(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
12 May 2011 (12.05.2011)

(10) International Publication Number
WO 2011/056591 A1

(51) International Patent Classification:
C07K 14/11 (2006.01) ***C12N 7/02*** (2006.01)
C12N 7/08 (2006.01)

(21) International Application Number:
PCT/US2010/054128

(22) International Filing Date:
26 October 2010 (26.10.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/254,795 26 October 2009 (26.10.2009) US

(71) Applicant *(for all designated States except US)*: **WARF - WISCONSIN ALUMNI RESEARCH FOUNDATION** [US/US]; P.O. Box 7365, Madison, Wisconsin 53707-7365 (US).

(72) Inventor; and
(71) Applicant : **KAWAOKA, Yoshihiro** [US/US]; 8722 Airport Road, Middleton, Wisconsin 53562 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **HORIMOTO, Taisuke** [JP/JP]; 2-7-2 Hakusan, Bankyotan, Tokyo 112-0001 (JP). **MURAKAMI, Shin** [JP/JP]; 2-6-9 Hiratsuka #203, Shinagawa-ku, Tokyo 142-0051 (JP).

(74) Agents: **PERDOK SHONKA, Monique M.** et al.; Schwegman, Lundberg & Woessner, P.A., P.O. Box 2938, Minneapolis, Minnesota 55402 (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, RO, RS, RU, 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, 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:
— *with international search report (Art. 21(3))*

WO 2011/056591 A1

(54) Title: HIGH TITER RECOMBINANT INFLUENZA VIRUSES WITH ENHANCED REPLICATION IN VERO CELLS

(57) Abstract: The invention provides a composition useful to prepare high titer influenza viruses, e.g., in the absence of helper virus, which includes internal genes from an influenza virus vaccine strain or isolate, e.g., one that is safe in humans, for instance, one that does not result in significant disease, and genes from vaccine seed virus isolates which include a HA gene segment with a HA2 sequence encoding a HA2 that confers enhanced growth in cells in culture, such as Vero cells.

HIGH TITER RECOMBINANT INFLUENZA VIRUSES WITH ENHANCED REPLICATION IN VERO CELLS

Cross-Reference to Related Applications

5 This application claims the benefit of the filing date of U.S. application Serial No. 61/254795, filed on October 26, 2009, the disclosure of which is incorporated by reference herein.

Statement of Government Rights

This invention was made with United States government support awarded by the National Institutes of 10 Health (grant NIH AI069274). The United States government has certain rights in this invention.

Background

Influenza is a major respiratory disease in some mammals including horses and is responsible for substantial morbidity and economic losses each year. In addition, influenza virus infections can cause severe systemic disease in some avian species, leading to death. The segmented nature of the influenza virus 15 genome allows for reassortment of segments during virus replication in cells infected with two or more influenza viruses. The reassortment of segments, combined with genetic mutation and drift, can give rise to a myriad of divergent strains of influenza virus over time. The new strains exhibit antigenic variation in their hemagglutinin (HA) and/or neuraminidase (NA) proteins, and in particular the gene coding for the HA protein has a high rate of variability. The predominant current practice for the prevention of flu is vaccination. Most 20 commonly, whole virus vaccines are used. As the influenza HA protein is the major target antigen for the protective immune responses of a host to the virus and is highly variable, the isolation of influenza virus and the identification and characterization of the HA antigen in viruses associated with recent outbreaks is important for vaccine production. Based on prevalence and prediction, a vaccine is designed to stimulate a protective immune response against the predominant and expected influenza virus strains (Park et al., 2004).

25 There are three general types of influenza viruses, Type A, Type B and Type C, which are defined by the absence of serological crossreactivity between their internal proteins. Influenza Type A viruses are further classified into subtypes based on antigenic and genetic differences of their glycoproteins, the HA and NA proteins. All the known HA and NA subtypes (H1 to H15 and N1 to N9) have been isolated from aquatic birds, which are though to act as a natural reservoir for influenza. The H1N1 "swine flu" virus has recently 30 been declared to be a pandemic. While this virus may be less virulent than some circulating influenza viruses in certain populations, it is ubiquitous and has become the subject of significant public health efforts. Unfortunately, this virus appears to be less amenable than other viruses to high titer productions which may lead to challenges in vacine manufacture.

35

Summary of the Invention

The invention provides isolated recombinant, e.g., reassortant, influenza viruses with selected amino acid residues at specified positions in HA2, NA and/or PB2. In one embodiment, the recombinant reassortant influenza virus has an amino acid residue at position 117 in HA2 (position is based on H1 HA2 numbering; for example, position 117 in H1 HