Iowa/US/06</sup>ΔV (P GS)-infected Vero cells, at an MOI of 0.5, were examined at 24 hpi. P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV (P GS) are less than 0.05. Fig. 12D shows NP and P expression ratio of rMuV<sup>Iowa/US/06</sup> (L gene). Ratios of MFI of P over NP in rMuV<sup>Iowa/US/06</sup>ΔV (L gene)-infected Vero cells, at an MOI of 0.5, were examined at 24 hpi. P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV (L gene) are less than 0.05.

Figure 13. HN expression level in rMuV<sup>Iowa/US/06</sup>ΔV. Vero cells were mock infected or infected with rMuVΔV or rMuV at an MOI of 0.5. Cells were collected at 24 hpi and stained for HN using flow cytometry.

Figures 14A-14C. Induction of cell death by rMuV<sup>Iowa/US/06</sup>ΔV. In Fig. 14A, rMuV<sup>Iowa/US/06</sup>ΔV induced a greater cytopathic effect in cell lines. HeLa cells, MDBK cells, or Vero cells were mock infected or infected with rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.5 and photographed at 72 hpi. Fig. 14B shows induction of apoptosis by rMuVΔV in HeLa cells. HeLa cells were infected as in Fig. 14A. At 24 hpi, cells were collected for TUNEL assay.

Percentages of TUNEL-positive cells out of total cells are shown. The P value of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV is less than 0.05. Fig. 14C shows induction of apoptosis in Vero cells. The Vero cells were infected with 0.5 MOI of viruses and processed for TUNEL assay as in Fig. 14B. P values between the wild type and rMuV<sup>Iowa/US/06</sup>ΔV are less than 0.05.

Figures 15A and 15B. Degradation of STAT1 and STAT3 in MuV<sup>Iowa/US/06</sup>-infected cells. As shown in Fig. 15A, rMuV<sup>Iowa/US/06</sup>ΔV failed to degrade STAT1 in Vero cells. Vero cells were infected at an MOI of 0.5. Cell lysates were immunoblotted with anti-NP, -P, -V, and anti-STAT1 recognizing both STAT1 isoforms (STAT1α and STAT1β). β-Actin was used as a loading control. As shown in Fig. 15B, rMuV<sup>Iowa/US/06</sup>ΔV failed to degrade STAT3 in Vero cells. Cell lysates were immunoblotted with anti-NP, -P, -V, -STAT3, and -STAT2.

Figures 16A and 16B. Induction of IFN-β and IL-6 by rMuV<sup>Iowa/US/06</sup>ΔV. Fig. 16A shows induction of IFN-β production by rMuV<sup>Iowa/US/06</sup>ΔV virus. 293T cells were infected with wild-type PIV5, rPIV5V C, rMuV<sup>Iowa/US/06</sup>, or rMuV<sup>Iowa/US/06</sup>ΔV or mock infected. The cellular supernatants were collected at 24 and 48 hpi and analyzed for IFN-β production by ELISA. The graph shows the average of three independent experiments, and error bars represent the standard deviation (SD). P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV at 24 and 48 hpi are less than 0.05. Fig. 16B shows induction of IL-6 production by rMuV<sup>Iowa/US/06</sup>ΔV virus. HeLa cells were infected with wild-type PIV5, rPIV5V C, rMuV<sup>Iowa/US/06</sup>, or rMuV<sup>Iowa/US/06</sup>ΔV or mock infected. The cellular supernatants were collected at 24 and 48 hpi and analyzed for IL-6 production by ELISA. The test samples were diluted to 1:10 in a sample diluent provided in the kit. The graph shows the average of two independent experiments, and error bars represent the SD. P values of rMuV<sup>Iowa/US/06</sup> versus rMuV<sup>Iowa/US/06</sup>ΔV at 24 and 48 hpi are less than 0.05.

Figure 17. Neurotoxicity of rMuV<sup>Iowa/US/06</sup>ΔV *in vivo*. The severity of hydrocephalus in rats inoculated with rMuV<sup>Iowa/US/06</sup> or rMuV<sup>Iowa/US/06</sup>ΔV was measured as described in Example 2. rMuVΔV-1 is rMuV<sup>Iowa/US/06</sup>ΔV rescue #4 (PX2-SP-48) and rMuVΔV-2 is rMuV<sup>Iowa/US/06</sup>ΔV rescue #5 (PX2-SP-51); as detailed in Fig. 10). P values of rMuV versus rMuVΔV-1 or rMuVΔV-2 are less than 0.05. The P value of rJL versus rMuVΔV-1 is less than 0.05. *n*=36 for MuV, *n*=16 for rMuVΔV-1, and *n*=18 for rMuVΔV-2.

Figures 18A and 18B. Titers of anti-MuV antibodies in the sera measured using ELISA. Figure 18A shows titers measured at serial dilutions of the sera. Figure 18B shows titers at a dilution of 1:1024.

Figure 19. Schematics of MuV-F and F-MuV RSV F can be inserted between F and SH to give rise to MuV-F or between leader sequence and NP to give rise to F-MuV. The insert is a more detailed diagram of F insertion. Sequences of gene start (GS), intergenic region (I) and gene end (GE), which are important for initiation and termination of viral mRNA synthesis, are indicated.

### DETAILED DESCRIPTION

In 2006, the U.S. experienced the largest mumps epidemic in nearly 20 years (Marin et al., 2008, *Vaccine*; 26(29–30):3601–3607). The outbreak originated at a university in Iowa and spread to eleven other states. With the present invention, the sequence of the complete genome of a clinical wild-type isolate from the Iowa mumps epidemic has been determined. This isolate, the Iowa strain, also referred to herein as MuV-IA, rMuV<sup>Iowa/US/06</sup>, MuV Iowa/US/06, MuV-Iowa/US/06, or MuV(Iowa/US/06) is a member of genotype G, not genotype A of the widely used Jeryl Lynn (JL) mumps vaccine. A reverse genetics system was generated for this mumps virus, and using this reverse genetics system, various recombinant MuV constructs were generated, including, but not limited to, recombinant MuV lacking the expression of the viral proteins SH (rMuVΔSH) and/or V (rMuVΔV). These recombinant viruses grow well in tissue culture cells such as Vero cells, which are WHO-approved cell line for vaccine production, but are attenuated in an animal model, demonstrating lower neurotoxicity than even the JL vaccine. These recombinant viruses and their derivatives are suitable for a new generation of MuV vaccines.

Mumps virus (MuV), a member of the family *Paramyxoviridae*, is a negative stranded, non-segmented RNA virus with a genome of 15,384 nucleotides. The viral genome has seven genes but encodes nine known viral proteins. The nucleocapsid protein (NP), phosphoprotein (P) and large RNA polymerase (L) protein are important for transcription and replication of the viral RNA genome (Elango et al., 1988, *J Gen Virol*; 69(Pt 11):2893–2900; Okazaki et al., 1992, *Virology*; 188:926–930; and Rima et al., 1980, *J Gen Virol*; 46(2):501–505). The V/P gene encodes three proteins, I, V and P (Paterson and Lamb, 1990, *J Virol*; 64:4137–4145). Mutations in the P gene have been associated with increased virulence of mumps virus (Saito et al., 1996, *Microbiol Immunol*; 40(4):271–275). The V protein plays important roles in inhibiting interferon

signaling in infected cells (Kubota et al., 2002, *J Virol*; 76(24):12676-12682; Takeuchi et al., 1990, *Virology*; 178:247-253; Ulane et al., 2003, *J Virol*; 77(11):6385-6393; and Yokosawa et al., 2002, *J Virol*; 76(24):12683-12690). The fusion (F) protein, a glycoprotein, mediates both cell-to-cell and virus-to-cell fusion in a pH-independent manner that is essential for virus entry into cells (Waxham et al., 1987, *Virology*; 159:381-388). The hemagglutinin-neuraminidase (HN), another viral glycoprotein, is also involved in virus entry (Tanabayashi et al., 1992, *Virology*; 187:801-804) and mutations in the HN gene have been implicated in mumps virus virulence (Cusi et al., 1998, *J Clin Microbiol*; 36(12):3743-3744). The matrix (M) protein plays an important role in virus assembly (Matsumoto, 1982, *Microbiol Immunol*; 26(4):285-320). The small hydrophobic (SH) protein is a 57-residue type 1, hydrophobic integral membrane protein (Elango et al., 1988, *J Gen Virol*; 69(Pt 11):2893-2900).

The present invention includes an isolated polynucleotide sequence representing a mumps viral genome as described herein, and fragments and derivatives thereof. Such mumps viral genomes include, but are not limited to, the wild type MuV-IA genome or a mumps viral genome lacking expression of the viral proteins SH (rMuV $\Delta$ SH) and/or V (rMuV $\Delta$ V), and derivatives and fragments thereof. MuV, as a member of the family *Paramyxoviridae*, has a negative stranded, non-segmented RNA genome. Thus, in preferred embodiments, an isolated polynucleotide sequence encoding the MuV-IA genome is a complementary DNA (cDNA). One such a cDNA sequence is represented by SEQ ID NO:1. The genomic sequence of the MuV-IA virus, as well as the amino acid sequence of each encoded protein may be found on the National Center for Biotechnology Information (NCBI) website (available on the world wide web at [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov)) under GenBank Accession No. JN012242; Version JN012242.1 (GI:338784246), which is herein incorporated by reference in its entirety. In some embodiments, an isolated polynucleotide representing the MuV-IA genome is an RNA molecule. An isolated polynucleotide representing the MuV-IA genome may be genome or antigenome RNA or cDNA. An isolated polynucleotide representing the MuV-IA genome may be a positive-sense version of the MuV genome corresponding to the replicative intermediate RNA, also referred to as an antigenome.

Also included in the present invention are derivatives of an isolated polynucleotide described herein. In some embodiments, a derivative thereof may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%,

at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to a polynucleotide sequence described herein. For example, a derivative thereof may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:1, or a fragment thereof. In some embodiments, a derivative thereof may encode an amino acid sequence with at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to an amino acid sequence described herein, or encoded by a mumps viral genome described herein. For example, a derivative thereof may encode a polypeptide sequence having at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity a polypeptide sequence encoded by SEQ ID NO:1. Two polynucleotide sequences may be compared using the Blastn program of the BLAST 2 search algorithm, as described by Tatusova and Madden, 1999, *FEMS Microbiol Lett*; 174:247-250), and available on the world wide web at [ncbi.nlm.nih.gov/gorf/bl2.html](http://ncbi.nlm.nih.gov/gorf/bl2.html). Preferably, the default values for all BLAST 2 search parameters are used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x\_dropoff = 50, expect = 10, wordsize = 11, and filter on.

In some embodiments, a derivative thereof hybridizes under "stringent conditions," also referred to herein as "high stringency conditions," to a polynucleotide sequence described herein. For example, a derivative thereof may hybridizes under stringent conditions to SEQ ID NO:1. Such a derivative thereof may further exhibit one or more of the various functional traits described herein. Stringency of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are

present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995). "Stringent conditions" or "high stringency conditions," as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1X SSC containing EDTA at 55°C.

In some aspects, a derivative thereof includes the deletion and/or addition of nucleotide sequences so that the derivative nucleotide sequences complies with "the rule of six." See, for example, Kolakofsky et al., 1998, *J Virol*; 72:891-899.

Also included in the present invention are fragments of isolated polynucleotides, and derivatives thereof. Such fragments may include only a portion of the MuV genome, for example, encoding only one, two, three, four, five, six, seven, or eight of the nine mumps viral proteins. In some aspects, a fragment may serve as a primer or probe.

A fragment thereof may include a fragment of a mumps virus genome determined by any of the primer pairs described in Table 1 or Table 2. For example the fragment determined by any one of PX1F, PX3F, PX5F, PX7F, PX9F, PX11F, PX13F, PX15F, PX17F, PX19F, PX21F, PX23F, PX25F, PX27F, PX29F, PX31F, or PX33F paired with any one of PX2R, PX4R, PX6R, PX8R, PX10R, PX12R, PX14R, PX16R, PX18R, PX20R, PX22R, PX24R, PX26R, PX28R, PX30R, PX32R, or PX34R used as a primer pair in a PCR reaction with a polynucleotide sequence described herein as a template. For example, a fragment of the present invention may

represent the PCR product obtained when any one of PX1F, PX3F, PX5F, PX7F, PX9F, PX11F, PX13F, PX15F, PX17F, PX19F, PX21F, PX23F, PX25F, PX27F, PX29F, PX31F, or PX33F is used as a forward primer, and any one of PX2R, PX4R, PX6R, PX8R, PX10R, PX12R, PX14R, PX16R, PX18R, PX20R, PX22R, PX24R, PX26R, PX28R, PX30R, PX32R, or PX34R is used as a reverse primer on SEQ ID NO:1, or another mumps virus genome, including, but not limited to, any of those described herein.

An isolated polynucleotide, derivative, or fragment thereof may include additional sequences not of mumps origin. Such heterologous sequences may, for example, encode additional antigenic determinants or other additional components, such as promoter, transcription initiation, and/or and termination sequences.

Included with the present invention are vectors and other constructs that incorporate an isolated polynucleotide sequence encoding a mumps virus genome, such as MuV-IA, or a derivative, or fragment thereof. Such a vector may be an expression vector. One such vector construct is a plasmid that includes the polynucleotide sequence encoding the complete genome of MuV, such as the MuV-IA. Such a plasmid is referred to herein as a "pMuV." The present invention includes a pMuV including any of mumps genomes described herein. In some embodiments, the genome sequence may be a cDNA sequence.

The present invention includes a reverse genetics system including a mumps virus described herein, such as the MuV-IA genomic sequence, or a mutant, or derivative thereof. Reverse genetics systems, as described in more detail in the examples included herewith, can be used to generate in vitro infectious virus particles. See also, He et al., 1997, *Virology*; 237(2):249-60 and Tompkins et al., 2007, *Virology*; 362(1):139-50. Such infectious viral particles are referred to herein as recombinant MuV, also referred to herein as rMuV. A rMuV is produced by recombinant means and is, thus, not naturally occurring. A rMuV may function as an infectious viral particle. Included in the present invention are rMuV that express any of the mumps viral genomes described herein. For example, a mumps viral genome unable to express a small hydrophobic (SH) protein product and/or unable to express a V protein product, including, but not limited to, the rMuV $\Delta$ SH, rMuV $\Delta$ V, or rMuV $\Delta$ SH $\Delta$ V constructs described herein.

A mumps viral genome as described herein, may belong to the G serotype or the A serotype. A mumps viral genome may, for example, be the mumps virus strain MuV-IA, Glouc1/UK96(AF280799), UK01-22, 87-1004 (AF314560), SIPAR 02 (AF314558), Biken

(AF314561), 87–1005 (AF314562), MuV(2001) (AF314559), Urabe 1004-10/2 (FJ375177), Urabe Gw7 (FJ375178), Hoshino (AB470486), Miyahara (1992) (NC\_002200), MuV Miyahara (1992) (2) (AB040874), Y213(AB576764), Dg1062/Korea/98 (32172464), L3/Russia/Vector (AY508995), L-Zagreb master seed (AY685921), L-Zagreb vaccine strain (AY685920), 9218/Zg98 (299766355), Novosbrisk genotype C (50404164), PetroNov genotype H (AY681495), 88–1961 (AF467767), Du/CRO05 (EU370207), SP-A (FJ556896), SP (EU884413), SP(2006) (DQ649478), JL2 (AF3452901), Jeryl Lynn sub strain (FN31985), Enders (GU9800521), Jeryl Lynn major component (AF338106), MuV(2000) (AF201473), JL1 (FJ211586), RIT4385 (FJ211585), or RIT4385(2) (FJ211584). In some preferred embodiments, the mumps viral genome is MuV-IA.

A mumps viral genome unable to express a small hydrophobic (SH) protein product may include a deletion of the open reading frame (ORF) encoding the SH protein or a mutation converting a start codon into a stop codon. For example, the deletion of the open reading frame (ORF) encoding the SH protein may include a deletion of about 156 nucleotides of the ORF encoding the SH protein.

A mumps viral genome unable to express a V protein product may include one or more mutations to the V/I/P gene abrogating expression of the V protein. In some aspects, one or more mutations to the V/I/P gene abrogating expression of the V protein may include the nucleotide sequence GAGGAGGG at the editing site in the P/V gene.

A genome of a mumps virus of the present invention may include one or more further mutations and/or deletions. In some aspects, a further mutation or deletion may include a mutation or deletion effecting phosphorylation of the P protein. In some aspects, a further mutation or deletion effecting phosphorylation of the P protein may include a mutation or deletion at T147 and/or S307 of the P protein. Also included in the present invention is a mumps virus genome, as described herein, further including sequences that allow for the expression of an I protein product. In some aspects, a further mutation or deletion may include a mutation or deletion of the L gene. In some aspects, a further deletions and/or mutations may be selected from any of those known to one of skill in the art.

The present invention also includes a mumps virus genome as described herein, wherein the mumps genome further encodes a heterologous polypeptide. Such a heterologous

polypeptide may be for example, an antigenic polypeptide of non-mumps origin, or a detectable marker, such as, for example GFP or luciferase.

Also included in the present invention are compositions including one or more of the isolated polynucleotide sequences, pMuV, rMuV, vector constructs, infectious viral particles, and/or viral constructs, as described herein. Such a composition may include a pharmaceutically acceptable carrier. As used, a pharmaceutically acceptable carrier refers to one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. Carriers include, for example, stabilizers, preservatives and buffers. Suitable stabilizers include, for example, SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers include, for example, alkali metal phosphates. Suitable preservatives include, for example, thimerosal, merthiolate and gentamicin. Diluents, include, but are not limited to, water, aqueous buffer (such as buffered saline), alcohols, and polyols (such as glycerol). Such compositions and/or carriers may be pyrogen free.

Compositions of the invention may include an adjuvant, including, but not limited to aluminum hydroxide; aluminum phosphate; QS-21 Stimulon; 3-O-deacylated monophosphoryl lipid A; IL-12; N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP); N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP); N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dip- almitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE); cholera toxin; and non-toxic derivatives of cholera toxin, including its B subunit; procholeraenoid, and fungal polysaccharides.

Compositions of the present invention may include additional active immunogens, including other immunologically active antigens against other pathogenic species. The other immunologically active antigens may be replicating agents or non-replicating agents. Replicating agents include, for example, attenuated forms of measles virus, rubella virus, varicella zoster virus (VZV), Parainfluenza virus (PIV), and Respiratory Syncytial virus (RSV). Such an additional agent may be one or more of those currently used in the combination measles-mumps-rubella (MMR) and measles-mumps-rubella-varicella (MMRV) vaccines. The formulation of such compositions is well known in the art.

The present invention also includes methods of making and using the viral vectors and compositions described herein. The compositions of the present disclosure may be formulated in pharmaceutical preparations in a variety of forms adapted to the chosen route of administration. One of skill will understand that the composition will vary depending on mode of administration and dosage unit. The agents of this invention can be formulated for administration in a variety of ways, including, but not limited to, intravenous, topical, oral, intranasal, subcutaneous, intraperitoneal, intramuscular, and intratumor deliver. In some aspects, a composition is formulated for needle-less administration to the mucosa, for example for intranasal administration to the upper respiratory tract. It is expected that mucosal administration of the pharmaceutical composition to a mammalian subject will stimulate an immune response in mucosal tissues, including mucosal tissues that are remote from the site of administration, in addition to producing a systemic immune response in the subject.

The present invention also includes methods of inducing an immune response in a subject by administering an isolated polynucleotide sequences, pMuV, rMuV, vector constructs, infections viral particles, viral constructs, or composition, as described herein to the subject. The immune response may or may not confer protective immunity. An immune response may include, for example, a humoral response and/or a cell mediated response. Such an immune response may be a humoral immune response, a cellular immune response, and/or a mucosal immune response. A humoral immune response may include an IgG, IgM, IgA, IgD, and/or IgE response. The determination of a humoral, cellular, or mucosal immune response may be determined by any of a variety of methods, including, but not limited to, any of those described herein. The induction of an immune response may include the priming and/or the stimulation of the immune system to a future challenge with an infectious agent, providing immunity to future infections. The induction of such an immune response may serve as a protective response, generally resulting in a reduction of the symptoms. The immune response may enhance an innate and/or adaptive immune response. Immunogenicity may be assayed in any of a variety of animal models, including, but not limited to, mouse, ferret, and/or non-human primates model systems.

The isolated polynucleotide sequences, pMuV, rMuV, vector constructs, infections viral particles, viral constructs, or composition of the present invention may demonstrate reduced neurotoxicity when administered to a subject, for example, in comparison to mumps vaccines in

current use, such as, for example, the JL vaccine. Neurotoxicity may be assayed by any of a variety of methods, including, but not limited to, those in conventional use and any of those described herein, including a neurotoxicity test involving intracerebral inoculation into neonatal rats (Rubin et al., 2000, *J Virol*; 74:5382-5384).

The present invention also includes methods of vaccinating a subject by administering an isolated polynucleotide sequences, pMuV, rMuV, vector constructs, infections viral particles, viral constructs, or composition, as described herein to the subject. Such vaccination may result in a reduction or mitigation of the symptoms of future infection and may prevent a future infection. Preferably, these compositions have therapeutic and prophylactic applications as immunogenic compositions in preventing and/or ameliorating mumps infection. In such applications, an immunologically effective amount of at least one attenuated recombinant mumps virus of this invention is employed in such amount to cause a substantial reduction in the course of the normal mumps infection. Again, immunogenicity may be assayed in any of a variety of animal models, including, but not limited to, mouse, ferret, and/or non-human primates model systems. The isolated polynucleotide sequences, pMuV, rMuV, vector constructs, infections viral particles, viral constructs, or composition of the present invention may demonstrate reduced neurotoxicity when administered to a subject, for example, in comparison to mumps vaccines in current use, such as, for example, the JL vaccine. Neurotoxicity may be assayed by any of a variety of methods, including, but not limited to, those in conventional use and any of those described herein, including a neurotoxicity test involving intracerebral inoculation into neonatal rats (Rubin et al., 2000, *J Virol*; 74:5382-5384).

With the methods of the present invention, any of a variety of modes of administration may be used. For example, administration may be intravenous, topical, oral, intranasal, subcutaneous, intraperitoneal, intramuscular, or intratumor. In some aspects, administration is the needleless administration to a mucosal membrane, for example, by the intranasal administration to the upper respiratory tract by spray, droplet or aerosol

An agent of the present disclosure may be administered at once, or may be divided into a number of multiple doses to be administered at intervals of time. For example, agents of the invention may be administered repeatedly, e.g., at least 2, 3, 4, 5, 6, 7, 8, or more times, or may be administered by continuous infusion. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using

known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that any concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

By a "therapeutically effective amount" is meant a sufficient amount of the compound to treat the subject at a reasonable benefit/risk ratio applicable to obtain a desired therapeutic response. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including, for example, the disorder being treated and the severity of the disorder, activity of the specific compound employed, the specific composition employed, the age, body weight, general health, sex and diet of the patient, the time of administration, route of administration, and rate of excretion of the specific compound employed, the duration of the treatment, drugs used in combination or coincidentally with the specific compound employed, and like factors well known in the medical arts.

In some therapeutic embodiments, an "effective amount" of an agent is an amount that results in a reduction of at least one pathological parameter. Thus, for example, in some aspects of the present disclosure, an effective amount is an amount that is effective to achieve a reduction of at least about 10%, at least about 15%, at least about 20%, or at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, compared to the expected reduction in the parameter in an individual not treated with the agent.

As used herein, the term "subject" includes, but is not limited to, humans and non-human vertebrates. In preferred embodiments, a subject is a mammal, particularly a human. A subject may be an individual. A subject may be an "individual," "patient," or "host." Non-human vertebrates include livestock animals, companion animals, and laboratory animals. Non-human subjects also include non-human primates as well as rodents, such as, but not limited to, a rat or a

mouse. Non-human subjects also include, without limitation, chickens, horses, cows, pigs, goats, dogs, cats, guinea pigs, hamsters, ferrets, mink, and rabbits.

As used herein "in vitro" is in cell culture and "in vivo" is within the body of a subject. As used herein, "isolated" refers to material that has been either removed from its natural environment (e.g., the natural environment if it is naturally occurring), produced using recombinant techniques, or chemically or enzymatically synthesized, and thus is altered "by the hand of man" from its natural state.

As used herein, an "isolated" substance is one that has been removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized. For instance, a polypeptide, a polynucleotide, or a cell can be isolated. Preferably, a substance is purified, i.e., is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded RNA and DNA. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. A polynucleotide can be linear or circular in topology. A polynucleotide may be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment. A polynucleotide may include nucleotide sequences having different functions, including, for instance, coding regions, and non-coding regions such as regulatory regions.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

The description exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

## EXAMPLES

### Example 1

Rescue of wild-type mumps virus from a strain associated with recent outbreaks  
defines role of the SH ORF in the pathogenesis of mumps virus

With this example, the complete genome of a representative strain from the epidemic (MuV-IA) was sequenced. MuV-IA is a member of genotype G, the same genotype of MuV that was associated with the outbreak in the UK in 2004–2005. A reverse genetics system was constructed for MuV-IA (rMuV-IA) and used to rescue a virus lacking the open reading frame (ORF) of the SH gene (rMuV $\Delta$ SH). rMuV $\Delta$ SH infection in L929 cells induced increased NF- $\kappa$ B activation, TNF- $\alpha$  production and apoptosis compared to rMuV-IA. rMuV $\Delta$ SH was attenuated in an animal model. These results indicated that the SH ORF of MuV plays a significant role in interfering with TNF- $\alpha$  signaling and viral pathogenesis during virus infection.

### Results

Sequence of the complete genome of MuV-IA. To better understand the genetic characteristics of viruses associated with recent outbreaks in the U.S., the complete genomic sequence of a representative isolate from the Iowa outbreak was determined. It is available as GENBANK Accession No. JN012242. A set of primers was designed based on the consensus sequence derived from comparison of the genomic sequences of Jeryl Lynn, Urabe, 88.1961 and PetroNov. These primers are shown in Table 1. Viral RNA of MuV-IA was reverse-transcribed into cDNA using random hexamers, PCR reactions were then carried out using the set of primers and the products were sequenced using the corresponding primers. A second set of primers based on the sequencing results were then used to perform RT-PCR and the products overlapping with those of first round of sequencing fragments were sequenced using the primers. This second set of primers is shown in Table 2. Leader and trailer sequences were determined by performing 5'/3' RACE.

Table 1. Mumps virus specific primers

| Primer | Approximate genomic location | primer sequence (5' → 3') | SEQ ID NO: |
|--------|------------------------------|---------------------------|------------|
| PX1F   | 100-300                      | ATGTCGTCCGTGCTCAAAG       | 48         |
| PX2R   | 1100-1300                    | CGGTCTCAACCCCAATCTG       | 49         |
| PX3F   |                              | GGGGGCTACCCATTGATATT      | 50         |
| PX4R   | 2100-2300                    | GAAAAGGGGCTCAGGAATCT      | 51         |
| PX5F   |                              | TTCAGTACCCCACTGCATCA      | 52         |
| PX6R   | 3100-3300                    | GGCTGGATTGGACTTGTGTT      | 53         |
| PX7F   |                              | CGAGGATGCCCTGAATGATA      | 54         |
| PX8R   | 4100-4300                    | GCATAGTCTGAGCCCTGGAG      | 55         |
| PX9F   |                              | CACATTCCGACAACCTGCAAA     | 56         |
| PX10R  | 5100-5300                    | TGAACCACTGCAGGTGTCAT      | 57         |
| PX11F  |                              | GCTTGCAACCTCCCTAGGAT      | 58         |
| PX12R  | 6100-6300                    | TGGCACTGTCCGATATTGTG      | 59         |
| PX13F  |                              | GTGTCTGATGATCTCATCAGGTACT | 60         |
| PX14R  | 7100-7400                    | ACCTCAAAGCACGCTGATCT      | 61         |
| PX15F  |                              | GGGAATTGGGCTACTTTGGT      | 62         |
| PX16R  | 7900-8300                    | GTGCATGAACCTGGGATTCT      | 63         |
| PX17F  |                              | GATACCGGTGATGCTAGTGTG     | 64         |
| PX18R  | 9100-9300                    | GAAAGAAAGCCAGGGTCTTCA     | 65         |
| PX19F  |                              | GCTCTACTCATGGGGGACAA      | 66         |
| PX20R  | 10100-10300                  | ATCAAGGTCAAGTTGGGTAGGA    | 67         |
| PX21F  |                              | CCAAGTCATCATCCCCTTTG      | 68         |
| PX22R  | 11100-11500                  | TTGCTGACAATGGTCTCACC      | 69         |
| PX23F  |                              | CATGCCCAATATACATTGATGG    | 70         |
| PX24R  | 12100-12300                  | TGAAGGGTACAGGAAGCAAAG     | 71         |
| PX25F  |                              | CTGGCCTTGCTTTAATTGAGA     | 72         |
| PX26R  | 13100-13300                  | AGAGATGCTGATTCGGATGAA     | 73         |
| PX27F  |                              | GAACCAAATTAAGTGCCTACCC    | 74         |
| PX28R  | 14100-14300                  | CCGCCTGAAGGATAATGTTG      | 75         |
| PX29F  |                              | CCCTGAATGAACAGGGGTTT      | 76         |
| PX30R  | 15100-15300                  | CTTTTGCTGGCCTTTTGCT       | 77         |
| PX31F  |                              | CTGCTAACAAAGCCCGAAAG      | 78         |
| PX32R  | 16100-16300                  | AAGTTGCAGGACCACTTCTG      | 79         |
| PX33F  |                              | TGACTCCCCGTCGTGTAGAT      | 80         |
| PX34R  | 17100-17300                  | AGACGTCAGGTGGCACTTTT      | 81         |

Table 2. MuV-IA primers

| Primer | Location          | Sequence (5' → 3')         | SEQ ID NO: |
|--------|-------------------|----------------------------|------------|
| 5F     | Iowa-L-upstream-1 | TGAATCATAGTGAATGCAGCAGG    | 26         |
| 6F     | Iowa-L-upstream-2 | GCCCTATTGGCGTGTCTCA        | 27         |
| 7R     | Iowa-L-downstream | TGGTGACGTATCGTGCCAGA       | 28         |
| 10F    | Iowa-NP-F         | AACAGTAAGCCCGGAAGTG        | 29         |
| 11R    | Iowa-NP-R         | CCAATGAGTACTGGTGCAAC       | 30         |
| 12F    | Iowa-P-F          | GCGACTGGGATGAGTAAA         | 31         |
| 13R    | Iowa-P-R          | TGGATTGGACTTGTGTTCG        | 32         |
| 14F    | Iowa-M-F          | GCGAGACATCATACGAAG         | 33         |
| 15R    | Iowa-M-R          | AAGCTTGACCACTATGTAGG       | 34         |
| 16F    | Iowa-F-F          | CCTCAATGAGCAACCTATG        | 35         |
| 17R    | Iowa-F-R          | TTAGTACCTGATGAGATCATCG     | 36         |
| 18F    | Iowa-SH-F-EcoRI   | GAATTCATGCCGCGATCCAAC      | 37         |
| 19R    | Iowa-SH-R-NheI    | GCTAGCTTAGAGTGAGTGATCGAAAC | 38         |
| 20F    | Iowa-HN-F         | ATGGAGCCCTCGAAATTCT        | 39         |
| 21R    | Iowa-HN-R         | AACGATGGGTGAGTTTAAATG      | 40         |
| 22F    | Iowa-NP-F2        | GGCTTGGGTGATGGTCTGTA       | 41         |
| 23R    | Iowa-NP-R2        | CATTTTGAATCCTGCACCT        | 42         |
| 24F    | Iowa-HN-F2        | TGCAAGGACCATACTTCGTC       | 43         |
| 25R    | Iowa-HN-R2        | GAGTTCATACGGCCACCAG        | 44         |
| 26F    | Iowa-P-F2         | CTCAACGCCGGTAACAGAAT       | 45         |
| 27F    | Iowa-F-F2         | ATGAAGGTTCTTTAGTTACTTGC    | 46         |
| 28F    | Iowa-P-F3         | AGCCAACCTGCTCAAATCCAC      | 47         |

There is only one conserved change in the putative transmembrane domain of the SH protein when the SH protein sequence of MuV-IA was compared to other strains of mumps virus in genotype G (Fig. 1A), confirming that MuV-IA belongs to genotype G (Rota et al., 2009, *J Med Virol*; 81(10):1819-1825). To further study the genomic divergence of MuV-IA, a phylogenetic tree was generated using the genomic sequence of MuV-IA and 32 full length genomic sequences from Genbank (Fig. 1B). Phylogenetic analysis indicated that MuV-IA is most closely related to the sequence of MuV Du/CRO05, a genotype G virus, which was isolated in Croatia in 2005 (Santak et al., 2006, *J Med Virol*; 78(5):638-643). A comparison of the predicted amino acid sequences between the protein coding regions of MuV-IA and Jeryl Lynn vaccine (major component) showed that while NP, M and L protein sequences are highly conserved with an identity of over 98%, there was more divergence among V, P, F, SH and HN proteins (Fig. 1C). The predicted SH protein sequences had only 85% identity.

Generation of an infectious cDNA clone for MuV-IA. To study the pathogenesis of MuV-IA, a reverse genetics system was derived. Because RNA viruses exist as a quasi-species, the consensus sequence of the genome was used as the base for the recombinant MuV. A plasmid containing a mini-genome with luciferase (Luc) reporter gene for mumps virus (pT7-MuV-Mini-Luc) similar to the PIV5 mini-genome expressing plasmid was constructed using rMuV-IA trailer and leader sequences (Lin et al., 2005, *Virology*; 338(2):270-280). In addition, plasmids encoding NP, P and L in the pCAGGS vector have been obtained and confirmed by sequencing. To test the functionality of the plasmids, the plasmids were transfected into BSRT7 cells. At 2 dpi, the cells were harvested and Luciferase (Luc) assays were performed. Luc activity was detected in the cell transfected with all plasmids, not ones missing P or L, indicating that the plasmids expressed functional P and L proteins. RT-PCR was conducted to amplify DNA fragments representing the complete genome and inserted into individual plasmid vectors before being assembled into a full-length genome. The plasmid with the full length genome of MuV-IA expressed under the control of a T7 (pMuV-IA) promoter (pMuV-IA) was similar to the plasmid used to generate infectious PIV5 (He et al., 1997, *Virology*; 237:249-260). pMuV-IA had changes in two nucleotides within the L ORF compared with consensus sequence of MuV-IA at positions of 11863 (T to C) and 12028 (C to T). However, neither of these nucleotide changes resulted in changes in the predicted L protein sequence. A recombinant MuV (rMuV-IA) was rescued using the plasmid containing the full-length genome of MuVIA. BSRT-7 cells were co-transfected with pMuV-IA and plasmids expressing viral RNA polymerase components. Individual plaques were selected and amplified in Vero cells. The entire genome of the rescued virus was sequenced and found to match the input cDNA genome sequence.

To compare time course of the growth of rMuV and MuV-IA, a multi-cycle growth assay was performed (Fig. 2A). Both viruses grew to similar peak titers in Vero cells. Viral titers in the supernatant of the infected cells increased exponentially during the first two days after infection, and reached a titer of  $10^7$  pfu/ml at 48 hpi. The growth of both viruses in HeLa cells (a human cell line), MDBK cells (a bovine cell line), and L929 cells (a murine cell line) was also compared, and no obvious differences between these two viruses were observed. The viral protein expression levels in cells were also examined using Western blot (Fig. 2B) and the protein levels were similar at different time points after infection, indicating that the replication of rMuV resembles MuV-IA in tissue culture cells.

In addition, infectious recombinant viruses expressing either EGFP or Renilla Luciferase (RL) protein as an extra gene were rescued. pMuVEGFP was constructed by inserting an EGFP gene, flanked by gene start (GS) of SH and gene end (GE) of NP, between F gene and SH gene in pMuV-IA, pMuV-RL was constructed through substitution of coding sequence of EGFP with that of renilla luciferase (RL) in pMuV-EGFP. Expression of EGFP or RL in the infected Vero cells was detected (Figs. 2C and 2D).

Rescue of a recombinant mumps virus lacking the SH ORF. To study the function of the SH protein of MuV, 156 nucleotides in the SH gene open reading frame (ORF) of the SH gene were deleted from pMuV-IA. The truncated SH ORF contained a short ORF encoding five amino acid residues flanked by the original SH ORF start and gene end (pMuV-IA $\Delta$ SH, Fig. 3A). An infectious MuV lacking the SH ORF was rescued (rMuV $\Delta$ SH) (Figs. 3B and 3C) and the genome was sequenced, which matched the input cDNA sequence. The rMuV $\Delta$ SH genome was of 15,228 nt in size, complying with “the rule of six” (Kolakofsky et al., 1998, *J Virol*; 72:891-899). To confirm that wtMuV and rMuV did express a SH protein and that rMuV $\Delta$ SH did not, cell lysates of infected Vero cells were examined by immunoblotting with anti-SH as well as anti-NP and anti-P (Fig. 3D). SH was detected in MuV-IA and rMuV-infected cells, but not in rMuV $\Delta$ SH-infected cells, confirming the lack of the SH protein expression in rMuV $\Delta$ SH-infected cells.

Analysis of rMuV and rMuV $\Delta$ SH. To investigate the growth rate of rMuV $\Delta$ SH, a multiple-cycle growth curve and protein expression levels were examined in Vero cells, and the titers of the viruses released from rMuV $\Delta$ SH-infected Vero cells remained similar to rMuV-infected Vero cells at all time points (Fig. 4A). When the infected cells were lysed and viral protein expression levels were compared using Western blot, the protein levels of NP and P in rMuV $\Delta$ SH and rMuV-infected cells were similar (Fig. 4B), indicating that the SH ORF was not essential for viral gene expression, or virus release in Vero cells, consistent with the previous findings. The HN gene is downstream of the SH gene. To examine whether there is any significant impact of the deletion of the SH ORF on the expression level of HN, expression levels of HN and NP of infected cells were examined using flow cytometry. As shown in Fig. 4C, relative expression level of HN in rMuV-infected cells and in rMuV $\Delta$ SH-infected cells were similar, suggesting that the deletion of the SH ORF sequence did not affect expression of the HN protein. Furthermore, mRNA expression levels of HN were examined using real-time RT-PCR.

No significant difference was observed between rMuV and rMuV $\Delta$ SH (Fig. 4D). Interestingly, rMuVSH formed larger plaques in Vero cells compared to rMuV (Fig. 4A).

rMuV $\Delta$ SH induced cytopathic effect in L929 cells. We compared infection of Vero, MDBK and HeLa cells with rMuV $\Delta$ SH and rMuV. At one day post infection, there were no observable differences in rMuV $\Delta$ SH- or rMuV-infected Vero and MDBK cells. Previous studies in our lab showed that the SH ORFs of PIV5 and RSV played a role in blocking TNF- $\alpha$  signaling. To test the hypothesis that mumps virus SH ORF has a role in regulating the TNF- $\alpha$  signaling pathway, the phenotype of rMuV $\Delta$ SH in L929 cells, which undergo apoptosis after TNF- $\alpha$  treatment, was investigated. rMuV $\Delta$ SH infection led to significantly more cell death than infections with rMuV or wtMuV. The phenotype was evident at 2-day post infection (Fig. 5B). To investigate whether the cytopathic effects (CPE) observed in rMuV $\Delta$ SH infected L929 cells was caused by apoptosis, TUNEL assay was performed. At 1 dpi, infection with rMuV $\Delta$ SH resulted in a higher percentage of infected cells with apoptosis than rMuV (Fig. 5C), indicating that the lack of SH led to increased apoptosis in infected cells.

TNF- $\alpha$  played a critical role in rMuV $\Delta$ SH-induced apoptosis. To test whether apoptosis in rMuV $\Delta$ SH infected L929 cells resulted from an elevated TNF- $\alpha$ , the activation of NF- $\kappa$ B in rMuV $\Delta$ SH-infected L929 cells was examined by examining nuclear translocation of p65, a key subunit of NF- $\kappa$ B. NF- $\kappa$ B factors are localized in the cytoplasm. On activation, for example by TNF- $\alpha$  stimulation, p65 is translocated into the nucleus (Baud and Karin, 2001, *Trends Cell Biol.*; 11(9):372–377). A higher level of p65 nuclear localization was observed in rMuV $\Delta$ SH-infected L929 cells (Fig. 6A), indicating activation of NF- $\kappa$ B. To investigate whether the production of TNF- $\alpha$  was increased in rMuV $\Delta$ SH-infected cells, supernatants of infected were collected and levels of TNF- $\alpha$  were measured using ELISA. TNF- $\alpha$  production level was up regulated in rMuV $\Delta$ SH-infected cells (Fig. 6B). To determine whether the increased TNF- $\alpha$  played a role in increased apoptosis in rMuV $\Delta$ SH infected cells, the infected cells were treated with neutralizing antibody against TNF- $\alpha$ . Anti-TNF- $\alpha$  reduced CPE in rMuV $\Delta$ SH infected cells, while the control antibody had no effect (Fig. 6C), indicating that TNF- $\alpha$  played a critical role in rMuV $\Delta$ SH induced cell death. This was confirmed with TUNEL assay (Fig. 6D). At 1 dpi, with control antibody treatment, rMuV $\Delta$ SH induced almost 4-fold higher apoptotic rate than rMuV. Treatment of anti-TNF- $\alpha$  antibody effectively blocked cell death in infected cells (Figs. 6C, D).

SH of MuV-IA blocked TNF- $\alpha$  signaling in vitro. To investigate whether MuV-IA SH expressed alone can block TNF- $\alpha$  signaling, a plasmid encoding SH of MuV-IA was co-transfected with a NF- $\kappa$ B promoter-luciferase reporter system into L929 cells. At one day post transfection, cells were treated with TNF- $\alpha$ . TNF- $\alpha$  signaling was blocked by SH of MuV-IA as well as SH of PIV5, but not by NP of MuV-IA (Fig. 7A) or the sequence of the SH ORF (Fig. 7B), indicating that the SH protein can block TNF- $\alpha$ -mediated signaling.

rMuV $\Delta$ SH was attenuated in vivo. MuV is a human virus and there is no ideal animal model in which to study viral pathogenesis. Intracerebral injection of MuV into newborn rats has been used to compare the relative pathogenicities of different strains of MuV (Rubin et al., 2005). To compare the neurotoxicity of the viruses, rMuV or rMuV $\Delta$ SH was injected intracerebrally into brains of newborn rats. Relative neurotoxicity score was calculated based on relative severity of hydrocephalus. As shown in Fig. 8, rMuV $\Delta$ SH had a lower neurotoxicity score than rMuV, indicating that deletion of the SH ORF resulted in attenuation in vivo.

## Discussion

Immunization against MuV is a part of a 2-dose MMR (mumps, measles and rubella) vaccine regimen that is administrated to children at 1 and 5 years of age in the U.S. Even with a two-dose vaccination schedule, large outbreaks have occurred in vaccinated populations. This example describes the rescue of a wild-type mumps virus that is representative of the strain associated with recent outbreaks in the U.S. and Europe. This example identifies the potential role of the SH protein in regulating TNF- $\alpha$ , and demonstrates that the deletion of the SH ORF resulted in attenuation in vivo, indicating that SH plays a role in viral pathogenesis. The attenuation of rMuV $\Delta$ SH in vivo suggests that deleting the SH ORF can be a possible strategy to develop attenuated mumps strains. Recombinant MuVs expressing foreign genes such as GFP and RL have been obtained, and interestingly, the expression level of RL in rMuV-RL in Vero cells remained relatively high after 20 passages, indicating that MuV can possibly be used as a vector.

The SH protein of paramyxoviruses was first identified in PIV5- infected cells (Hiebert et al., 1985, *J Virol*; 55:744–751). A similar gene was predicted basing on sequence analysis of the Enders strain of MuV. However, due to a mutation in the intergenic sequence of the putative SH gene, the SH protein of the Enders strain MuV is not expressed in infected cells (Takeuchi et al.,

1991, *Virology*; 181:364-366). Thus, the SH protein of MuV has never been detected in MuV-infected cells. Wilson et al. replaced the SH ORF within the genome of PIV5 with the SH ORF of MuV Enders strain and found that the MuV SH can functionally replace the SH ORF of PIV5 (Wilson et al., 2006, *J Virol*; 80(4):1700-09). Thus, it is thought that the function of MuV SH is the same as the function of the SH ORF of PIV5, a closely related paramyxovirus. In this example, the expression of SH was detected in MuV-infected cells for the first time, confirming the existence of the SH protein in MuV-infected cells. Furthermore, taking advantage of the new reverse genetics system, a recombinant MuV lacking the SH ORF (rMuV $\Delta$ SH) was obtained and analyzed.

One interesting observation was that rMuV $\Delta$ SH produced larger plaques. A possible explanation is that the deletion of the SH ORF resulted in a virus that promotes cell-to-cell fusion better than the wild type virus. Because there was no change of total number of ORFs or the overall order of genes, we expect that the relative amounts of viral mRNAs and the expression levels of viral proteins of rMuV $\Delta$ SH should be similar to those of wild type virus (Figs. 4B, 4C, and 4D). Thus, it is unlikely that the bigger plaque formation by rMuV $\Delta$ SH was due to a higher level of viral protein expression. Further, a fusion assay using cells transfected with MuV HN and F was performed in the presence or absence of MuV SH, and no difference in the extent of cell-to-cell fusion was observed, suggesting that the SH does not have a role in promoting cell-to-cell fusion. It is possible that the larger plaques formed by rMu $\Delta$ SH are due to a higher level of induction of cell death by rMuV $\Delta$ SH. The viruses infected cells at the same rate; however, the cells infected by rMuV $\Delta$ SH induced more cell death than rMuV resulting in more rapid cell death at the edge of a plaque.

It is possible that the mRNA of from some ORFs may have biologic functions. For example, the mRNA of the L ORF of PIV5 is capable of activating IFN- $\beta$  expression (Luthra et al., 2011, *Proc Natl Acad Sci USA*; 108(5):2118-2123). In this example, the ORF of SH was deleted, and the function of the polypeptide encoded by the SH ORF cannot be differentiated from SH mRNA itself. While the SH polypeptide was needed to block TNF- $\alpha$  mediated signaling, not the sequence of the SH ORF, and we favor a critical role of the SH polypeptide in mumps virus pathogenesis; however, it is possible that the small mRNA potentially expressed from the deleted SH gene could have contributed to the phenotype of rMuV $\Delta$ SH. The reduced neurotoxicity of rMuV $\Delta$ SH in neonatal rat brain indicates that the SH ORF plays a critical role in

viral pathogenesis. We propose that infection with rMuV $\Delta$ SH induced a higher level of proinflammatory cytokine expression, resulting in a more rapid resolution of infection, thus limiting damage in the infected brain.

#### Material and methods

**Plasmids, viruses and cells.** All molecular cloning was conducted according to standard procedures as previously described (He et al., 1997, *Virology*; 237:249–260). MuV-IA NP, P and L genes were cloned into the pCAGGS expression vector (Niwa et al., 1991, *Gene*; 108:193–200). MuV-IA SH gene was cloned into the pCAGGS expression vector. MuV-IA SH (stop codon) was constructed by introducing three continues stop codon sequence into the SH ORF, six nucleotides downstream of the start codon. Construction of MuV-IA full-length cDNA in pUC19 was analogous to the PIV5 reverse genetics system (He et al., 1997, *Virology*; 237:249–260). To construct pMuV $\Delta$ SH, the region of the SH ORF from the 4th amino acid to the 57th (156 nt) was substituted with a short six nucleotide sequence designed to facilitate subcloning and to maintain the length of the genome a multiple of six (known as the “rule of six”). pMuV-EGFP and pMuV-RL were constructed by inserting either an EGFP or a renilla luciferase gene between F and SH gene flanked by F gene start and SH gene end.

To rescue an infectious virus from cDNA, plasmid (5  $\mu$ g) containing a full-length genome or a mutated MuV genome was co-transfected with plasmids pCAGGS-L (1  $\mu$ g), pCAGGS-NP (1.5  $\mu$ g) and pCAGGS-P (200 ng) into BSRT-7 cells. Usually four to seven days post-transfection, syncytia formation could be observed in transfected BSRT-7 cells. Supernatants were plaqued in Vero cells. Plaques could be visualized at 4 to 7 dpi. One or two plaques from each independent rescue were amplified in Vero cells and their genomes were sequenced.

Vero, HeLa, MDBK and L929 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S)(Mediatech Inc., Holu Hill, FL), BSRT-7 cell were maintained in DMEM supplemented with 10% FBS, 1% P/S and 10% tryptose phosphate broth (TPB) plus G418 at 400  $\mu$ g/ml. Cells were cultured at 37 °C with 5% CO<sub>2</sub> and passed the day before infection or transfection at appropriate dilution factors to archived 80.90% confluence the next day. For virus infection, cells were infected with viruses in DMEM plus 1% bovine serum albumin (BSA) at MOI of

0.01, 3 or 5 and incubated for 1 to 2 h at 37 °C with 5% CO<sub>2</sub>. The culturing medium was then replaced with DMEM supplied with 2% FBS and 1% P/S. For transfection, cells were transfected with plasmids using PLUS<sup>™</sup> and Lipofectamine<sup>™</sup> reagents from Invitrogen following the manufacturer provided protocol.

MuV-IA (MuV/Iowa/US/2006) was isolated at the Iowa Hygienic Laboratory from a buccal swab obtained from a mumps case during the early phase of the outbreak in 2006. Genotype analysis was performed at the CDC (Rota et al., 2009, *J Med Virol*; 81(10):1819-1825) and the accession number for the SH sequence is DQ661745. All mumps viruses were grown in Vero cells and were harvested 4 to 7 dpi. Virus titers were measured in Vero cells by plaque assay followed by Giemsa staining as described before (He and Lamb, 1999, *J Virol*; 73:6228–6234; and He et al., 1997, *Virology*; 237:249–260).

Sequencing of viruses. Viral RNA was extracted from cell culture supernatants using QIAampR viral RNA extraction mini kit from QIAGEN following manufacturer's protocol. Isolated total RNA was reverse transcribed into cDNA using Super ScriptR III reverse transcriptase from Invitrogen with random hexamers. Synthesized cDNA was then served as templates for PCR using mumps virus genome specific primers (Table 1) and Taq polymerase from Invitrogen. Fifteen sets of primers (shown in Table 2), each contained a forward and a reverse primer, were designed as to divide the genome into fifteen overlapped fragments. The primers were used for the subsequent sequencing of the PCR products (Li et al., 2011, *J Virol*; 85(1):32-42). Leader and Trailer sequences were sequenced following standard protocol of Rapid Amplification of cDNA Ends (RACE) (Li et al., 2011, *J Virol*; 85(1):32-42).

Generation of monoclonal and polyclonal antibodies against mumps NP, P and SH. To generate monoclonal antibodies against MuV-IA, the virus was grown in Vero cells. The medium of infected Vero cells was collected, and clarified with low-speed centrifugation at 3 K rpm for 10 min. The clarified media containing virus was overlaid onto a 10 ml 20% sucrose solution and centrifuged at 40 K rpm for 1.5 h at 4 °C. The pellet was resuspended in 0.5 ml 10X PBS, mixed with 1.3 ml 80% sucrose solution and overlaid by a decreasing sucrose gradient from bottom to top: 1.8 ml 50% sucrose solution and 0.6 ml 10% sucrose solution. The sucrose gradient with virus at the bottom was centrifuged at 45 K rpm for 3 hours (h) at 4 °C. 1 ml fractions were collected, mixed with 10 ml 1X TEN buffer (100 mM NaCl, 10 mM Tris-base, 1 mM EDTA) and spun down at 40 K rpm for 1.5 h at 4 °C. The pellet containing virus was

suspended in 50  $\mu$ l of 1X TEN buffer plus 1% NP-40 and used for generation of mouse hybridoma cells. Mouse hybridoma cells generating monoclonal antibodies against MuV-IA NP and P were engineered by the core facility in the Pennsylvania State University. The hybridomas were culture in D-MEM supplied with sodium pyruvate, with addition of 20% FBS and 0.1% Gentamicin at 37 °C with 5% CO<sub>2</sub>.

To generate polyclonal antibodies against MuV-IA SH, two peptides (N-terminal MPAIQPPLYLTFLLC (SEQ ID NO:10) and C-terminal CYQRSFFHWSFDHSL (SEQ ID NO:11)) were purchased from GenScript Corporation. Two peptides (QFIKQDETGDLIETC (SEQ ID NO:12) and CSRPDNPRGGHRREW (SEQ ID NO:13)) were used to generate polyclonal antibodies against MuV-IA V (GenScript Corporation) in rabbits.

Treatment of infected cells with anti-TNF- $\alpha$ . L929 cells in six well plates were infected with rMuV $\Delta$ SH or rMuV at a MOI of 5 and cultured in DMEM supplemented with 2% FBS and 1% P/S with neutralizing anti-TNF- $\alpha$  antibody or control antibody (BD Pharmingen) at 50  $\mu$ g/ml for 1 or 2 days. At 1 day or 2 dpi, cells were photographed with a microscope with a digital camera, and then collected for MuV-NP staining or TUNEL assay.

Flow cytometry and TUNEL assay. Flow cytometry was performed as previously described (Timani et al., 2008, *J Virol*; 82(18):9123-9133). L929 cell in 6 well plates were infected with rMuV $\Delta$ SH or rMuV or mock infected at MOI of 3 or 5. At 1 or 2 dpi, attached cells were trypsinized and combined with floating cells in the culture media. Cells were centrifuged and resuspended in 0.5% formaldehyde in phosphate buffered saline (PBS) for one hour at 4 °C. The fixed cells were then washed with PBS, permeabilized in 50% FCS-50% DMEM plus three volumes of 70% ethanol overnight. Permeabilized cells were subjected for either TUNEL staining for apoptotic cells according to manufacturer's protocol or MuV-NP staining for infection rate. When cells were for NP staining, monoclonal MuV-NP antibody was diluted to 1:200 followed by PE anti-mouse secondary antibody staining at a dilution factor of 1:100.

Vero cells were mock infected or infected with rMuV $\Delta$ SH or rMuV at a MOI of 0.5 or 0.01. At 24 or 48 hpi, attached cells were collected in combination with floating cells, fixed. For HN surface staining, cells were directly stained with anti-HN at a dilution factor of 1:50; for total staining of HN and NP staining, fixed cells were permeabilized with 0.1% saponin in PBS and stained with anti-NP at a dilution factor of 1:200 or anti-HN at a dilution factor of 1:50.

Assays for detection of activation of NF- $\kappa$ B. L929 cells on glass cover slips in six well plates were infected with rMuV $\Delta$ SH, or rMuV at MOI of 0.01, or mock infected. At 2 dpi the cover slips were washed with PBS, and fixed in 0.5% formaldehyde. The fixed cells were permeabilized with PBS plus 0.1% saponine and then incubated with mouse anti-P65 (Santa Cruz Biotechnology) in PBS with 0.1% saponine followed by secondary FITC labeled goat antimouse antibody (Jackson Laboratory). The cells were photographed using a fluorescence microscope with a digital camera.

The NF- $\kappa$ B reporter assay system was performed as described previously (Wilson et al., 2006, *J Virol*; 80(4):1700-09). L929 cells were plated into 24 well plates and transfected using PLUS<sup>TM</sup> and Lipofectamine<sup>TM</sup> reagents with either empty vector, pCAGGS-MuV SH, pCAGGS-MuV SH(stop), pCAGGS-PIV5 SH or pCAGGS-MuV NP, plus a  $\kappa$ B-TATA-Luc (a reporter plasmid containing a NF- $\kappa$ B promoter region followed by TATA box enhancer and a firefly luciferase gene) and a pCAGGS-RL (a transfection control plasmid expressing renilla luciferase protein). On the second day post transfection, half of the cells were treated with TNF- $\alpha$  (Alexis, San Diego) at a concentration of 10 ng/ml in Optima (Invitrogen) for 4 h at 37 °C with 5% CO<sub>2</sub>; half of the cells were treated with Optima only. Cells were then lysed with 100  $\mu$ l 1X passive lysis buffer (Promega, Madison, WI) and 10  $\mu$ l of the lysate were subjected for dual luciferase assay using a dual luciferase assay kit (Promega, Madison, WI). The ratio of TNF- $\alpha$  stimulated cells over no TNF- $\alpha$  stimulation is used as “induction of luciferase activity.”

Immunoblotting. Vero cells in 6 well plates at about 90% confluence were infected with mock, MuV-IA, rMuV or rMuV $\Delta$ SH at a MOI of 0.05. Cells were collected and lysed at 0 h, 24 h, 48 h, or 72 h post-infection in 0.5 ml WCEB buffer (50 mM Tris.HCl PH 8.0, 120 mM NaCl, 0.5% NP-40, 0.00076% EGTA, 0.2 mM EDTA, 10% Glycerol) with a mixture of protease inhibitors as described before (Luthra et al., 2008, *J Virol*; 82(21):10887-10895). Cell lysates were briefly centrifuged to remove cell debris. Cell lysates were loaded into 10% or 17.5% polyacrylamide gel and subjected for SDS-PAGE. Protein were transferred to Immobilon-FL transfer membrane (Millipore), incubated with primary antibody (anti-MuV SH 1:250, anti-MuV V 1:500, anti-MuV NP 1:5000, anti-MuV P 1:2000) and corresponding secondary antibodies conjugated to horseradish peroxidase, and detected by Amersham ECL<sup>TM</sup> western blotting detection kit (GE Healthcare).

Time course of rMuV $\Delta$ SH, rMuV and MuV-IA infection in cell culture. Cells in 6 cm plates were infected with MuV-IA, rMuV, or rMuV $\Delta$ SH at MOI of 0.01. 100  $\mu$ l of supernatant were collected at 0 h, 24 h, 48 h, 72 h postinfection and frozen down at -80 °C supplemented with 1% BSA. Virus titers were determined by plaque assay using Vero cells in 24 well plates in triplicates. After one to two hours incubation with the viruses, growth media were changed into semisolid DMEM with 2% FBS, 1% P/S and 1% low melting point agarose. 4 to 7 dpi, 24 well plates of Vero cells were stained with Giemsa stain and plaques were counted.

Enzyme-linked immunosorbent assay (ELISA) of TNF- $\alpha$ . L929 cells in 6 well plates were infected with mock, rMuV or rMuV $\Delta$ SH at MOI of 5. Culturing media were collected at 1 dpi, 2 dpi and 3 dpi. The amount of TNF- $\alpha$  secreted into the culturing media was measured using a murine TNF- $\alpha$  detection kit (Amersham Pharmacia) following the procedures described before (Li et al., 2011, *J Virol*; 85(1):32-42).

Real time RT-PCR. Vero cells were mock infected or infected with rMuV $\Delta$ SH or rMuV at a MOI of 0.005. Viral RNA was extracted from infected cells at 4 dpi using QIAGEN RNeasymini kit and reverse transcribed into cDNA using Oligo-dT as primers. MuV F and HN mRNA specific FAM tagged probes were purchased from Applied Biosystems™. Real time PCR was assembled using TaqMan® Gene Expression Master Mix, according to manufacturer's protocol. Ratio between HN mRNA verses F mRNA was calculated using  $\Delta$ ct.

Examination of MuV neurotoxicity. The rat neurotoxicity test was performed as described before (Rubin et al., 2000, *J Virol*; 74:5382-5384). Newborn rats were inoculated intracerebrally with 100 pfu of rMuV (n=36), or rMuV $\Delta$ SH (n=24) in 20  $\mu$ l EMEM. Animals were sacrificed at one month after injection and the brains were removed, immersion fixed and embedded in paraffin. One 10  $\mu$ m sagittal section at a constant distance from the anatomical midline from each hemisphere of brain was selected, and stained with haematoxylin and eosin. The neurotoxicity score was calculated based on the cross-sectional area of the brain (excluding the cerebellum) as a percentage of the lateral ventricle on tissue sections from paired brain using Image- Pro Plus image analysis software (Media Cybernetics). The neurotoxicity score was defined as the mean ratio (percentage) of these two measurements on each of the two tissue sections per rat brain. Any rats with signs of pain or distress prior to the planned 1 month end point were humanely euthanized immediately and included in analyses. The NIH Guidelines for the Care and Use of Laboratory Animals were strictly adhered to throughout.

The results of this example can now also be found in Xu et al., "Rescue of wild-type mumps virus from a strain associated with recent outbreaks helps to define the role of the SH ORF in the pathogenesis of mumps virus," *Virology*; 417(1):126-36 (published August 15, 2011; Epub 2011 Jun 14).

## Example 2

### The V Protein of Mumps Virus Plays a Critical Role in Pathogenesis

Mumps virus (MuV) causes an acute infection in humans characterized by a wide array of symptoms ranging from relatively mild manifestations, such as parotitis, to more-severe complications, such as meningitis and encephalitis. Widespread mumps vaccination has reduced mumps incidence dramatically; however, outbreaks still occur in vaccinated populations. The V protein of MuV, when expressed in cell culture, blocks interferon (IFN) expression and signaling and interleukin-6 (IL-6) signaling. In this example, a recombinant MuV incapable of expressing the V protein (rMuV $\Delta$ V) was generated. The rescued MuV was derived from a clinical wild-type isolate from a recent outbreak in the United States (MuV<sup>Iowa/US/06</sup>, G genotype). Analysis of the virus confirmed the roles of V protein in blocking IFN expression and signaling and IL-6 signaling. It was also found that the rMuV<sup>Iowa/US/06</sup> $\Delta$ V virus induced high levels of IL-6 expression in vitro, suggesting that V plays a role in reducing IL-6 expression. In vivo, the rMuV<sup>Iowa/US/06</sup> $\Delta$ V virus was highly attenuated, indicating that the V protein plays an essential role in viral virulence.

The RNA genome of MuV is 15,384 nucleotides long. It encodes nine known viral proteins. The V protein of MuV has 224 amino acid residues and contains a cysteine (Cys)-rich C terminus that is conserved among all paramyxoviruses. The V protein interrupts the interferon (IFN) signaling pathway through degradation of STAT1, a critical transcription factor for IFN-activated gene expression (Kubota et al., 2002, *J Virol*; 76:12676-12682). A tryptophan-rich motif within the Cys-rich C terminus of the MuV V protein is essential in the ubiquitination and degradation of STAT1 (Kubota et al., 2002, *J Virol*; 76:12676-12682; Kubota et al. al., 2001, *Biochem Biophys Res Commun*; 283:255-259; and Nishio et al., 2002, *Virology*; 300:92) through the N-terminal region of STAT1 (Yokosawa et al., 2002, *J Virol*; 76:12683-12690). The V

protein has also been demonstrated to associate with receptor-activated C kinase (RACK1), which contains Trp-Asp (WD) repeats and mediates interactions between the IFN receptor and STAT1. The V-RACK1 interaction results in the disassociation of STAT1 and RACK1, contributing to the blockade of IFN signaling by V protein (Kubota et al., 2002, *J Virol*; 76:12676-12682). This interaction may be important to block IFN signaling before the complete degradation of STAT1 occurs (Kubota et al., 2005, *J Virol*; 79:4451-4459). The V protein of MuV also interacts with MDA5, a RNA helicase that plays a critical role in the activation of IFN expression in infected cells (Andrejeva et al., 2004, *Proc Natl Acad Sci USA*; 101:17264-17269) and blocks the activation of IFN expression. The Cys-rich C terminus of V protein is essential for its interaction with MDA5 through its helicase C domain (Parisien et al., 2009, *J Virol*; 83:7252-7260; Ramachandran and Horvath, 2010, *J Virol*; 84:11152-11163). The V protein can serve as a substrate for inhibitor of  $\kappa$ B kinase  $\epsilon$  (IKKe)/tumor necrosis factor receptor associated factor (TRAF) family member-associated NF- $\kappa$ B activator (TANK)-binding kinase 1 (TBK1), resulting in inhibition of the activation of interferon regulatory factor 3 (IRF3). The interaction between V protein and TBK1/IKKe inhibits the activation of IRF3, a critical transcription factor for IFN expression, resulting in the blockade of IFN expression (Lu et al., 2008, *J Biol Chem*; 283:14269-14276). The V protein causes degradation of STAT3, a critical transcription factor for interleukin-6 (IL-6)-mediated signaling and oncogenesis (Ulane et al., 2003, *J Virol*; 77:6385-6393). A point mutation within the V protein (E to D at position 95) results in a V protein that is capable of STAT1 degradation without affecting its ability to target STAT3 for degradation. The ability of V protein to block IFN signaling is thought to be important for viral pathogenesis (Rosas-Murrieta et al., 2010, *Virol J*; 7:263). In this Example a recombinant MuV that it was no longer capable of expressing the V protein (rMuV<sup>Iowa/US/06</sup> $\Delta$ V) was generated. The rescued MuV was derived from a clinical wild-type (WT) isolate from a recent outbreak in the United States (MuV<sup>Iowa/US/06</sup>, G genotype). This is the first study of the functions of the V protein of MuV in the context of viral infection.

## Materials and Methods

Plasmids, viruses, and cells. The MuV strain, MuV<sup>Iowa/US/06</sup>, was obtained from a patient during the 2006 Midwest mumps outbreak in the United States. A full-length cDNA clone of the virus (pMuV<sup>Iowa/US/06</sup>) was constructed as described in Example 1 (see also Xu et al., 2011,

*Virology*; 417:126-136). This plasmid was modified to not express the V protein by changing the editing site of the P/V gene (GGGGGG; nucleotides 1-6 of SEQ ID NO:14) to GAGGAGGG (nucleotides 1-8 of SEQ ID NO:15) and the addition of another four base pairs (CTAG; nucleotides 3-6 of SEQ ID NO:16) to the 3' untranslated region (3' UTR; SEQ ID NO:16) of the gene to comply with "the rule of six" (Kolakofsky et al., 1998, *J Virol*; 72:891-899).

To rescue an infectious virus, plasmid pMuV<sup>Iowa/US/06</sup> (5 µg), along with plasmids pCAGGS-L (1 µg), pCAGGS-NP (1.5 µg), and pCAGGS-P (200 ng), were transfected into BSRT-7 cells. Three days later, transfected BSRT-7 cells were mixed with Vero cells at 1:1. Ten to 14 days later, when syncytium formation was observed, supernatants containing rMuV<sup>Iowa/US/06</sup>ΔV were collected and plaque purified in Vero cells. Plaques (developing 4 to 7 days postinfection [dpi]) were amplified in Vero cells, and their genomes were sequenced. The rescue procedure was repeated to produce independent stocks of rMuV<sup>Iowa/US/06</sup>ΔV.

Vero, HeLa, MDBK, and L929 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) (Mediatech Inc., Holu Hill, FL). BSRT-7 cells were maintained in DMEM supplemented with 10% FBS, 1% P/S, and 10% tryptose phosphate broth (TPB), plus 400 µg/ml Geneticin G418 antibiotic. Cells were cultured at 37°C with 5% CO<sub>2</sub> and passaged the day before infection or transfection at appropriate dilution factors to achieve 80 to 90% confluence the next day. For virus infection, cells were inoculated with viruses in DMEM plus 1% bovine serum albumin (BSA) at a multiplicity of infection (MOI) of 0.01, 3, or 5 and incubated for 1 to 2 h at 37°C with 5% CO<sub>2</sub>. The inocula were then replaced with DMEM supplemented with 2% FBS and 1% P/S. Cells were transfected with plasmids using PLUS and Lipofectamine reagents (Invitrogen, Carlsbad, CA) following the manufacturer-provided protocols.

All mumps viruses were grown in Vero cells and were harvested at 4 to 7 dpi. Virus titers were measured in Vero cells by plaque assay as described previously (He and Lamb, 1999, *J Virol*; 73:6228-6234; He et al., 1997, *Virology*; 237:249-260). Parainfluenza virus 5 (PIV5) and recombinant PIV5 lacking the expression of the C terminus of the V protein (rPIV5VΔC) were grown as described before (He et al., 2002, *Virology*; 303:15-32).

Sequencing of viruses. Viral RNA was extracted from cell culture supernatants by using the QIAamp viral RNA extraction minikit (Qiagen Inc., Valencia, CA) following manufacturer's protocol. Isolated viral RNA was reverse transcribed into cDNA by using SuperScript III

reverse transcriptase with random hexamers (Invitrogen). Synthesized cDNA then served as templates for PCR using mumps virus genome-specific primers (shown in Table 1) and Taq polymerase (Invitrogen). Fifteen sets of primers (shown in Table 2), each containing a forward and reverse primer, were designed to divide the genome into 15 overlapping fragments. The primers were then used for the subsequent sequencing of the PCR products (Li et al., 2006, *Virology*; 346:219-228). Leader and trailer sequences were sequenced following the standard protocol of rapid amplification of cDNA ends (RACE) (Li et al., 2011, *J Virol*; 85:32-42).

**Flow cytometry and TUNEL assay.** Flow cytometry was performed as previously described (36). HeLa or Vero cells in 6-well plates were mock infected or infected with rMuV<sup>Iowa/US/06</sup>ΔV, rMuV<sup>Iowa/US/06</sup>, or MuV<sup>Iowa/US/06</sup> at an MOI of 0.1 or 0.5. At 24 h postinfection (hpi), 48 hpi, 72 hpi, or 96 hpi, attached cells were trypsinized and combined with floating cells in the culture media. Cells were centrifuged and resuspended in 0.5% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C. The fixed cells were then washed with PBS and permeabilized in 50% fetal calf serum (FCS)-50% DMEM plus three volumes of 70% ethanol overnight. Permeabilized cells were subjected to either terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining or MuV<sup>Iowa/US/06</sup>-NP, MuV<sup>Iowa/US/06</sup>-P, or MuV<sup>Iowa/US/06</sup>-HN staining for protein expression level. For NP staining, monoclonal MuV<sup>Iowa/US/06</sup>-NP antibody was diluted 1:200; for P staining, monoclonal MuV<sup>Iowa/US/06</sup>-P antibody (as described in Example 1; see also Xu et al., 2011, *Virology*; 417:126-136) was diluted 1:50 followed by fluorescein isothiocyanate (FITC) anti-mouse secondary antibody (Jackson ImmunoResearch) staining at a dilution of 1:10,000. For HN staining, polyclonal MuV<sup>Iowa/US/06</sup>-HN was diluted 1:50 followed by FITC anti-rabbit secondary antibody staining at a dilution factor of 1:10,000. TUNEL staining was performed as described before following the manufacturer's protocol (Roche) (Sun et al., 2009, *PLoS Pathog*; 5:e1000525; Sun et al., 2004, *J Virol*; 78:5068-5078).

**Immunoblotting.** Vero cells in 6-well plates at approximately 90% confluence were mock infected or infected with rMuV<sup>Iowa/US/06</sup> or rMuV<sup>Iowa/US/06</sup>ΔV at an MOI of 0.01 or 0.5. Cells were lysed and collected at different time points postinfection in 0.5 ml WCEB buffer (50 mM Tris-HCl [pH 8.0], 120mMNaCl, 0.5% NP-40, 0.00076% EGTA, 0.2mM EDTA, 10% glycerol) with a mixture of protease inhibitors as described previously (Rubin et al., 2011, *Vaccine*; 29:2850-2855; Rubin et al., 2000, *J Virol*; 74:5382-5384). Cell lysates were briefly

centrifuged to remove cell debris and loaded onto a 10% or 17.5% polyacrylamide gel and subjected to SDS-PAGE. Proteins were transferred to an Immobilon-FL transfer membrane (Millipore, Billerica, MA), incubated with primary antibody (anti-MuV<sup>Iowa/US/06</sup> V, 1:500; anti-MuV<sup>Iowa/US/06</sup> NP, 1:5,000; anti-MuV<sup>Iowa/US/06</sup> P, 1:2,000 [43], anti-STAT1, 1:200 (#B2410; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-STAT2, 1:200 (#07-224; Millipore, Billerica, MA); anti-STAT3, 1:200 (#F300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and corresponding secondary antibodies conjugated to horseradish peroxidase, and detected using an Amersham ECL Western blotting detection kit (GE Healthcare Bioscience, Piscataway, NJ).

Growth curve of rMuV<sup>Iowa/US/06</sup>  $\Delta$ V and rMuV<sup>Iowa/US/06</sup>. Cells in 6-cm plates or 6-well plates were infected with rMuV $\Delta$ V or rMuV at an MOI of 0.01. One milliliter (6-cm plates) or 100  $\mu$ l (6-well plates) of supernatant were collected at 0 h, 24 h, 48 h, and 72 h (24 h, 48 h, 72 h, 120 h, 168 h, 216 h, and 264 h in HeLa) postinfection, supplemented with 1% BSA, and stored at 80°C. Virus titers were determined by plaque assay using Vero cells in 6-well plates in triplicate. After one to two hour (h) incubations with the viruses, the growth medium was changed to DMEM with 2% FBS, 1% P/S, and 1% low-melting-point agarose. Four to 7 dpi, 6-well plates of Vero cells were stained with Giemsa stain, and plaques were counted.

ELISA for IFN- $\beta$  and IL-6. HeLa cells or 293T cells were mock infected or infected with PIV5-WT (MOI 5), rPIV5-V $\Delta$ C (MOI- 5), rMuV<sup>Iowa/US/06</sup> (MOI 0.5), or rMuV<sup>Iowa/US/06</sup>  $\Delta$ V (MOI 0.5) virus in 12-well plates. The supernatants were collected at 24 h and 48 h postinfection. The amount of secreted IL-6 in the medium was measured using the OptEIA human IL-6 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA), and IFN- $\beta$  was measured using the VeriKine human IFN- $\beta$  ELISA kit as described before (16, 18) (PBL InterferonSource, Piscataway, NJ) according to the manufacturer's instructions.

Neurotoxicity test. The neurovirulence phenotype of the rescued viruses was assessed by measuring the extent of MuV-induced hydrocephalus, the major neuropathologic outcome of MuV infection in rats, as previously described (Rubin et al., 2000, *J Virol*; 74:5382-5384). Briefly, three litters of 8 to 10 newborn Lewis rats were inoculated intracerebrally with 10  $\mu$ l of DMEM containing 100 PFU of each of the two virus stocks rescued from plasmid pMuV<sup>Iowa/US/06</sup> and each of the two virus stocks rescued from plasmid pMuV<sup>Iowa/US/06</sup>  $\Delta$ V. On day 30 postinoculation, the rats were humanely sacrificed by CO<sub>2</sub> asphyxiation following the NIH Guidelines for the Care and Use of Laboratory Animals. Brains were removed and immersion

fixed in 10% neutral-buffered formalin at 4°C for 4 to 5 days, followed by paraffin embedding. Sagittal sections obtained at a standard distance from either side of the rostral-caudal midline were stained with hematoxylin and eosin.

The neurovirulence score was determined by calculating the ratio between the cross-sectional area of the brain (excluding the cerebellum) and the cross-sectional area of the lateral ventricle (which is enlarged following infection with neurovirulent MuV strains), measured using Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). The mean ratio (given in percent) of these two measurements on each of the two tissue sections per rat brain is the neurovirulence score for that particular brain. The neurovirulence score for each virus is the mean neurovirulence score for all brains within the treatment group. All comparisons were made using a *t* test or, with nonnormal data (failed Shapiro-Wilk test), the Mann-Whitney rank sum test ( $\alpha = 0.05$ ).

## Results

Recovery of a recombinant MuV lacking expression of V protein (rMuV $\Delta$ V). To investigate the role of the V protein in viral pathogenesis in the context of viral infection, we constructed a cDNA of the MuV<sup>Iowa/US/06</sup> genome containing mutations to ablate the V protein expression (pMuV<sup>Iowa/US/06</sup> $\Delta$ V) (the accession number for MuV<sup>Iowa/US/06</sup> genome is JN012242) (Xu et al., 2011, *Virology*; 417:126-136). Ablation of the V protein expression from the genome was achieved by changing the editing site (GGGGGG; nucleotides 1-6 of SEQ ID NO:14) in the P/V gene into GAGGAGGG (nucleotides 1-8 of SEQ ID NO:15). Therefore, only a transcript encoding the P protein is generated from P/V gene transcription (Fig. 9A). Infectious viruses abolishing the expression of the V protein (rMuV<sup>Iowa/US/06</sup> $\Delta$ V) were rescued from the cloned DNA through transfection of pMuV<sup>Iowa/US/06</sup> $\Delta$ V into BSRT-7 cells. Rescued viruses were further plaque purified and amplified in Vero cells. To confirm the presence of the genetic changes to shut off the V protein expression in the rescued virus genome, viral RNAs were extracted from virus stocks and reverse transcribed into cDNA for sequencing (Fig. 9B and 9C).

Sequencing of the genome of the rescued virus revealed the presence of nucleotide substitutions in the NP gene end (GE) sequence and at the P/V gene start (GS) sequence comparing to input cDNA sequence as well as the changes that would ablate the expression of the V protein (Fig. 9C). Immunoblotting of infected cells was performed to confirm the absence

of the V protein expression in rMuV<sup>Iowa/US/06</sup>ΔV-infected Vero cells (Fig. 9D). To further investigate, the virus was rescued from the cDNA plasmid seven more times (Fig. 10A). Viruses from seven out of the total eight rescued viruses contained a point mutation at either the NP GE (six) or P/V GS region (one), while one contained a point mutation in the L gene (Fig. 10B). All of the rescued rMuV<sup>Iowa/US/06</sup>ΔV viruses contained at least one point mutation in their genome, and the most frequent point mutation was at position 1899 in the genome; thus, this virus was used as a representative virus and designated as rMuV<sup>Iowa/US/06</sup>ΔV for this work, unless otherwise noted.

**Analysis of rMuVΔV in tissue culture cell lines.** To analyze the growth rate of rMuV<sup>Iowa/US/06</sup>ΔV in cell lines, Vero cells or HeLa cells were infected with rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.01, medium was collected at multiple time points postinfection, and viral titers were determined using plaque assay (Fig. 11A). rMuV<sup>Iowa/US/06</sup>ΔV grew at a rate comparable to that of rMuV<sup>Iowa/US/06</sup>V in Vero cells during the first 48 h postinfection (hpi), and then the growth of rMuV<sup>Iowa/US/06</sup>ΔV decreased and remained approximately 1 log lower in titer than rMuV<sup>Iowa/US/06</sup> throughout the studied time course (Fig. 11A). Plaque size of rMuV<sup>Iowa/US/06</sup>ΔV in Vero cells showed no significant differences from that of rMuV<sup>Iowa/US/06</sup> (Fig. 11B). Protein expression levels of rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> low-MOI-infected Vero cells were examined by immunoblotting with anti-NP, P, and V or anti-β-actin (Fig. 11C). Consistent with the time course, the viral protein expression levels of rMuV<sup>Iowa/US/06</sup>ΔV were similar to those of rMuV<sup>Iowa/US/06</sup> at 48, 72, and 96 hpi (adjusting for the levels of β-actin). In HeLa cells, the growth of rMuV<sup>Iowa/US/06</sup>ΔV was reduced (Fig. 11D). The absence of a functional V protein reduced the virus titer of rMuV<sup>Iowa/US/06</sup>ΔV by almost 2 log<sub>10</sub> from 72 hpi to 168 hpi compared with rMuV<sup>Iowa/US/06</sup>. Nevertheless, the both viruses reached similar titers at later time points.

**Expression of viral genes in rMuV<sup>Iowa/US/06</sup>ΔV-infected cells.** Mutations at either the NP GE or P/V GS in recovered rMuV<sup>Iowa/US/06</sup>ΔV viruses suggested that a modulation of viral protein expression levels between NP and P might be critical for the recovery of rMuV<sup>Iowa/US/06</sup>ΔV from cDNA. To investigate the viral protein expression pattern in rMuV<sup>Iowa/US/06</sup>ΔV, Vero cells infected with a high MOI were stained for NP and P proteins at different time points postinfection and assessed by flow cytometry (Fig. 12). To quantify the possible changes in the NP and P expression pattern, P protein expression levels were

normalized to that of the corresponding NP levels (Fig. 12A and 12B). The P/NP ratio of rMuV $\Delta$ V was significantly higher than that of rMuV at 12, 16, 24, and 48 hpi, indicating an elevated P protein expression in the rMuV<sup>Iowa/US/06</sup> $\Delta$ V virus. This difference was no longer evident by 72 hpi.

To investigate if this altered NP and P expression pattern was unique for this rMuV<sup>Iowa/US/06</sup> $\Delta$ V strain, an rMuV<sup>Iowa/US/06</sup> $\Delta$ V containing a P GS mutation (rMuV<sup>Iowa/US/06</sup> $\Delta$ V [P GS]) and an rMuV<sup>Iowa/US/06</sup> $\Delta$ V containing a L open reading frame (ORF) mutation (rMuV<sup>Iowa/US/06</sup> $\Delta$ V [L gene]) were also examined (Fig. 12C and 12D). Similar to rMuV<sup>Iowa/US/06</sup> $\Delta$ V, both rMuV<sup>Iowa/US/06</sup> $\Delta$ V (PGS) and rMuV<sup>Iowa/US/06</sup> $\Delta$ V (L gene) had a P protein expression level greater than that of rMuV<sup>Iowa/US/06</sup>, suggesting that this altered NP and P expression pattern was typical for the recovered rMuV<sup>Iowa/US/06</sup> $\Delta$ V viruses.

To examine if downstream viral protein expression was affected by either deletion of the V protein or the point mutation in NP GE, HN expression levels were examined using flow cytometry. Vero cells were either mock infected or infected with rMuV<sup>Iowa/US/06</sup> $\Delta$ V or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.5, and then cells were collected at 24 hpi and subjected to NP and HN staining (Fig. 13). No significant changes in the HN-to-NP ratio were observed.

rMuV<sup>Iowa/US/06</sup> $\Delta$ V-induced accelerated CPE in tissue culture cell lines. rMuV<sup>Iowa/US/06</sup> $\Delta$ V-induced cytopathic effects (CPE) were compared in three different cell culture lines from three different organisms. HeLa (human), Vero (monkey), and MDBK (bovine) cells were infected with rMuV<sup>Iowa/US/06</sup> $\Delta$ V or rMuV<sup>Iowa/US/06</sup> at an MOI of 0.5, and the cells were photographed at 72 hpi. rMuV<sup>Iowa/US/06</sup> $\Delta$ V caused the most-severe CPE in HeLa cells. More and larger syncytia were observed in rMuV<sup>Iowa/US/06</sup> $\Delta$ V-infected HeLa cells, which may be a major contributing factor to cell death (Fig. 14A). To examine whether the cell death was caused by apoptosis, the TUNEL assay was performed (Fig. 14B). HeLa cells infected with rMuV<sup>Iowa/US/06</sup> $\Delta$ V at an MOI of 0.5 showed at least a 2-fold higher level of apoptosis than cells infected with rMuV. Similarly, rMuV<sup>Iowa/US/06</sup> $\Delta$ V induced a higher level of apoptosis in Vero cells (Fig. 14C). That the lack of V led to an increase in apoptosis in infected cells suggests that the V protein might play a role in blocking induction of apoptosis in infected cells.

Status of STAT proteins in MuV<sup>Iowa/US/06</sup>-infected cells. Previous studies have shown that the V protein is involved in blocking the IFN signaling pathway by targeting STAT proteins for degradation. To determine whether MuV<sup>Iowa/US/06</sup> V protein is the only virus-encoding

antagonist of the IFN pathway, STAT family protein levels were examined in Vero cells infected with rMuV<sup>Iowa/US/06</sup>ΔV or rMuV<sup>Iowa/US/06</sup> (Fig. 15A and 15B). Consistent with previous in vitro transfection studies, STAT1 and STAT3, but not STAT2, were completely degraded in rMuV<sup>Iowa/US/06</sup>-infected Vero cells, while rMuV<sup>Iowa/US/06</sup>ΔV, which lacks expression of the V protein, failed to target any STAT proteins for degradation, indicating that the V protein might be essential and necessary for STAT protein degradation by MuV<sup>Iowa/US/06</sup>. There is a time lag between the occurrence of a detectable V protein and the degradation of STAT protein (Fig. 15A and 15B), implying that the degradation of STATs might require accumulation of the V protein.

PIV5, a paramyxovirus closely related to MuV, prevents induction of IFN-β in infected cells, while recombinant PIV5 lacking the expression of the conserved C terminus of the V protein does not (He et al., 2002, *Virology*; 303:15-32; Poole et al., 2002, *Virology*; 303:33-46). To compare IFN-β inductions by rMuV<sup>Iowa/US/06</sup>ΔV and rMuV<sup>Iowa/US/06</sup>, IFN-β concentration in the medium of infected 293T cells was measured by using ELISA. At 48 hpi, rMuV<sup>Iowa/US/06</sup>ΔV induced IFN-β production higher than that induced by rMuV<sup>Iowa/US/06</sup> (Fig. 16A), indicating that the V protein of MuV<sup>Iowa/US/06</sup> plays a role in limiting IFN-β expression.

rMuV<sup>Iowa/US/06</sup>ΔV led to a higher level of IL-6 induction. To investigate whether the absence of a functional V protein in MuV<sup>Iowa/US/06</sup> infection would lead to induction of other cytokines, IL-6 production levels in the medium of rMuV<sup>Iowa/US/06</sup>ΔV and rMuV<sup>Iowa/US/06</sup>-infected cells were examined. At 48 hpi, rMuV<sup>Iowa/US/06</sup>ΔV led to a higher level of IL-6 production than rMuV<sup>Iowa/US/06</sup> in HeLa cells (Fig. 16B), indicating that IL-6 induction was reduced by the presence of the V protein. Intriguingly, rMuV<sup>Iowa/US/06</sup> infection also induced a significant amount of IL-6 production. This is consistent with MuV being an inflammatory disease.

Neurotoxicity of rMuV<sup>Iowa/US/06</sup>ΔV. To examine the effect of the V protein on virus neurovirulence, viruses from two independent rescues using plasmid pMuV<sup>Iowa/US/06</sup>ΔV (rMuV<sup>Iowa/US/06</sup>ΔV) (Fig. 10A) were tested in rats, along with rMuV<sup>Iowa/US/06</sup> and the highly attenuated Jeryl Lynn (JL) vaccine virus as controls. As shown in Fig. 17, the ΔV viruses were highly attenuated compared to rMuV<sup>Iowa/US/06</sup> and JL vaccine virus.

## Discussion

The results of this example confirm these findings through the study of a recombinant virus derived from a clinical isolate (genotype G) ablating the expression the V protein in the context of in vitro infection.

The lack of V protein expression also led to the induction of a higher level of IL-6, a proinflammatory cytokine, suggesting that the V protein plays a role in suppressing IL-6 expression. The lack of V protein expression in infected cells likely resulted in the attenuation of this strain in an animal model, suggesting that the V protein plays an essential role in viral virulence. It is possible that the inability of rMuV<sup>Iowa/US/06</sup>ΔV to counter IFN action limited the replication of the virus in vivo, and the induction of a higher level of IL-6 by rMuV<sup>Iowa/US/06</sup>ΔV attracted monocytes to clear the infection quickly, resulting in the attenuation of rMuV<sup>Iowa/US/06</sup>ΔV in vivo.

Genetically, the closest virus to MuV is parainfluenza virus 5 (PIV5). The V proteins of MuV and PIV5 share many identical functions, including blocking IFN expression through MDA5, blocking IFN signaling through degradation of STAT1, and inhibiting expression of IL-6 in virus-infected cells. Interestingly, a recombinant PIV5 lacking the entire V protein has never been obtained in tissue culture cells, suggesting that the V protein of PIV5 plays a more critical role in virus replication (Dillon and Parks, 2007, *J Virol* 81:11116-11127; He et al., 2002, *Virology*; 303:15-32) than the V protein does for MuV. The viability of rMuV<sup>Iowa/US/06</sup>ΔV suggests that the role of MuV<sup>Iowa/US/06</sup> V protein in virus replication is dispensable, at least in tissue culture cells.

In this example, a recombinant virus incapable of producing the V protein (rMuV<sup>Iowa/US/06</sup>ΔV) was generated using a reverse genetics system for MuV based on a clinical isolate from a recent outbreak. This virus grew to titers similar to those for wild-type virus in Vero cells, a cell line that is used for vaccine production, as well as in other cell types. Most importantly, the virus exhibited low neurotoxicity in rats, supporting it as a vaccine candidate.

The V/P gene of MuV encodes three proteins, V, I, and P, through a process of “RNA editing,” in which nontemplate G residues are inserted into mRNA during transcription at a specific site to generate mRNAs that can be translated into three different ORFs (Saito et al., 1996, *Microbiol Immunol*; 40:271-275). The V protein is translated from the “unedited” copy of mRNA, P from the mRNA with two G residue insertions, and the I protein from the mRNA with

one or four G residue insertions. All of these proteins have identical N termini of 155 amino acid residues. The P protein has 391 amino acid residues and plays an essential role in viral RNA synthesis. The I protein has 170 amino acid residues, and its function is unclear. It is possible that the I mRNA is a by-product of RNA editing and it may not have any significant functions. The strategy we used to generate rMuV<sup>Iowa/US/06</sup>ΔV also eliminated expression of the I protein. Because the mRNA for I counts only for less than 2% of total V, I, and P transcripts, and its sequence is very similar to the N termini of V and P (I has about 170 amino acid residues and 155 of them are identical to the N termini of V and P) (Paterson and Lamb, 1990, *J Virol*; 64:4137-4145; Takeuchi et al., 1990, *Virology*; 178:247-253), the phenotypes of rMuV<sup>Iowa/US/06</sup>ΔV is attributed to the lack of V protein. However, a possible role for the I protein cannot be excluded.

All changes except the one in the L gene occurred in the gene junction between NP and P/P genes to generate viable infectious MuV incapable of expressing the V protein. It is interesting that a mutation in the L gene was able to allow the rescue of a virus lacking the V protein. While the possibility that the mutation in the L gene occurred fortuitously cannot be excluded and is immaterial to the function of L, one can speculate that the particular mutation may play a role in modulating interactions between NP-P and L, considering that all other viruses rescued had mutations to modulate the levels of NP and P. Further analysis of the virus may lead to a better understanding of the function of L.

The results of this example can now also be found in Xu et al., "The v protein of mumps virus plays a critical role in pathogenesis," *J Virol*; 86(3):1768-76 (February 2012; Epub 2011 Nov 16).

### Example 3

#### Immunogenicity of MuV $\Delta$ SH and MuV $\Delta$ V in Mice

The immunogenicity of rMuV $\Delta$ SH and rMuV $\Delta$ V in mice was determined and MuV-specific immune responses measured. Mice in a group of 10 were inoculated with PBS, or  $10^6$  pfu of MuV, rMuV $\Delta$ V or rMuV $\Delta$ SH intranasally. At 21 days post inoculation, blood samples from the mice were collected. Titers of anti-MuV antibodies in the sera were measured using ELISA. The 96-well plates for ELISA were coated with purified MuV virion. P values for MuV and rMuV $\Delta$ SH, MuV and MuV $\Delta$ V at highest dilution and lowest dilution of sera were lower than 0.05. The results are shown in Fig. 18.

Further humoral immunity (antibody) analysis will include a determination of anti-MuV antibodies in bronchoalveolar lavage (BAL), as measured by MuV-specific ELISA. In ELISA assays, the isotypes (IgA, IgG1, IgG2a, IgG2b, and IgG3) of the antibodies will also be determined using appropriate secondary antibodies. MuV-specific antibody titers will also be measured by virus. Neutralization assays against heterologous JL or homologous MuV-IA will be performed on serum and BAL wash samples.

Cell mediated immunity (T cell) may be measured by antigen-specific IFN $\gamma$  production. Specifically, lymphocytes from the BAL, spleen and/or draining lymph node will be assayed for MuV-specific T cell responses by restimulation with MuV- infected APCs or with purified MuV virions that are disrupted with mild detergent. IFN- $\gamma$  responses will be determined by intracellular cytokine staining and/or ELISPOT assays.

Since the site of induction of immune responses can alter the nature of the immune response and dramatically impact protective efficacy, local and systemic immunity to MuV will be measured at various time points after immunization. Intranasal (IN) MuV immunization has the potential to induce local MuV-specific T cell and immunoglobulin responses that mediate protection against MuV challenge. Local (i.e. lung) MuV- specific immune responses will be assessed by analysis of BAL samples collected at time points after immunization or challenge. Infiltrating lymphocyte populations will be collected by centrifugation and the bronchoalveolar lavage (BAL) will be analyzed for mucosal Ig. Systemic responses will be assessed by analysis of serum antibody and splenic or mediastinal lymph node (MLN) lymphocytes.

Current MMR vaccination regimen calls for two-dose intramuscular (IM) inoculation. A similar regimen was used in a mouse model to evaluate efficacies of vaccines (Cusi et al., 2001, *Arch Virol*; 146(7):1241-862). Initially, immunogenicity may be assayed using such a two dose/IM regiment. The mice will be injected with a primary dose and followed by an injection at two weeks after initial injection. At one month after last immunization, the mice will be sacrificed for immunological assays as described above. This experiment will generate a baseline of immune responses after inoculation with the vaccine candidates, along with the JL vaccine in our hand.

The immunogenicity of a rMuV vaccine construct as described herein may be examined using a two-dose/intranasal (IN) protocol. Both humoral and cell-mediated immune responses will be measured. It has been reported that the IN route generated better immune responses for some vaccines, including a robust cell mediated immune responses. In addition, IN inoculation has the benefit of generating mucosal immune responses and higher titers of IgA. Because of the success of using the IN route for influenza virus vaccination, the IN route will be feasible route for the new vaccine to be introduced to a large human population.

In a similar fashion, a three-dose inoculation regimen will also be tested. As the most likely target for initial Phase I clinical trials will be healthy individual who have already been vaccinated with two-dose MMR, the immune responses after two dose/IM inoculation with the JL vaccine followed by a third dose of JL (as a control) or a third dose of MuV vaccine either by IM or IN will be examined. If a MuV vaccine construct as described herein used as a boost (third dose) is safe and generates robust anti-genotype G immune responses, it may be used to replace the second dose of MMR and may eventually replace JL in the two-dose MMR.

The immunogenicity of any of the rMuV constructs described herein may be assayed in mice, as described in this example. Similar immunogenicity and efficacy studies may also be undertaken in additional animal model systems, including, but not limited to, ferret and non-human primate model systems.

## Example 4

Generation and Analysis of rMuV $\Delta$ SH $\Delta$ V

Because MuV vaccine is used in 1-year old infants, safety is a paramount consideration in developing a new vaccine. While both rMuV $\Delta$ SH and rMuV $\Delta$ V demonstrate attenuation in a rat brain-based neurotoxicity test, to further reduce any potential risk, a recombinant virus lacking both SH and V (rMuV $\Delta$ SH $\Delta$ V) was generated using the reverse genetics system.

Briefly, following protocols described in more detail in Example 1 (see, for example, Fig. 3A), the ablation of SH protein expression from the MuV $\Delta$ SH $\Delta$ V genome was achieved by deleting 156 nucleotides in the SH gene open reading frame (ORF) of the SH gene from pMuV-IA. And, following protocols described in more detail in Example 2 (see, for example, Fig. 9A), the ablation of the V protein expression from the MuV $\Delta$ SH $\Delta$ V genome was achieved by changing the editing site (GGGGGG) in the P/V gene into GAGGAGGG. Therefore, only a transcript encoding the P protein is generated from P/V gene transcription. Infectious viruses abolishing the expression of both the SH protein and the V protein (rMuV $\Delta$ SH $\Delta$ V) were rescued from the cloned DNA through transfection of pMuV $\Delta$ SH $\Delta$ V into BSRT-7 cells. Rescued viruses were further plaque purified and amplified in Vero cells. To confirm the presence of the genetic changes to shut off both SH and V protein expression in the rescued virus rMuV $\Delta$ SH $\Delta$ V genome, viral RNAs were extracted from virus stocks, reverse transcribed into cDNA, and sequenced.

Following procedures described in more detail in Example 1 and Example 2, immunoblotting of infected cells will be performed to confirm the absence of SH and V protein expression in rMuV $\Delta$ SH $\Delta$ V-infected cells. The expression, function, immunogenicity, and pathogenicity of rMuV $\Delta$ SH $\Delta$ V will be analyzed by a variety of methods, including, but not limited to, any of those described herein, for example, as described in the Examples included herewith. Studies may include examination of the neurotoxicity of rMuV $\Delta$ SH $\Delta$ V in the neonatal rat brain and examination of immunogenicity in mice.

### Example 5

#### Improving recombinant MuV as a vaccine candidate

In this example, rMuV constructs will be further mutated using the reverse genetics system to introduce mutations at desirable locations. The resultant MuV mutants will be analyzed in tissue culture cells. Neurotoxicity will be evaluated in a rat model and immunogenicity of the viruses examined in mice, ferrets, and primates. It is likely that rMuV lacking the V and SH plus additional point mutations will be the most attenuated and the least likely to be reverted.

Generation and analysis of additional MuV mutants. The closest virus to MuV is parainfluenza virus 5 (PIV5). These two viruses have identical number of genes and gene order. In recent studies of PIV5, residues within PIV5 proteins have been identified that are capable of enhancing viral gene expression and inducing expression of cytokines such as type I interferon (Sun et al., 2009, *PLoS Pathog*; 5(7):e1000525). It was found that the residue of S157 of the P protein of PIV5 is a binding site for host kinase PLK1 and the residue of S308 of the P protein of PIV5 is a phosphorylation site of PLK1. Mutating S157 or S308 to amino acid residue A, results in a virus that increases viral gene expression as well as induction of interferon- $\beta$  expression. Increasing viral gene expression will potentially increase immune responses because of increased amount of antigens and increasing IFN expression will likely cause attenuation because of anti-viral effects of IFN. Corresponding residues within the P protein of MuV are T147 and S307. These residues will be mutated and the impact of changing these residues on viral gene expression and induction of interferon will be examined.

Generation and analysis of rMuV lacking I and rMuV $\Delta$ V expressing I. The strategy used in Example 2 to generate rMuV $\Delta$ V also eliminated expression of the I protein besides the expression of V. The I protein is an editing product of VI/P gene. Its function is not known. Because its expression level is very low compare with V or P, and its sequence is very similar to the N-terminal of V and P (I has about 170 amino acid residues and 155 of them are identical to the N-terminal of V and P), the effect of deleting the I protein has often been overlooked. For the purposes of developing an effective vaccine, deleting I along with V may be advantageous for attenuation. However, it is possible that the I protein does have a role in viral pathogenesis

and contributes to efficacy of a vaccine. To investigate the role of the I protein in virus life cycle in general, and in generating immune responses in particular, a recombinant virus lacking the I protein will be generated. The rMuV $\Delta$ V genome will be used as a backbone to insert V between P and M. As a result of the mutations at the editing site, no I or V will be made from the P gene, but the V protein will be made from the newly inserted V. Similarly, the I gene will be inserted between HN and L in the backbone of the rMuV $\Delta$ V genome to generate a recombinant rMuV $\Delta$ V expressing I (rMuV $\Delta$  V+I). The reason for two different gene junctions to be inserted is that the V protein expression level ought to be high to reflect the wild type virus infection and expression level of I should be low as in wild type virus infected cells. Gene junction closer to the leader sequence (P-M junction) will give higher viral gene expression levels than the distant one (HN-L junction). The resultant viral construct will be analyzed as described in the previous examples.

Generation and analysis of revertants. In the case of rMuV $\Delta$ V, several point mutations were introduced into the genome of MuV to give rise to the V protein deletion phenotype. It is possible that mutations that will revert the phenotype may be generated over a period of time. While a revertant of rMuV $\Delta$ V has not been obtained after passing the virus in Vero cells over 20 passages, this experiment will be repeated in interferon competent cell lines. Vero cells are WHO and FDA-approved for vaccine production and do not produce type I IFN. That rMuV $\Delta$ V has been stable in this cell line is encouraging for future mass production of rMuV $\Delta$ V as a vaccine. However, Vero cells are defective in IFN production due to a deletion of IFN gene locus. Thus, the rate of revertant of rMuV $\Delta$ V in an interferon competent environment will be examined. A549 cells, a human lung cell line that produces and responds to interferons, will be infected with rMuV $\Delta$ V at a MOI of 0.1 and at 4 days post infection, media of the infected cells will be collected and used to infect fresh A549 cells at about 0.1 MOI. In preliminary studies, it was observed that rMuV $\Delta$ V reached about  $10^6$  pfu/ml and this titer will be used as a rough estimation for our experiment. Virus will be collected at every passage from the media of rMuV $\Delta$ V-infected A549 cells and initially sequence viruses from passage 5, 10, 15 and 20. Similarly, other MuV mutants such as rMuV $\Delta$ SH and rMuV-P-T147A will be examined.

Recombinant viruses that demonstrate enhanced viral gene expression and/or increased interferon induction will be tested for neurotoxicity and immunogenicity, as described in the previous example. Even mutations that do not achieve attenuation equal to rMuV $\Delta$ V will be tested, because of their potential in induction of type I interferon. While Type I interferon is well

known for its anti-viral activities, it also plays a positive role in inducing adaptive immunity (Iwasaki et al., 2004, *Nat Immunol*; 5(10):987-95). It promotes proliferation of memory T cells and prevents apoptosis of T cell. It plays a critical role in antigen cross presentation. It enhances humoral immunity and stimulates dendritic cells. It will be of significant interest if these IFN inducing MuV mutants generate more robust immune responses than its parent. If these mutations indeed produce better immune responses, these mutations will be incorporated into the rMuV genome.

It is possible that the I mRNA is a by-product of RNA editing and it may not have any significant functions. Investigating whether the I protein has a role in virus replication and pathogenesis will not only reveal potential novel functions about the I protein, it will also be important for vaccine development. In case rMuV $\Delta$ V is too attenuated, expressing I in the backbone of rMuV $\Delta$ V may help to design a virus with desirable level of attenuation. In the case of human PIV2 vaccine development, deleting the V protein resulting in a virus that is too attenuated to be effective (Schaap-Nutt et al., 2010, *Virology*; 397(2):285-98). Thus, adding V or I back may be result in a more appropriately attenuated MuV vaccine.

It is possible that the residues in the P protein of MuV that are responsible for PLK1 binding and phosphorylation may be different than predicted above. They will be searched using an approach similar to that used for PIV5: there are two PLK1 binding motifs within the P of MuV. Analogous residues in MuV will be examined. In preliminary studies, it has been found that the P protein and PLK1 interacted, indicating that a PLK1 binding site is within the P protein. In addition, mutations within P have been identified that enhanced its ability to facilitate viral gene expression, i.e., increased viral gene expression phenotype. Besides mutating the P protein, mutations will also be made in other genes. For instance, mutations in the L gene of PIV5 that enhances viral gene expression have been identified. The same mutations will be incorporated into the L gene of MuV.

### Example 6

#### Immunogenicity of Recombinant Mumps Viruses in Ferrets

The immunogenicity and efficacy as a vaccine candidate of any of the MuV described herein will be tested in ferrets. The ferret is a small animal model system for the study of the pathogenesis of MuV infection. There is a remarkable similarity in the lung physiology and morphology between ferrets and humans. Ferrets are highly susceptible to infection with respiratory viruses. Ferrets have been established as an animal model for several other respiratory pathogens. Most importantly, MuV has been isolated from infected ferrets and pathological changes were observed in the lungs of infected animals (Gordon et al., 1956, *J Immunol*; 76(4):328-33). Studies may include the infection of ferrets with a rMuV construct, the determination of immunogenicity of a rMuV construct in ferrets, and an examination of the efficacies of a such vaccine candidate in reducing virus load and pathological changes in lungs after challenge. As previously described (Gordon et al., 1956, *J Immunol*; 76(4):328-33), ferrets in a group of 5 will be infected with  $10^7$  pfu of wild type MuV or a rMuV construct in 1 ml volume. Animals will be monitored for fever daily in the first week and every other day in second week after infection. At 3, 4, 5, 7, 9 and 11 days after inoculation, nasal washes and blood samples will be collected and titers of virus in them will be determined using plaque assay. At 3, 4, 7, 11 and 14 days after inoculation, ferrets will be sacrificed and lungs and turbinates will be collected and titers of virus will be determined. Pathological changes in lungs and turbinates will be examined using H&E staining. The immunogenicity of MuV mutants in ferrets will be examined, as described in the previous examples. Humoral immunity and cellular immunity against MuV will be examined after inoculation with the vaccine candidates as well as the JL vaccine and wild type MuV. Two-dose IN inoculation and three-dose (two-dose IM inoculation with JL followed by a single IN inoculation of the vaccine candidates) may be used. Besides immunological tests, vaccinated animals will be challenged with wild type MuV. Reagents for immunological assays in ferrets, including reagents for assaying cellular immune responses, will be generated. Such reagents may include monoclonal antibodies against CD3, CD4, CD8, IFN- $\beta$ , IFN- $\gamma$ , IL-6, and IL-8.

### Example 7

#### Mumps Virus as a Vector for Respiratory Syncytial Virus Vaccine Development

Respiratory syncytial virus (RSV) is the most important cause of pediatric viral respiratory infection and is a major cause of morbidity and mortality among infants as well as immunocompromised subjects and the elderly (Collins, P.L., R.M. Chanock, and B.R. Murphy, Respiratory syncytial virus, in Fields Virology, D.M. Knipe and P.M. Howley, Editors. 2001, Lippincott, Williams and Wilkins: Philadelphia. p. 1443-1485.). In addition, severe RSV infection can result in wheezing and asthma later in life. Unlike infection by other respiratory viruses, RSV does not induce long-lasting protective immunity against subsequent infection. Thus, most individuals are infected multiple times throughout the course of their lives. Currently, there is no vaccine for RSV, nor are there effective curative treatments for severe RSV disease although aerosolized ribavirin and prophylactic immunoglobulin therapy are used in the clinical setting. However, the high cost of palivizumab prophylaxis raises the question of cost-effectiveness relative to health benefits due to the need for monthly injections during RSV season. Therefore, there is a pressing need for safe and effective vaccine for RSV.

As a negative non-segmented single-stranded RNA virus (NNSV), MuV is a good viral vector candidate for vaccine development because it does not have a DNA (or nuclear) phase in its life cycle, and thus the possible unintended consequences of genetic modifications of host cell DNA through recombination or insertion are avoided. In comparison to positive strand RNA viruses, the genome structure of MuV is stable. Thus, MuV is better suited as a vaccine vector than positive strand RNA viruses since the genomes of positive strand RNA viruses recombine and often delete the inserted foreign genes quickly.

Generation and analysis of MuV-F containing RSV F. The F gene of RSV (A2 strain) will be inserted between F and SH of MuV genome using the same strategy as for the generation of MuV-GFP (MuV-F). Briefly, the F gene will be combined with the gene end (GE), intergenic region (I) and gene start (GS) (which are important for viral mRNA synthesis), using a four-primer PCR approach (He et al., 1995, *Gene*; 164:75-79). The sequences will be inserted between GS of NP and the coding sequence of the NP gene. Although the "rule of six", which viral RNA genome requires to be multiple of six to be effective, is not absolute for MuV, the

length of the genome with F will be maintained to be a multiple of six. Expression levels of F in MuV-F-infected cells will be examined using immunoprecipitation in comparison to RSV-infected cells. Growth rates of the virus at high and low MOI will be compared to MuV.

Generation and examination of MuV-F containing 3'-proximal F as a vaccine candidate. Negative strand RNA viruses, such as MuV, initiate transcription from the 3' end leader sequence, and transcription levels of the viral genes are affected by their distances to the leader sequence. For example, the NP gene of MuV, which is the closest to the leader sequence, is the most abundantly transcribed, whereas the L gene that is the located most distant from the leader sequence is least transcribed (Fig. 19). It is expected that the efficacy of the vaccine candidate will be enhanced by increasing the expression level of the F protein (as has been shown for recombinant RSV (Krempl et al., 2002, *J Virol*; 76(23):11931-42)). To increase the expression level of the F gene, the F gene will be inserted immediately downstream of the leader sequence and upstream of the NP gene (F-MuV) (Fig. 19).

The RSV G protein can be similarly expressed as the RSV F protein using mumps virus as a vector.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

## Sequence Listing Free Text

|                  |                                                                                                                                     |
|------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| SEQ ID NO:1      | Mumps virus genome including V/P gene encoding a V protein and SH gene encoding a small hydrophobic protein                         |
| SEQ ID NO:2      | Mumps virus V protein                                                                                                               |
| SEQ ID NO:3      | Mumps virus small hydrophobic protein                                                                                               |
| SEQ ID NO:4      | SH protein sequence for Mumps virus strain Glouc1/UK96                                                                              |
| SEQ ID NO:5      | SH protein sequence for Mumps virus strain UK01-22                                                                                  |
| SEQ ID NO:6      | SH protein sequence for Mumps virus strain MuV-IA                                                                                   |
| SEQ ID NO:7      | nucleic acid sequence upstream of SH gene                                                                                           |
| SEQ ID NO:8      | recombinant nucleic acid sequence resulting from deletion of SH gene                                                                |
| SEQ ID NO:9      | PCR product sequenced to confirm deletion of SH protein                                                                             |
| SEQ ID NO:10     | MuV-IA SH N-terminal peptide sequence used to generate antibody                                                                     |
| SEQ ID NO:11     | MuV-IA SH C-terminal peptide sequence used to generate antibody                                                                     |
| SEQ ID NOs:12-13 | MuV-IA V protein peptide sequence used to generate antibody                                                                         |
| SEQ ID NO:14     | nucleic acid editing sequence within the P/V gene                                                                                   |
| SEQ ID NO:15     | recombinant nucleic acid sequence eliminating V protein expression                                                                  |
| SEQ ID NO:16     | recombinant nucleic acid sequence resulting from modification of P/V gene                                                           |
| SEQ ID NO:17     | PCR product sequenced to confirm deletion of V protein                                                                              |
| SEQ ID NO:18     | nucleic acid sequence from the end of the NP gene to the start of the P/V gene a Mumps virus having a V protein deletion            |
| SEQ ID NO:19-25  | rescued nucleic acid sequence from the end of the NP gene to the start of the P/V gene in a Mumps virus having a V protein deletion |
| SEQ ID NO:26-81  | synthetic oligonucleotide primer                                                                                                    |

What is claimed is:

1. An isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus, wherein the isolated nucleotide sequence encodes a mumps virus unable to express a small hydrophobic (SH) protein product and/or express a V protein product.
2. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1 comprising a deletion of the open reading frame (ORF) encoding the SH protein, a mutation converting a start codon into a stop codon, or a mutation in the region between the ORF encoding the F polypeptide and the ORF encoding the SH polypeptide that disrupts transcription of the SH gene.
3. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 2 comprising a deletion of the ORF encoding the SH protein.
4. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 3 comprising a deletion of 156 nucleotides of the ORF encoding the SH protein.
5. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1 comprising one or more mutations to the V/I/P gene abrogating expression of the V protein.
6. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 5, wherein the one or more mutations to the V/I/P gene abrogating expression of the V protein comprise the nucleotide sequence GAGGAGGG at the editing site in the P/V gene.

7. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1 comprising:  
a deletion of the open reading frame (ORF) encoding the SH protein, a mutation converting a start codon into a stop codon, or a mutation in the region between the ORF encoding the F polypeptide and the ORF encoding the SH polypeptide that disrupts transcription of the SH gene; and  
one or more mutations to the V/I/P gene abrogating expression of the V protein.
8. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 7 wherein the one or more mutations to the V/I/P gene abrogating expression of the V protein comprise the nucleotide sequence GAGGAGGG at the editing site in the P/V gene.
9. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of any one of claims 1 to 8 comprising a further mutation and/or deletion.
10. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 9 comprising a mutation or deletion effecting phosphorylation of the P protein.
11. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 9 comprising a mutation at T147 and/or S307 of the P protein.
12. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of any one of claims 1 to 11 further comprising a mutation of the L gene.

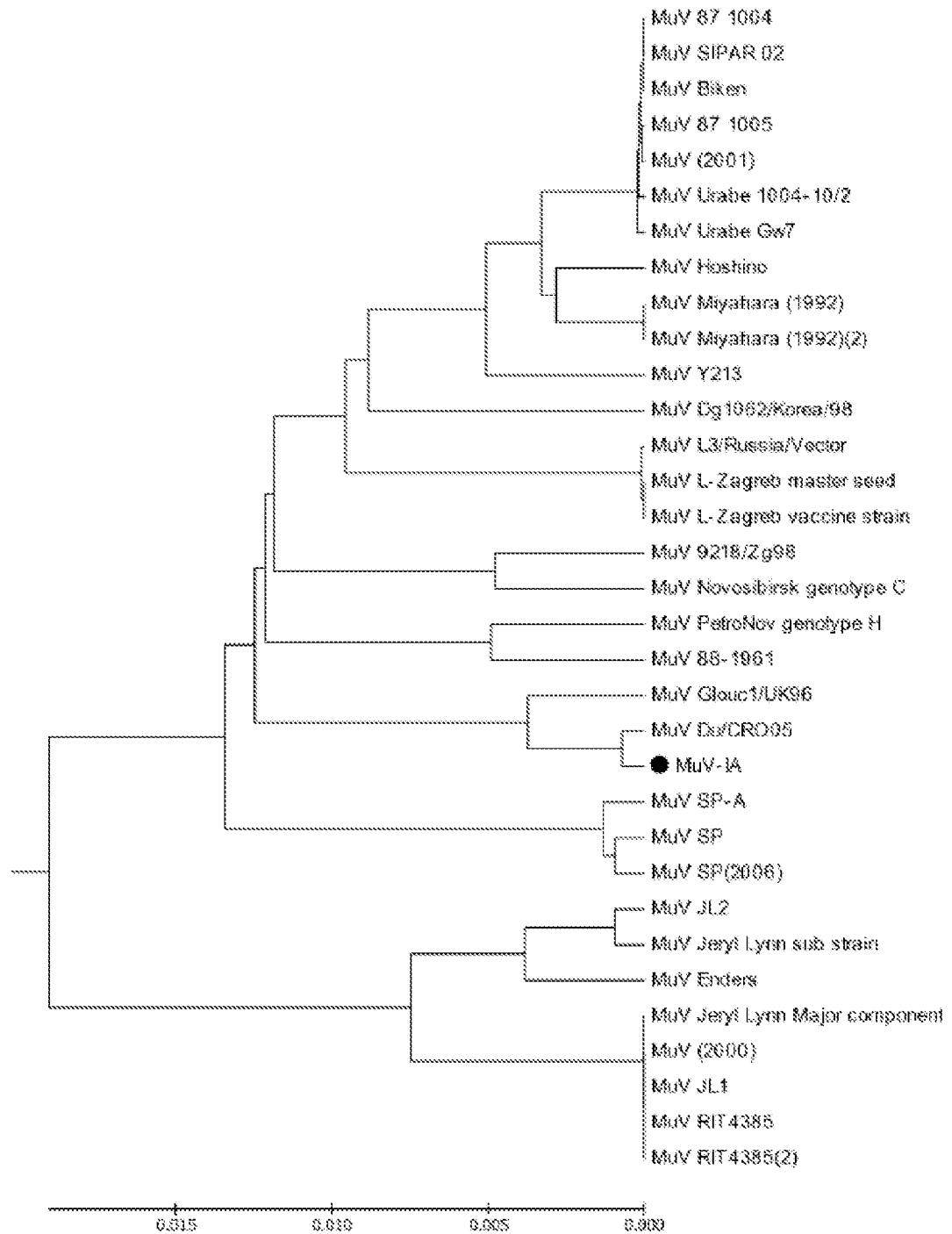
13. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of any one of claims 1 to 12 further comprising expression of an I protein product.
14. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1, wherein the mumps genome further encodes a heterologous polypeptide.
15. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1 wherein the mumps virus belongs to genotype G.
16. The isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of claim 1 wherein the mumps virus is MuV/IowaUS/2006 (MuV-IA).
17. An isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of the MUV/IowaUS/2006 (MuV-IA) strain of the mumps virus.
18. The isolated nucleotide sequence of claim 17, wherein the nucleotide sequence comprises SEQ ID NO:1.
19. A recombinant mumps virus (rMuV) or plasmid encoding a measles virus genome (pMuV) comprising an isolated nucleotide acid sequence of any one of claims 1 to 18.
20. A plasmid comprising the isolated nucleotide sequence of any one of claims 1 to 18, or a fragment thereof.
21. A plasmid encoding a measles virus genome (pMuV) comprising an isolated nucleotide acid sequence of any one of claims 1 to 18.

22. A viral expression vector comprising an isolated nucleotide sequence comprising the cDNA sequence encoding the full length RNA genome of a mumps virus of any one of claims 1 to 18, or a fragment thereof.
23. An infectious viral particle comprising an isolated nucleotide sequence or plasmid of any one of claims 1 to 22.
24. A composition comprising an isolated nucleotide sequence, plasmid, pMuV, rMuV, or infectious viral particle of any one of claims 1 to 23.
25. The composition of claim 24 further comprising a rubella and/or measles antigenic determinant.
26. A composition of claim 24 or 25 formulated for intranasal, oral, intradermal, or intramuscular administration.
27. A method of inducing an immune response to mumps virus in a subject, the method comprising administering an effective amount of an isolated nucleotide sequence plasmid, pMuV, rMuV, viral particle, or composition of any one of claims 1 to 26 to the subject.
28. A method of vaccinating a subject against mumps, the method comprising administering an effective amount of an isolated nucleotide sequence plasmid, pMuV, rMuV, viral particle, or composition of any one of claims 1 to 26 to the subject.
29. The method of claim 27 or 28 comprising intranasal, oral, intradermal, or intramuscular administration.
30. A method of expressing a heterologous RNA or polypeptide, the method comprising expressing an isolated nucleotide sequence of claim 14.

Figure 1A

| Strain       | SH Protein Sequence                                            |
|--------------|----------------------------------------------------------------|
| Glouc1/UK96* | MPAIQPPLY LTFLLLLILLYLIITLYVWIIILTIT YKTA VRHAALYQRSFFHWSFDHSL |
| UK01-22      | MPAIQPPLY LTFLLLLILLYLIITSYVWIIILTIT YKTA VRHAALYQRSFFHWSFDHSL |
| MuV-1A       | MPAIQPPLY LTFLLLLILLYLIITLYVWIIILTV YKTA VRHAALYQRSFFHWSFDHSL  |

2 / 27

*Figure 1B*

3 / 27

*Figure 1C*

| <b>Gene</b> | <b><i>Nucleotide identities</i></b> | <b><i>Amino Acid identities</i></b> |     |
|-------------|-------------------------------------|-------------------------------------|-----|
| <b>NP</b>   | 94%                                 |                                     | 98% |
| <b>P/V</b>  | 93%                                 | <b>P Protein</b>                    | 94% |
|             |                                     | <b>V Protein</b>                    | 93% |
| <b>M</b>    | 94%                                 |                                     | 99% |
| <b>F</b>    | 94%                                 |                                     | 95% |
| <b>SH*</b>  | 87%                                 |                                     | 85% |
| <b>HN</b>   | 92%                                 |                                     | 94% |
| <b>L</b>    | 94%                                 |                                     | 98% |

4 / 27

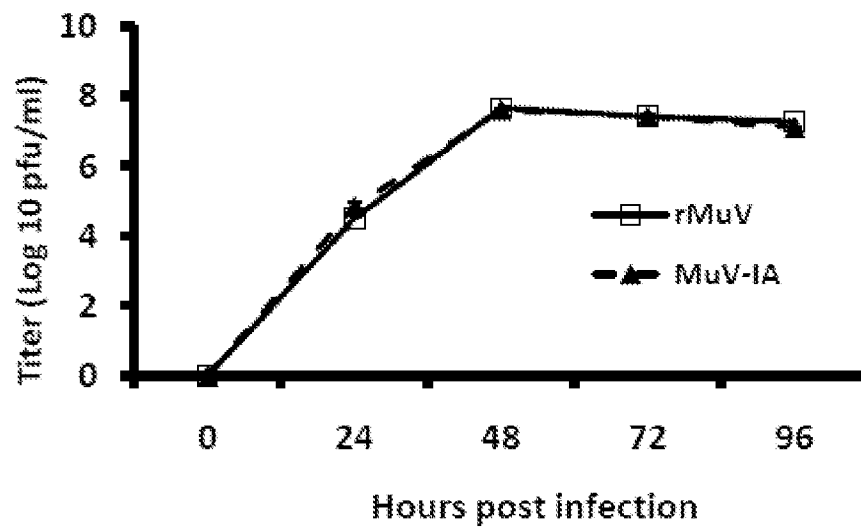
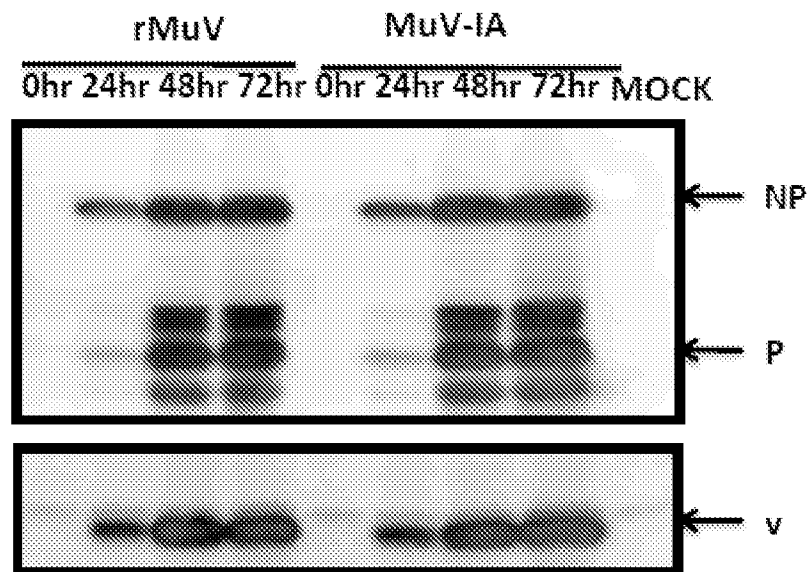
*Figure 2A**Figure 2B*

Figure 2C

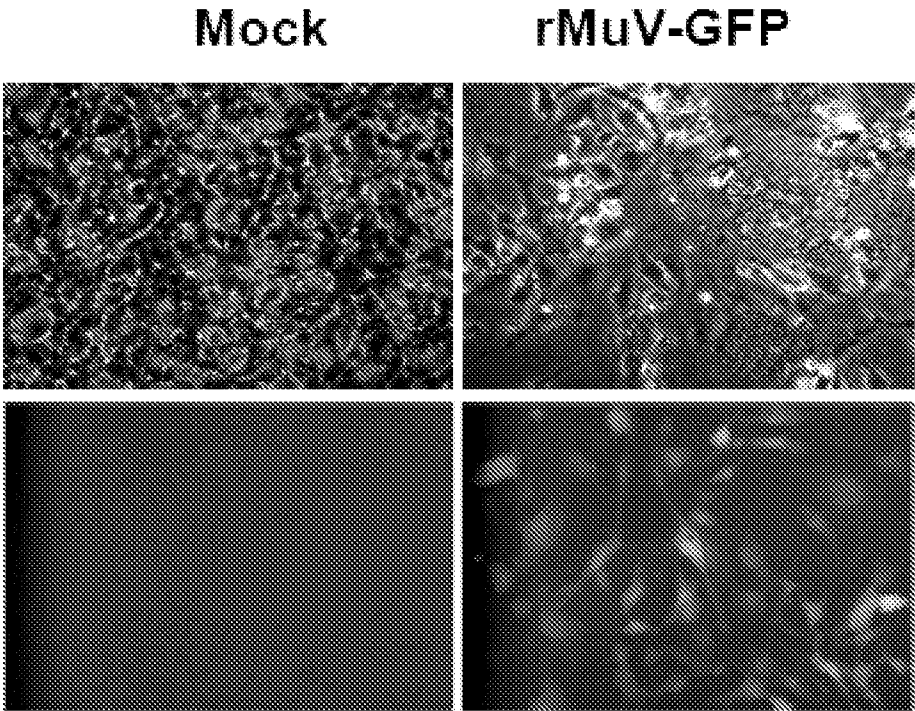
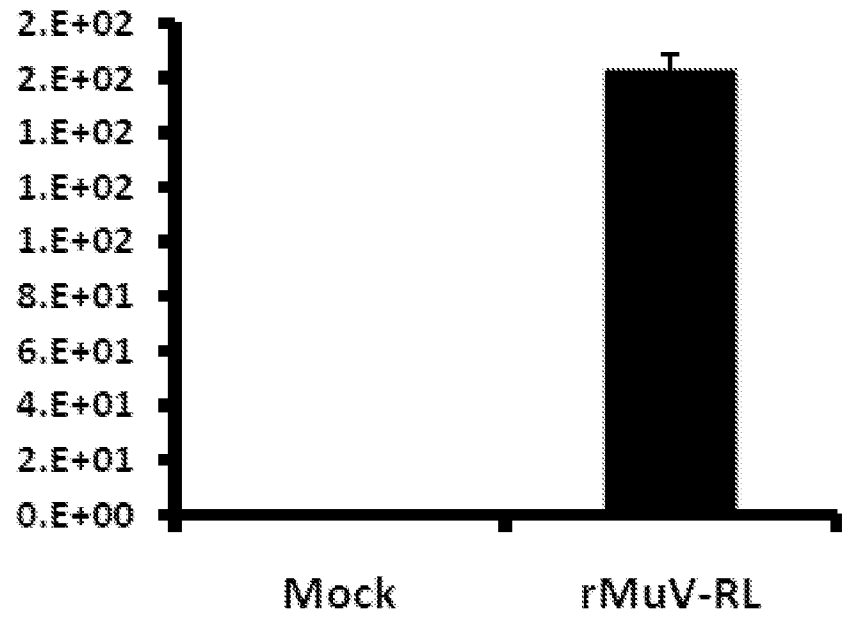


Figure 2D



6 / 27

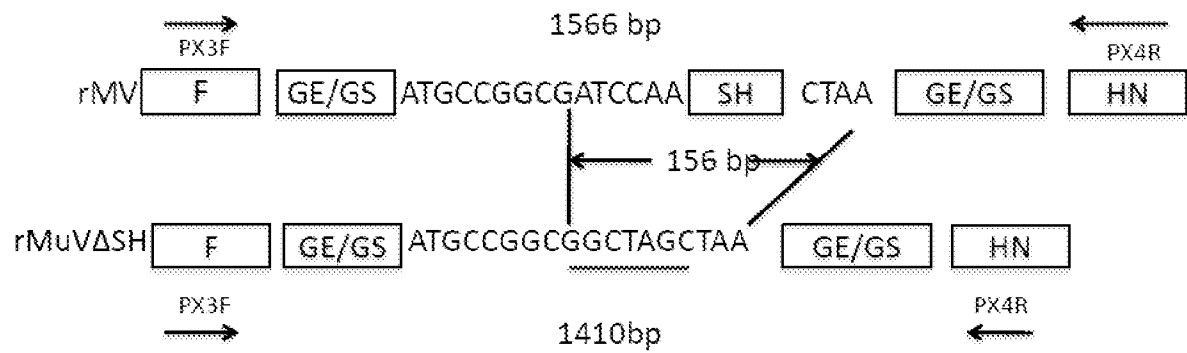
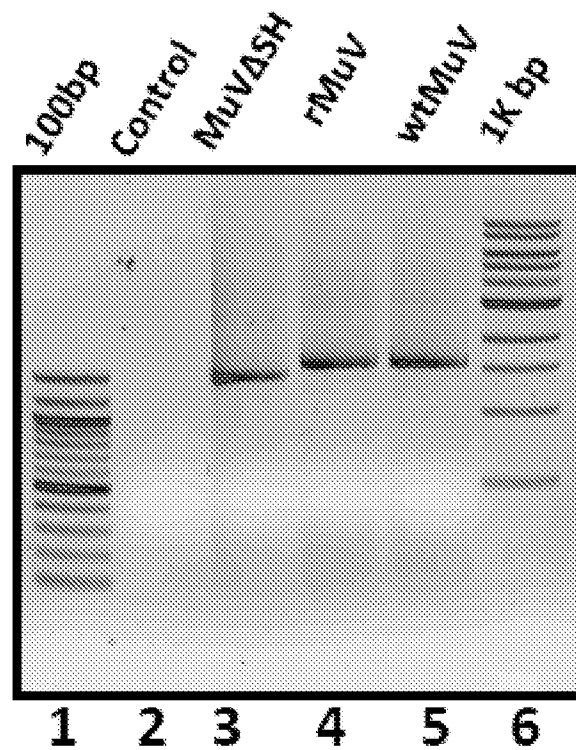
*Figure 3A**Figure 3B*

Figure 3C

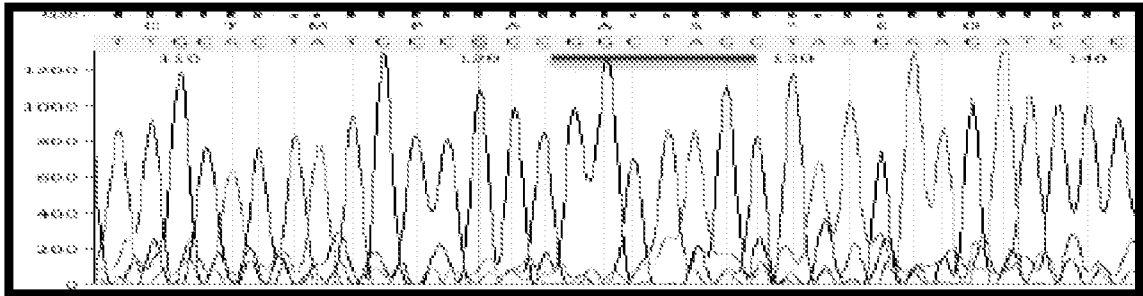
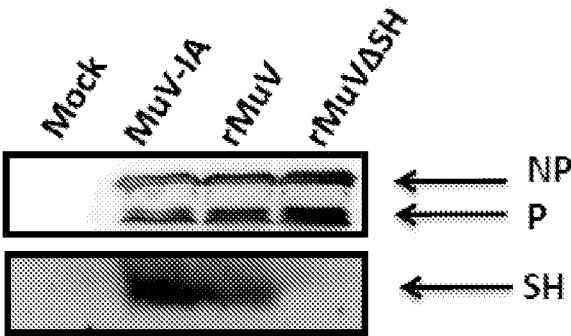
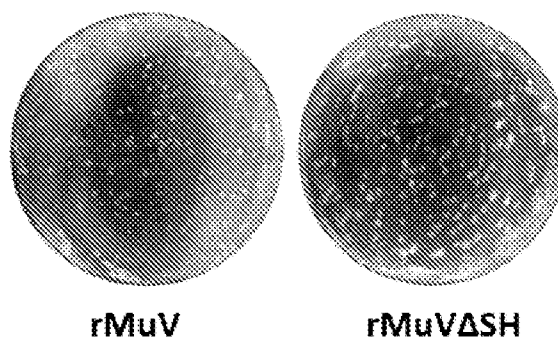
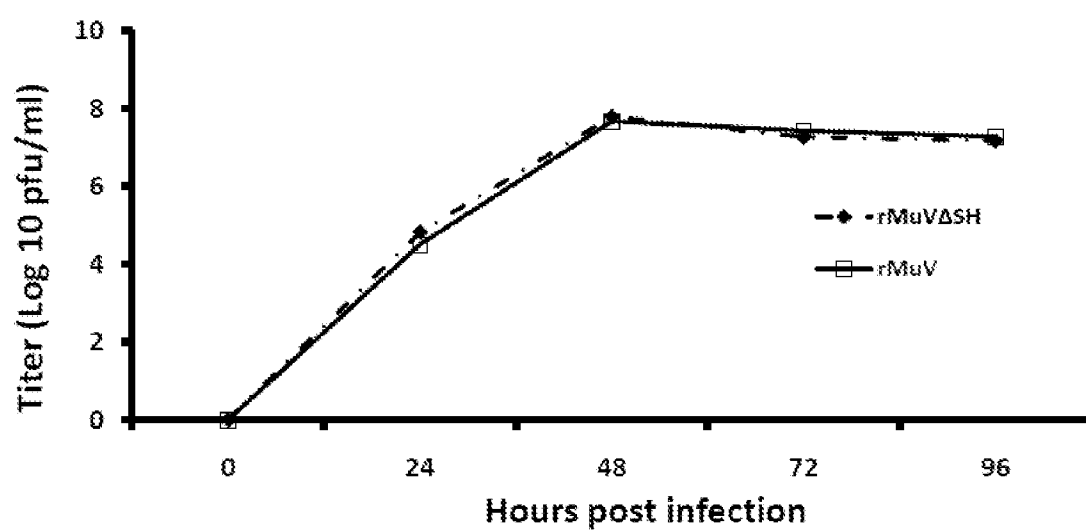


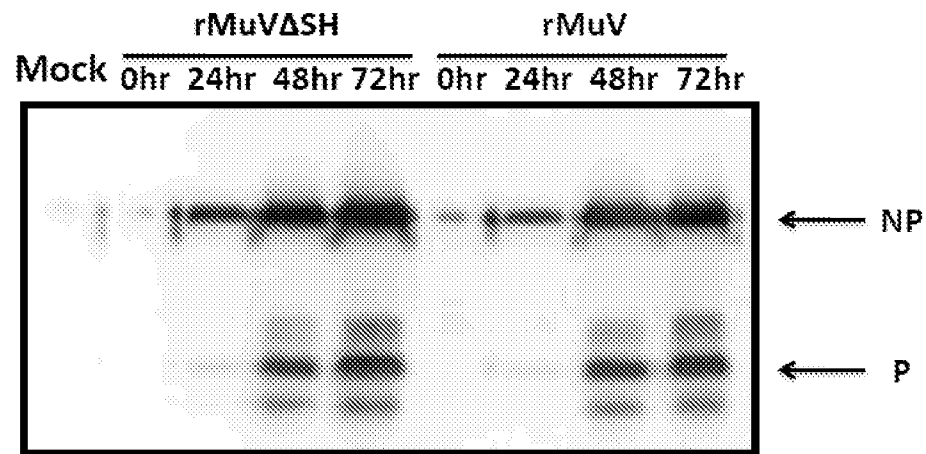
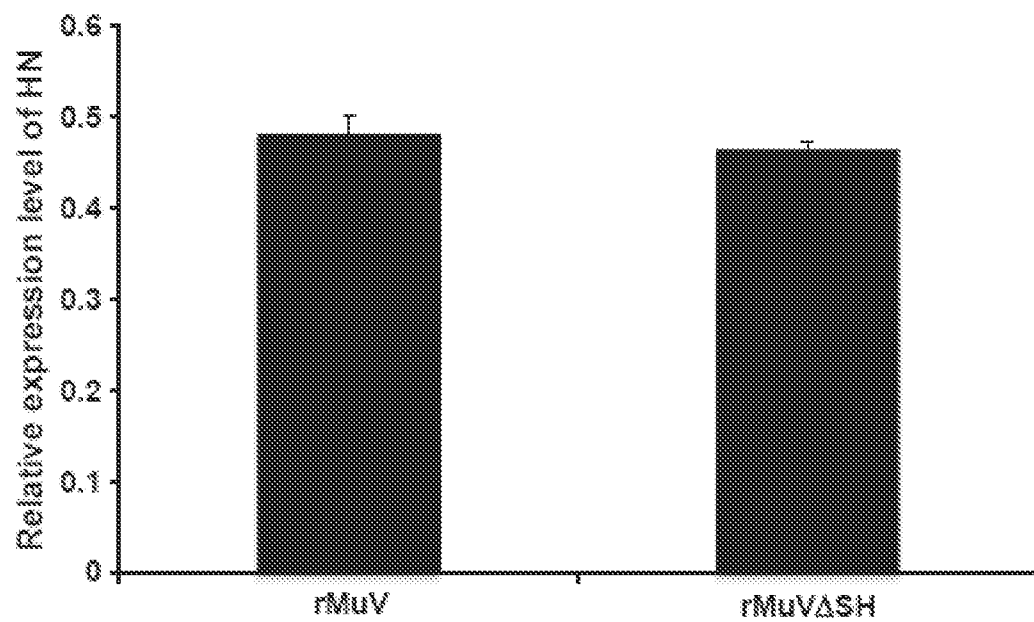
Figure 3D



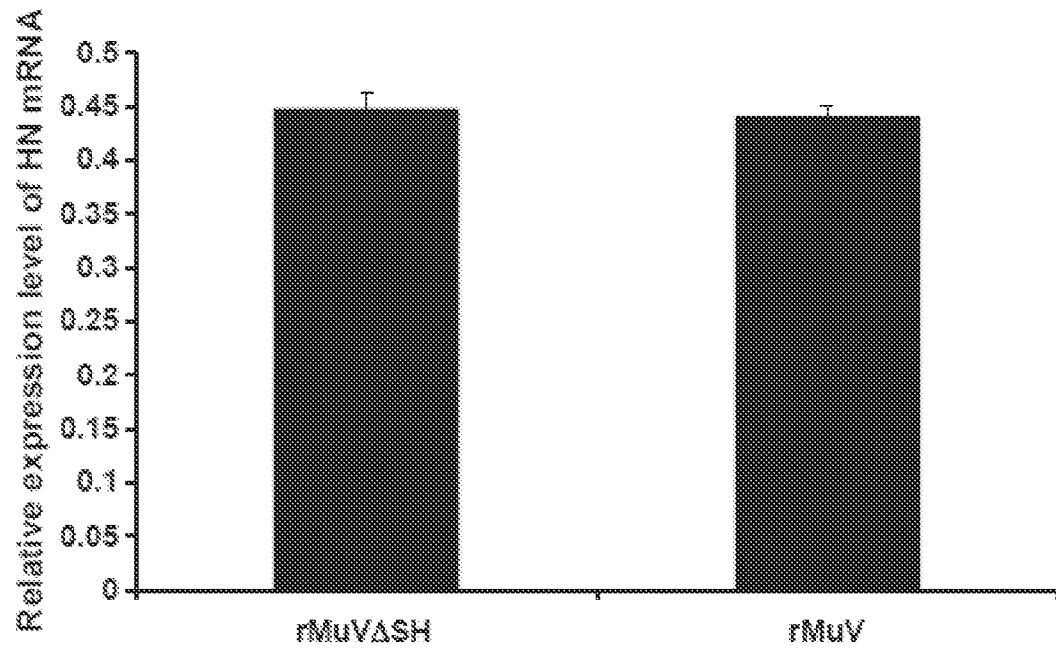
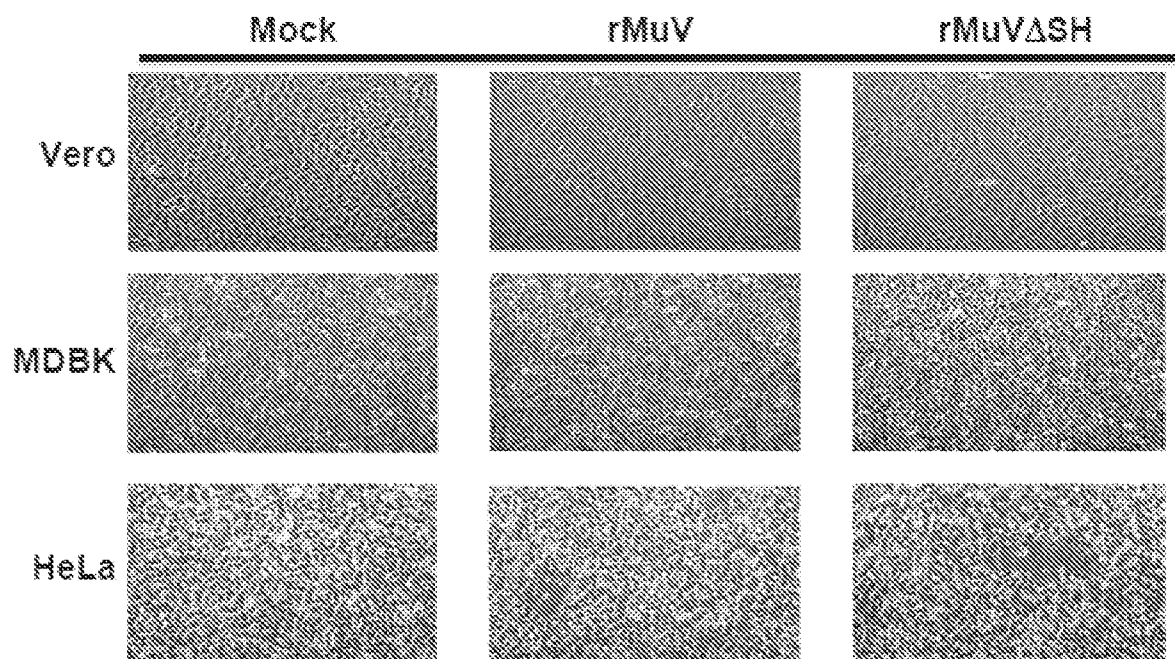
8 / 27

*Figure 4A*

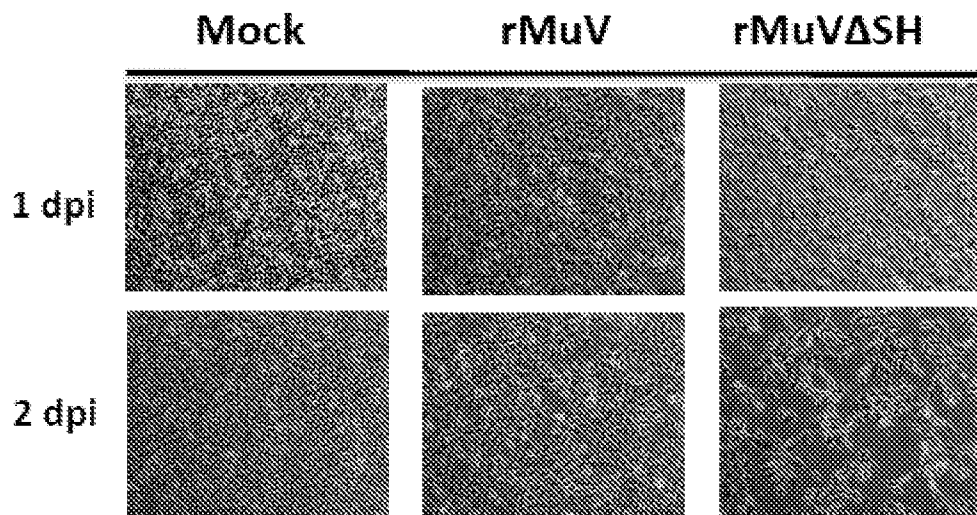
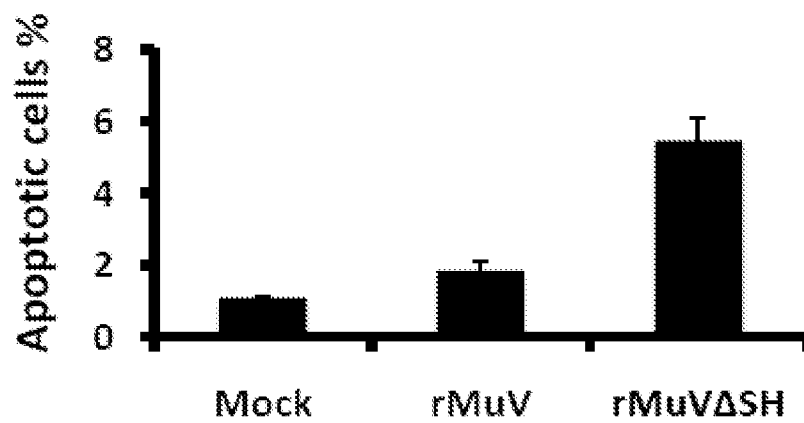
9 / 27

*Figure 4B**Figure 4C*

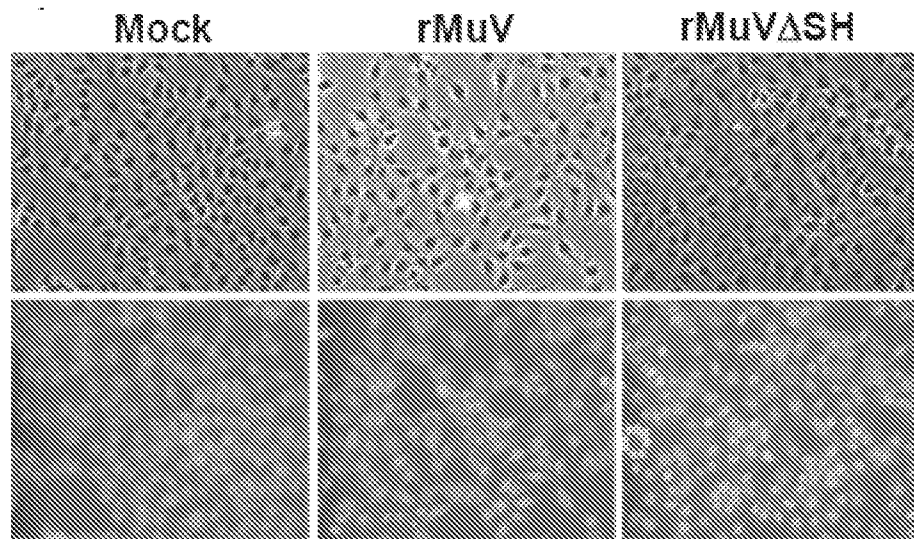
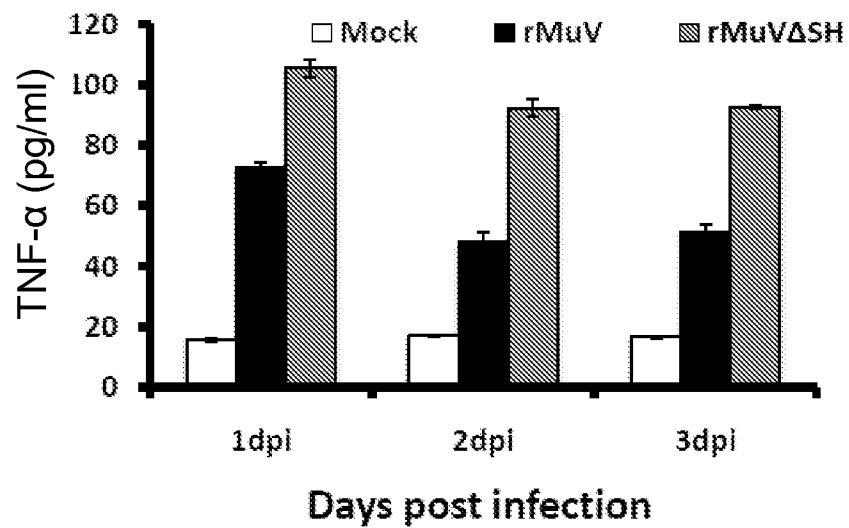
10 / 27

*Figure 4D**Figure 5A*

11 / 27

*Figure 5B**Figure 5C*

12 / 27

*Figure 6A**Figure 6B*

13 / 27

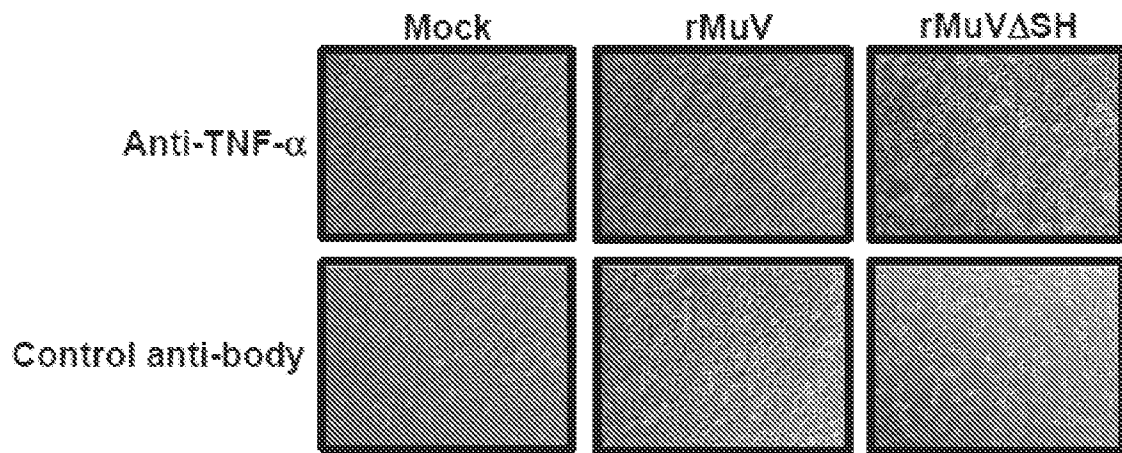
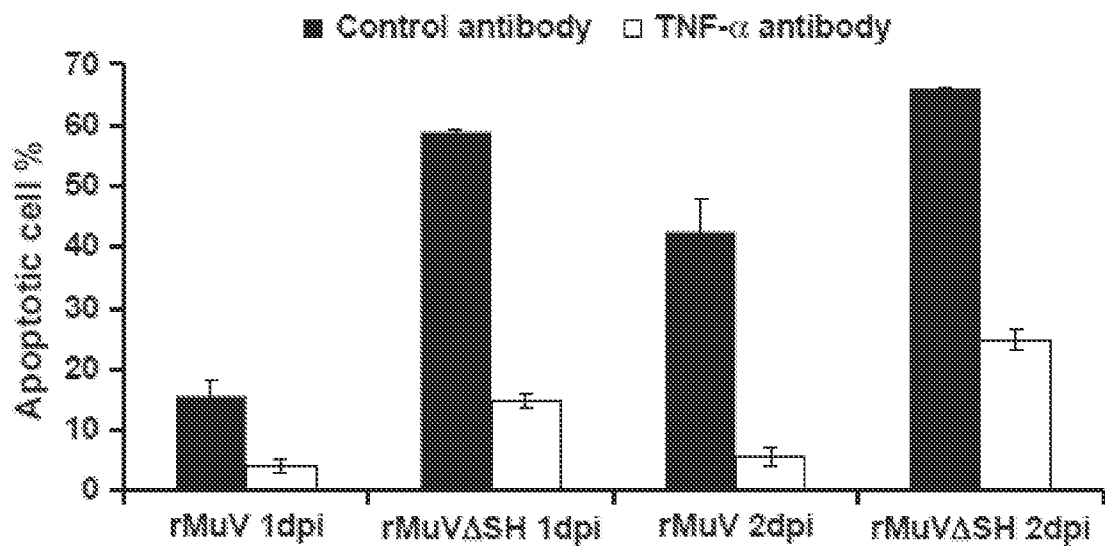
*Figure 6C**Figure 6D*

Figure 7A

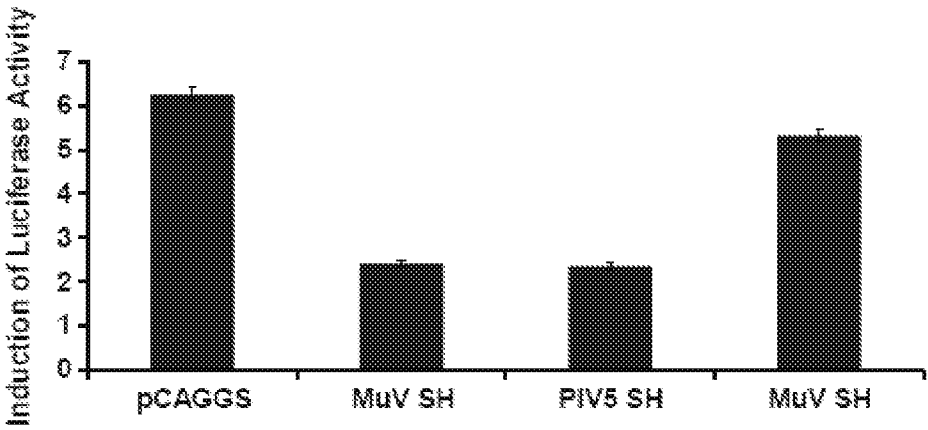
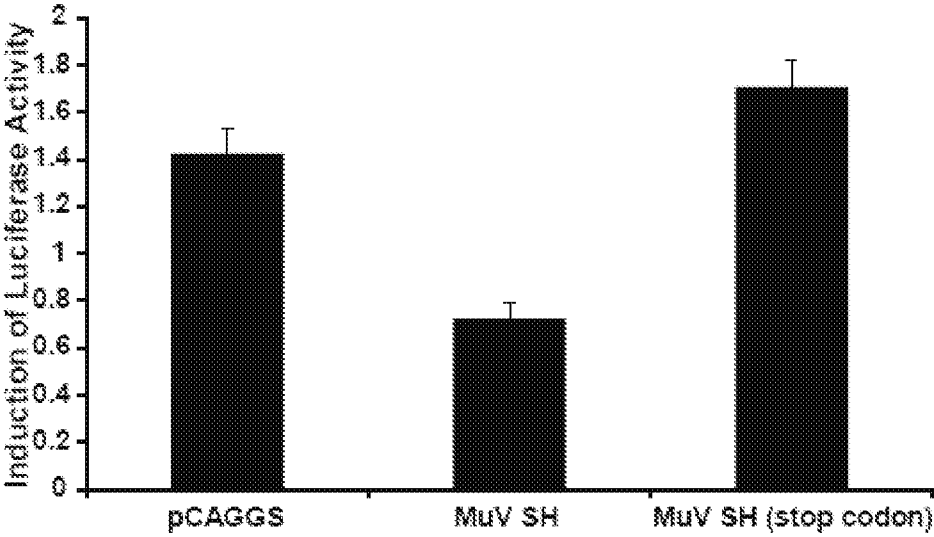
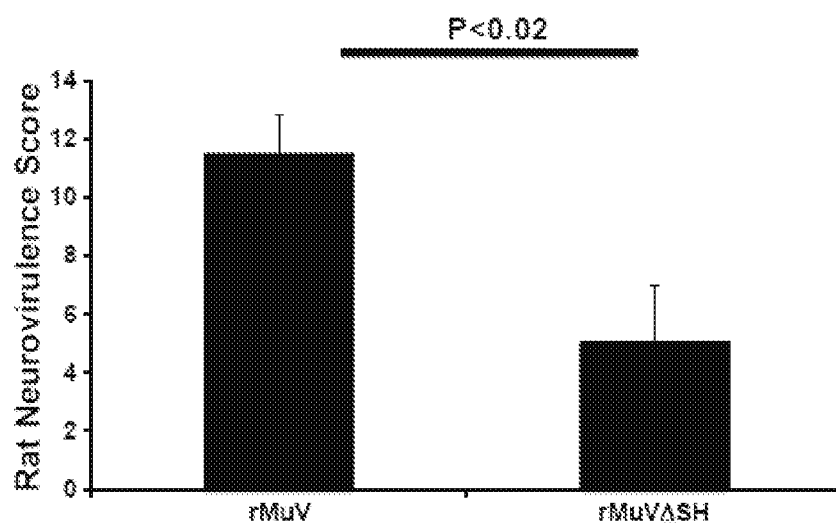


Figure 7B



15 / 27

*Figure 8*

16 / 27

Figure 9A

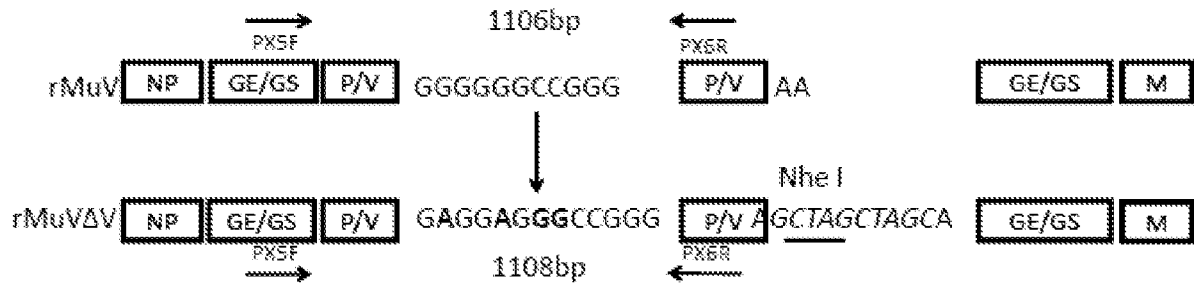


Figure 9B

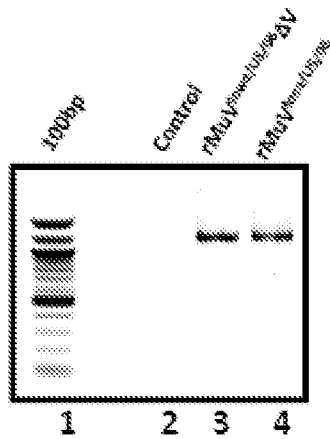


Figure 9C

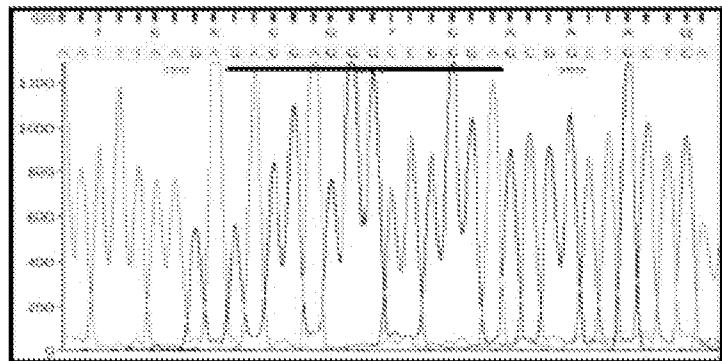
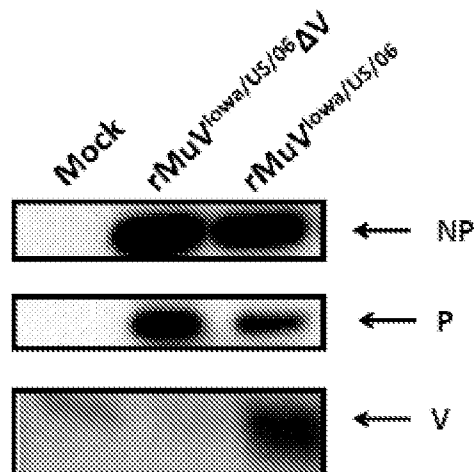


Figure 9D



| virus strain      | Number of rescue | Negative control | Positive control | Nucleotide change | Amino acid residue change | Region         |
|-------------------|------------------|------------------|------------------|-------------------|---------------------------|----------------|
| <i>px2-sp-2</i>   | 1                | -                | +                | 13704 C>A         | A1756D                    | L gene         |
| <i>px2-sp-48</i>  | 4                | -                | +                | 1899 A >T         | No                        | NP gene end    |
| <i>px2-sp-51</i>  | 5                | -                | +                | 1913 C>T          | No                        | P/V gene start |
| <i>Px2-sp-61</i>  | 6                | -                | +                | 1898 A>G          | No                        | NP gene end    |
| <i>PX2-SP-81</i>  | 8                | -                | +                | 1899 A>G          | No                        | NP gene end    |
| <i>PX2-SP-91</i>  | 9                | -                | +                | 1896T>G           | No                        | NP gene end    |
| <i>PX2-SP-101</i> | 10               | -                | +                | 1899A>G           | No                        | NP gene end    |
| <i>PX2-SP-106</i> | 11               | -                | +                | 1897T>G           | No                        | NP gene end    |

*Figure 10B*

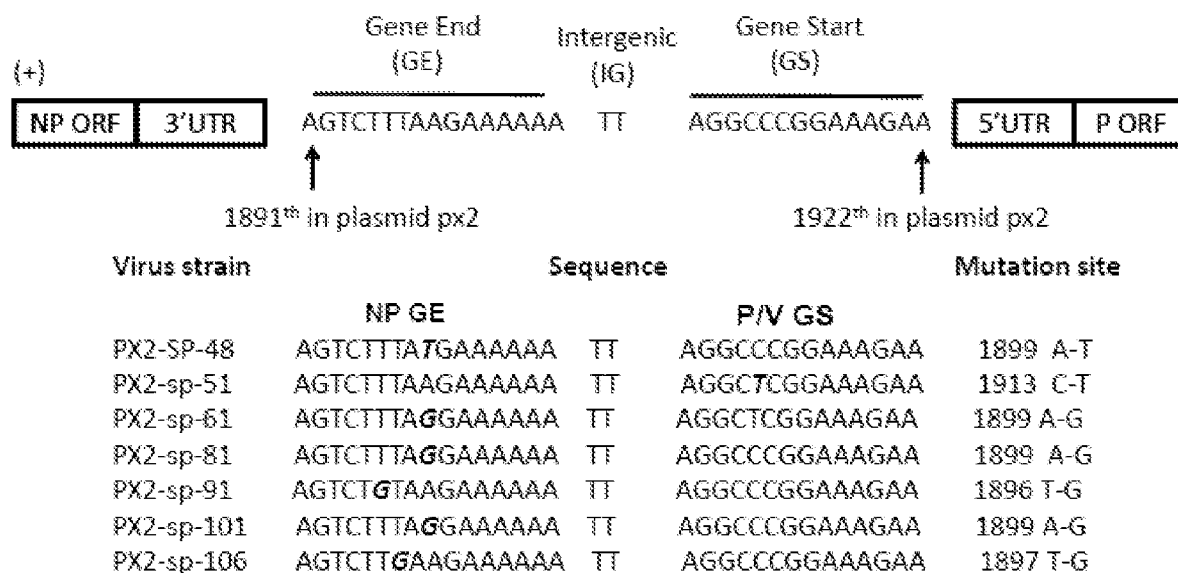


Figure 11A

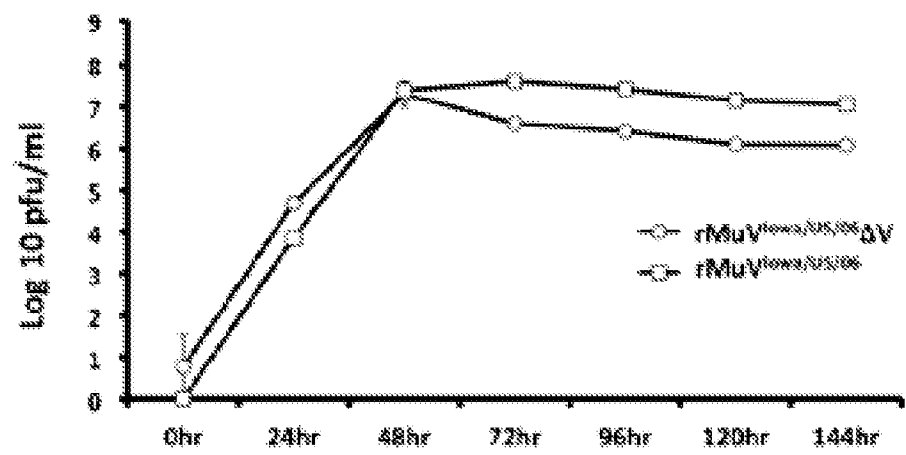


Figure 11B

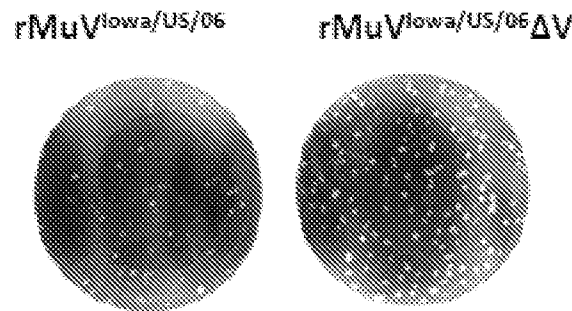
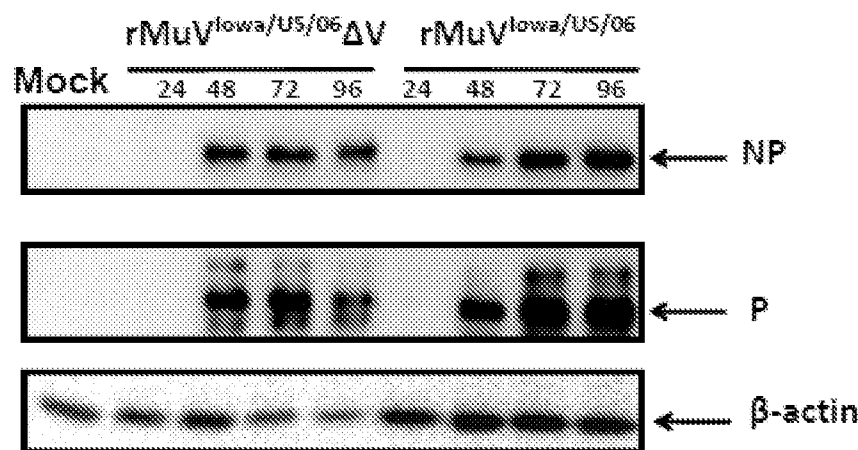
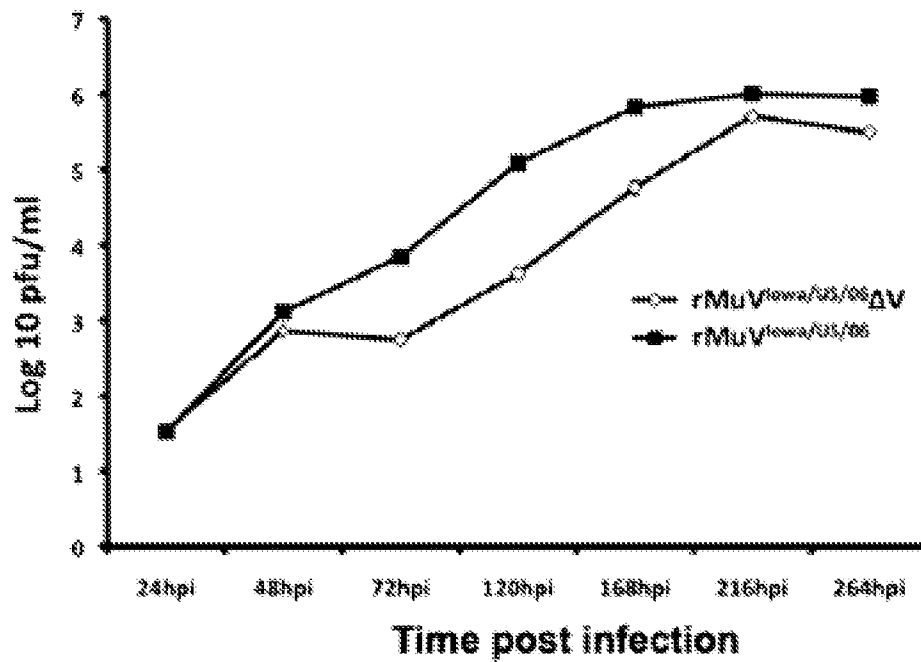
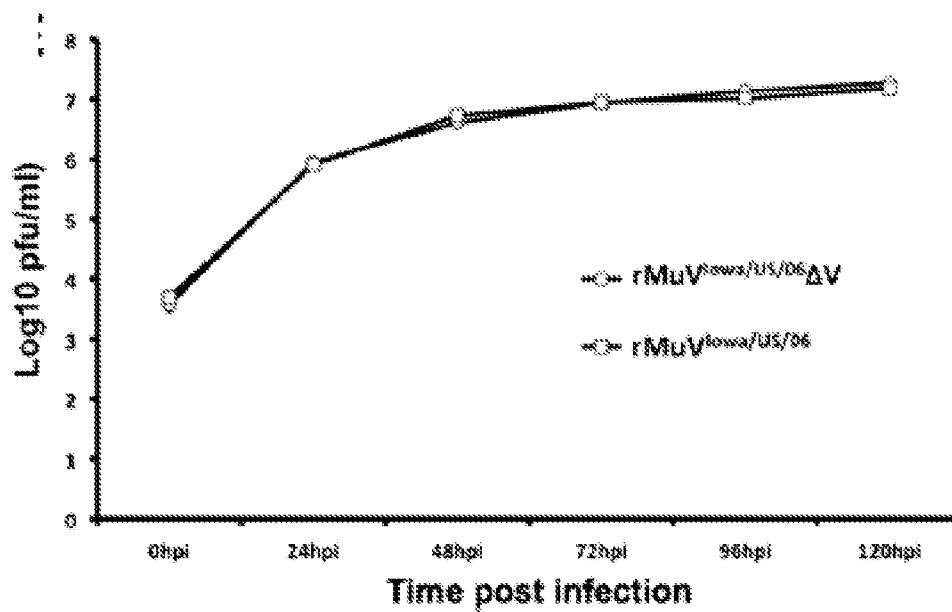


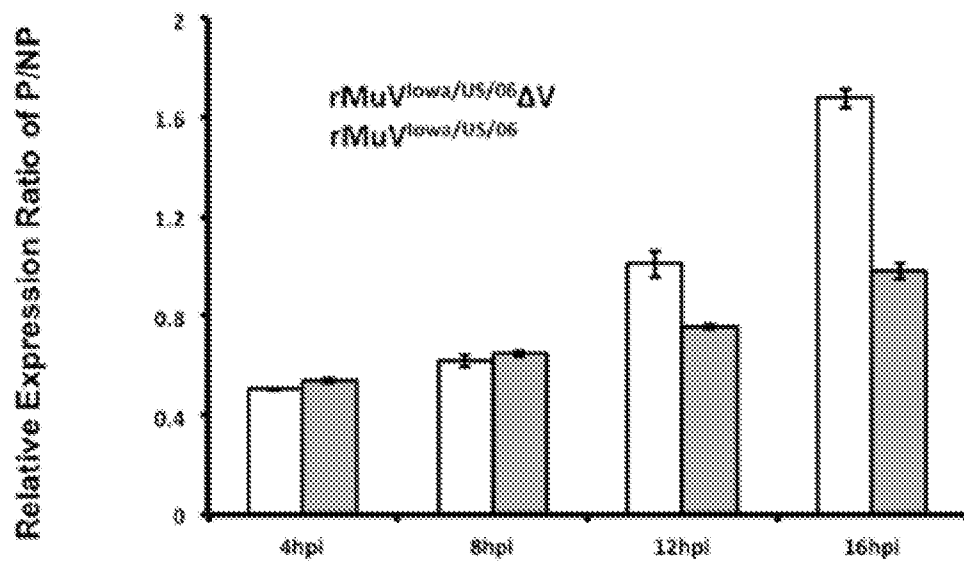
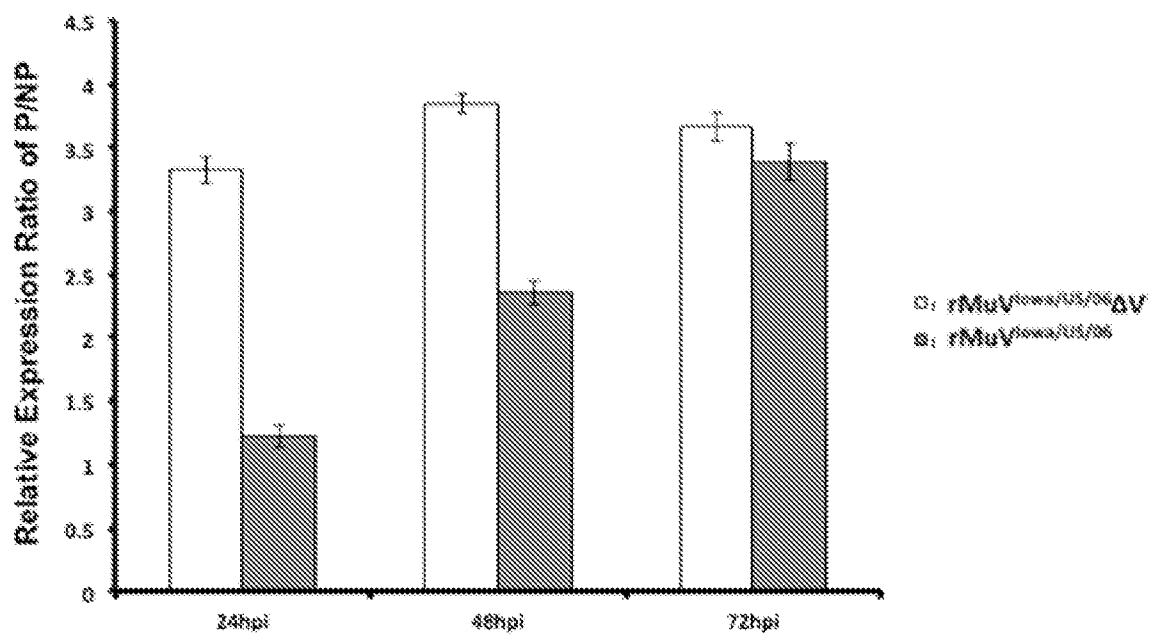
Figure 11C



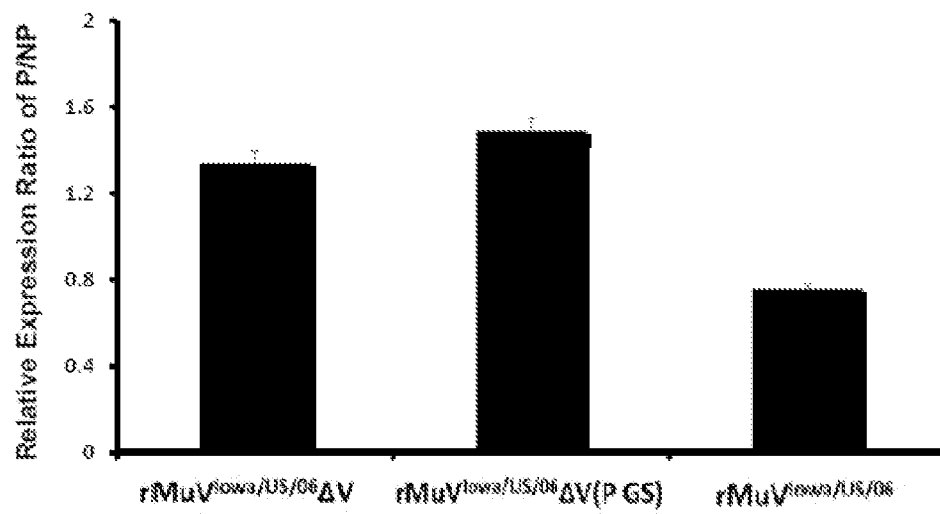
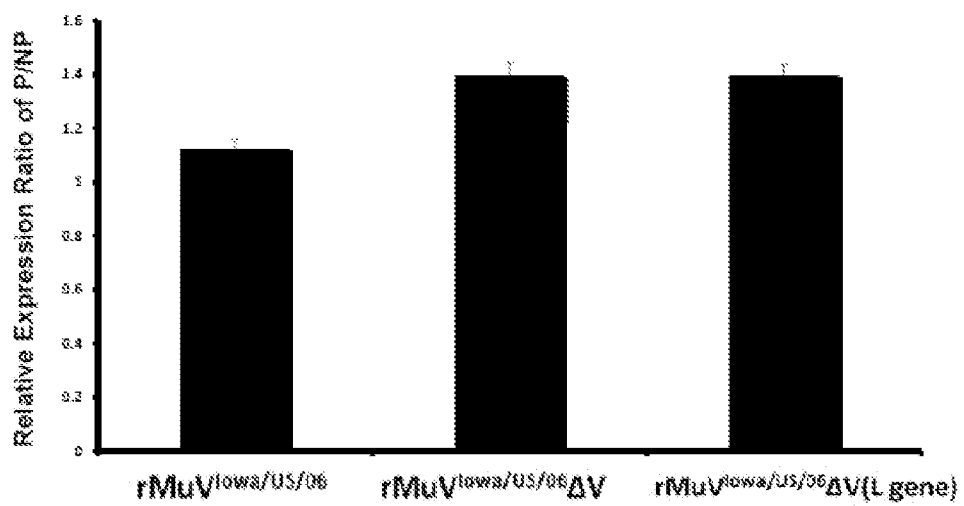
19 / 27

*Figure 11D**Figure 11E*

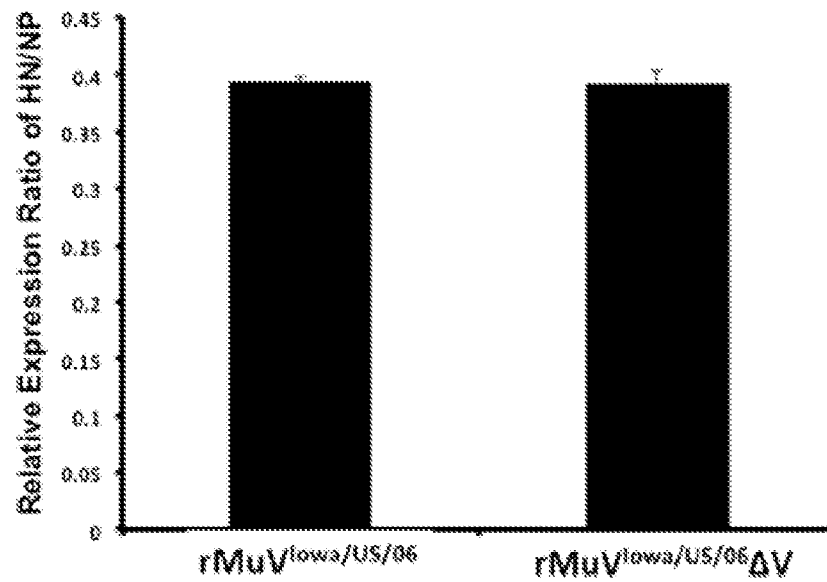
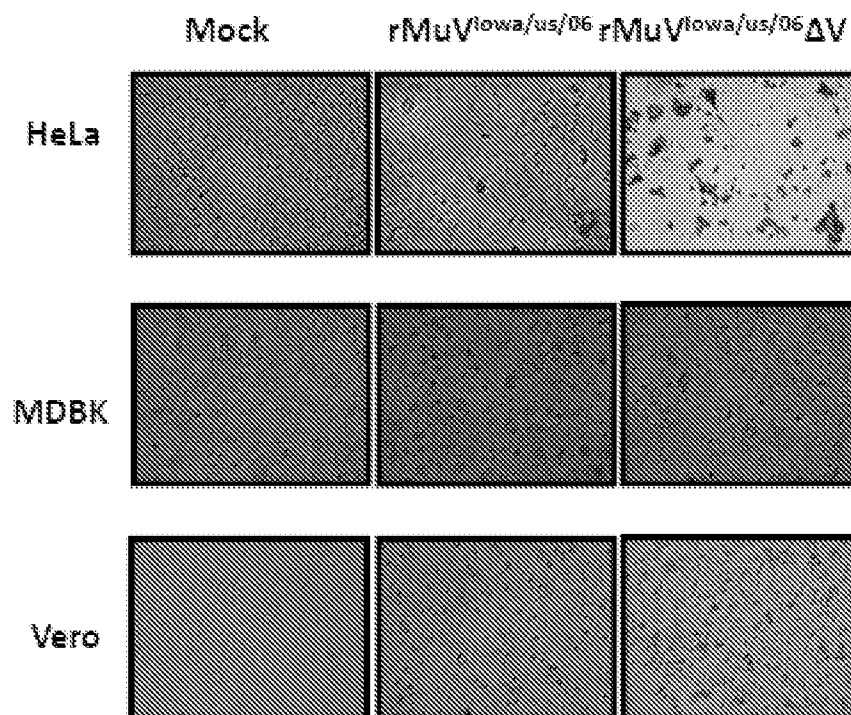
20 / 27

*Figure 12A**Figure 12B*

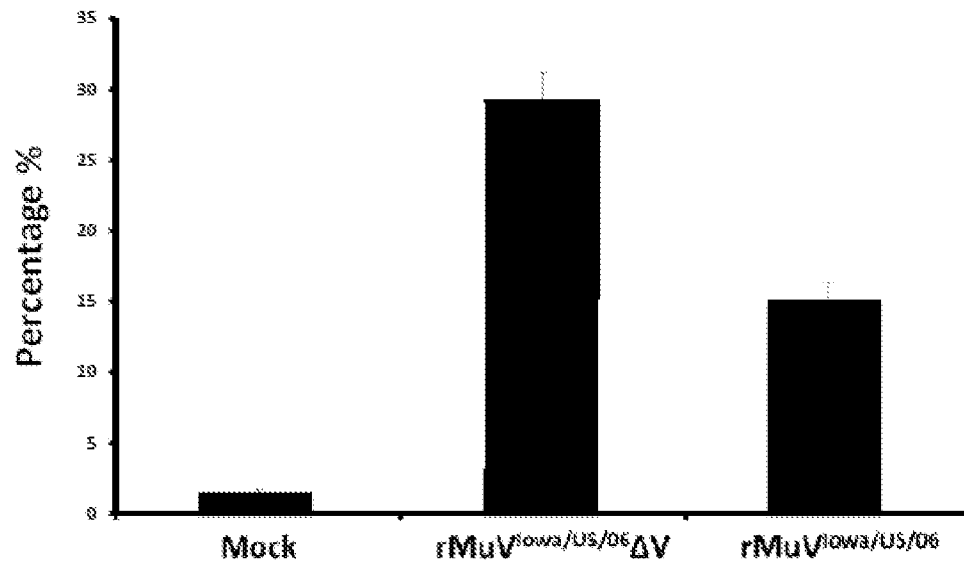
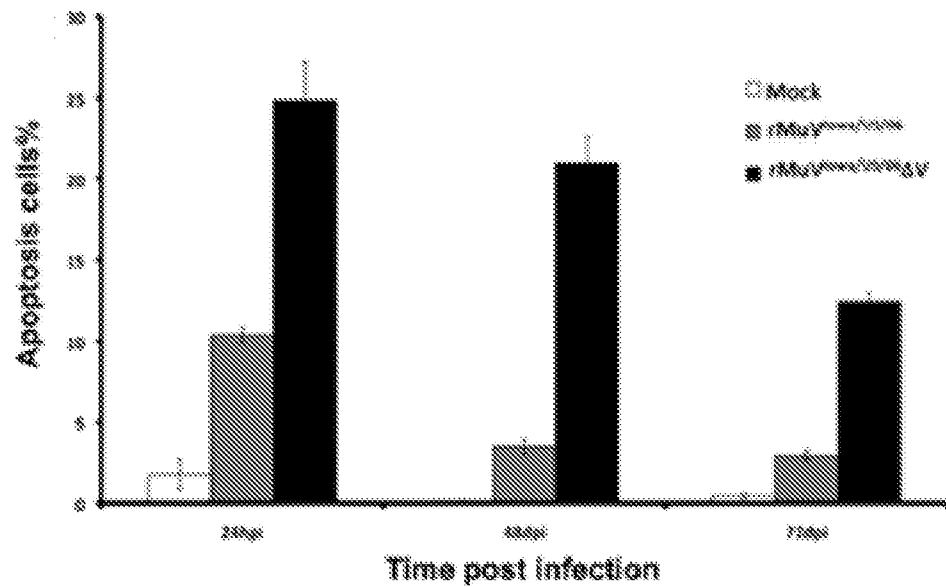
21 / 27

*Figure 12C**Figure 12D*

22 / 27

*Figure 13**Figure 14A*

23 / 27

*Figure 14B**Figure 14C*

24 / 27

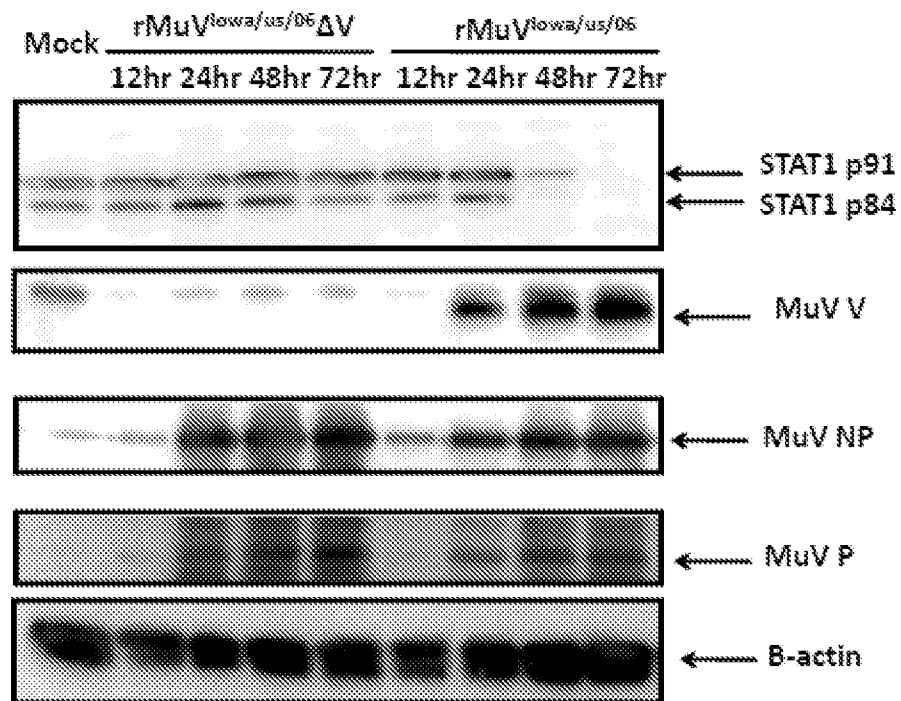
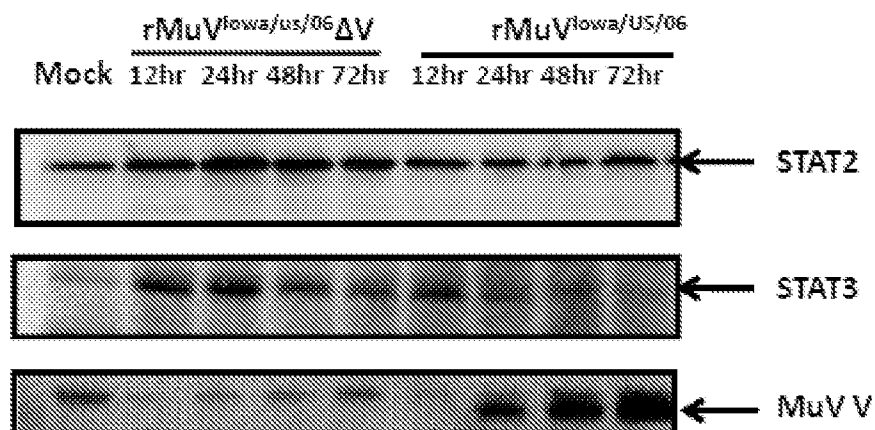
*Figure 15A**Figure 15B*

Figure 16A

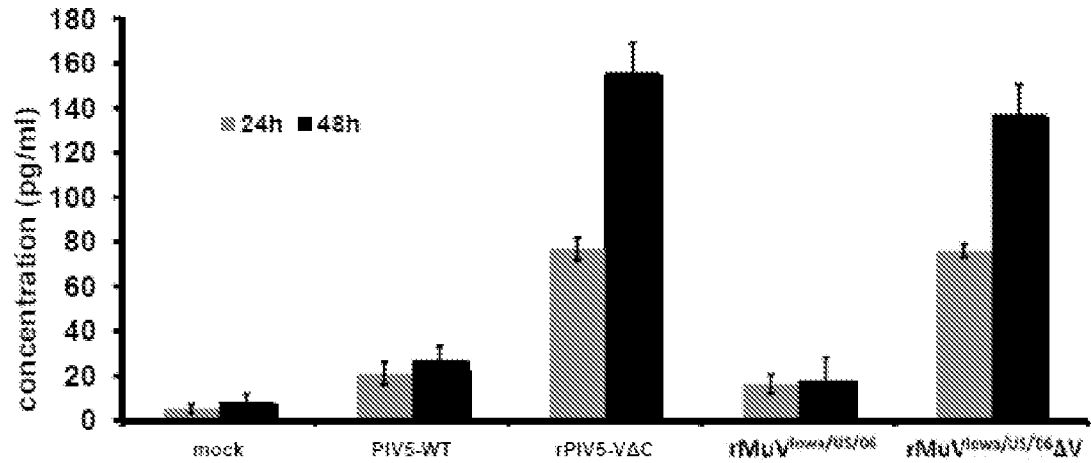
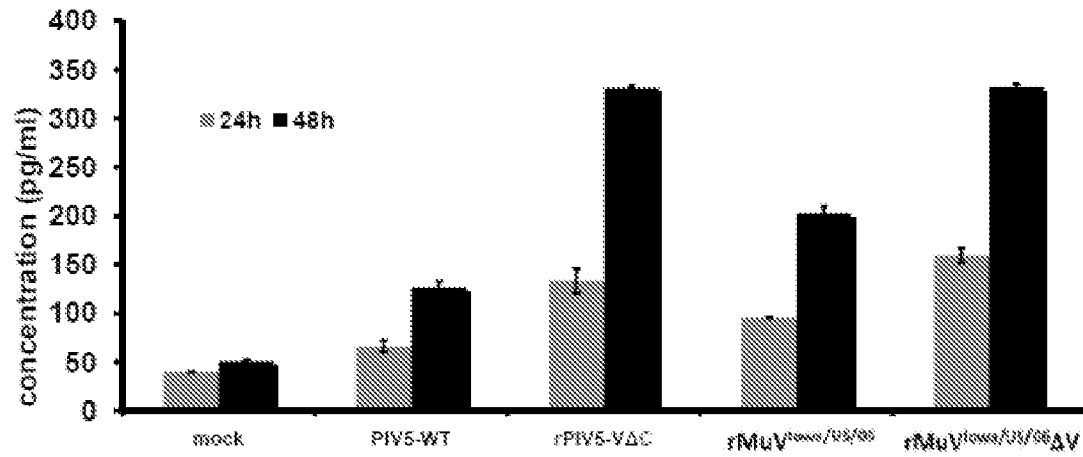


Figure 16B



26 / 27

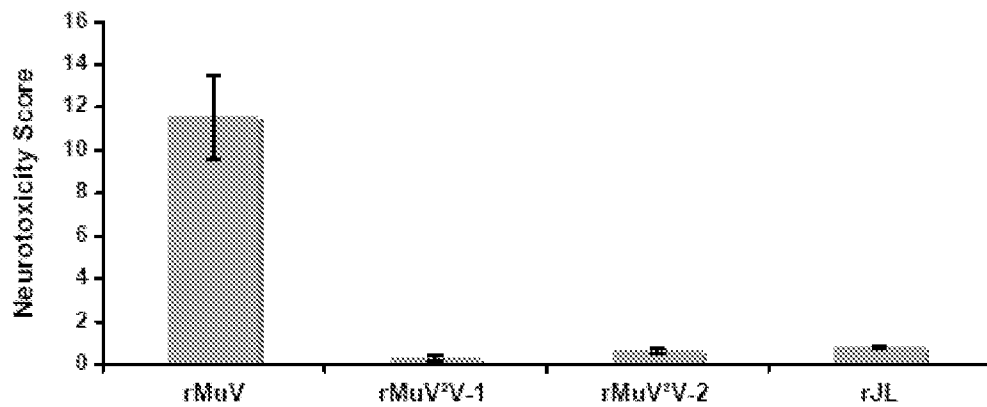
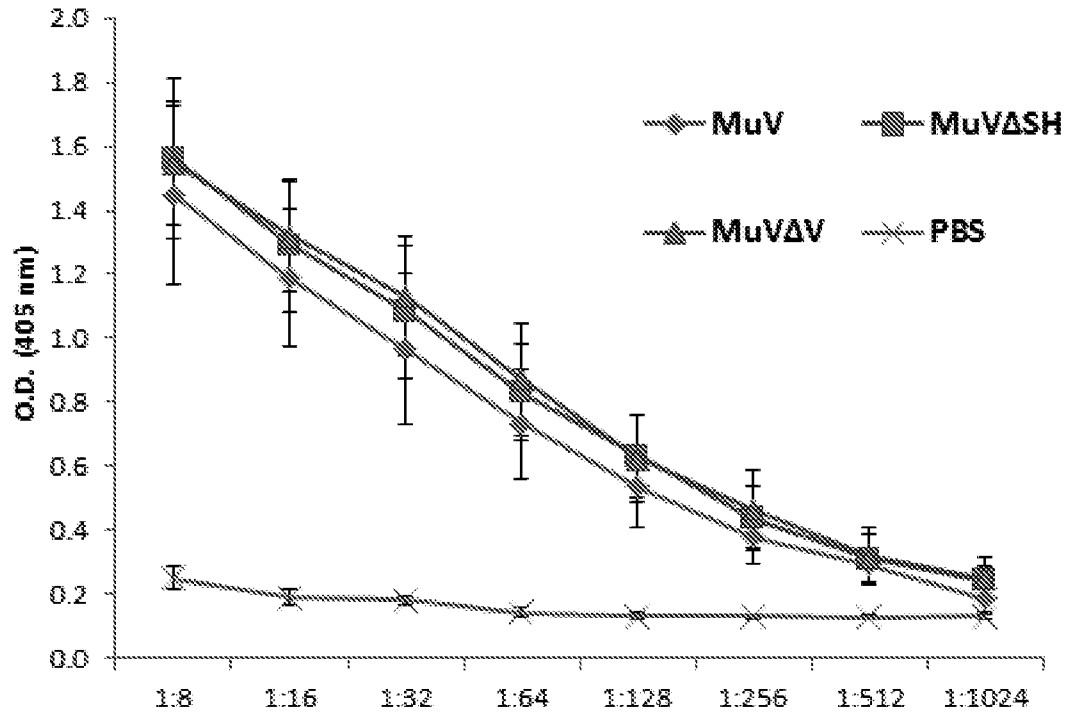
*Figure 17**Figure 18A*

Figure 18B

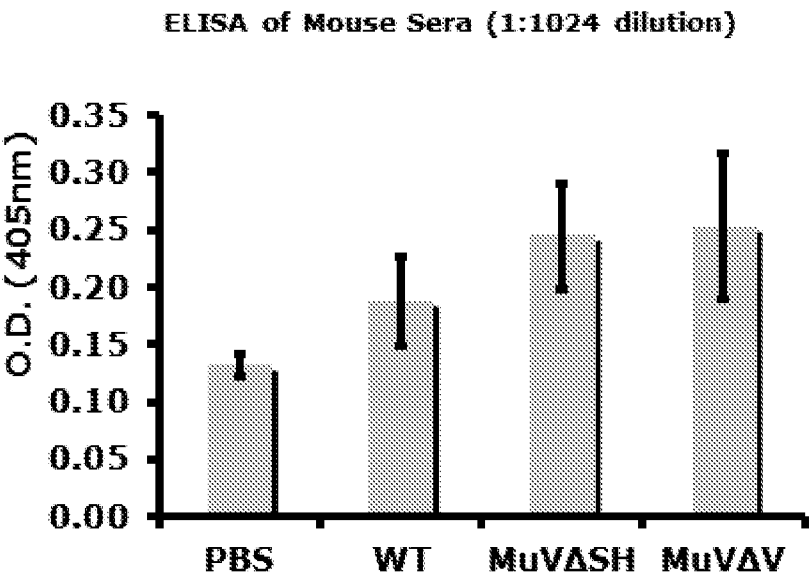


Figure 19

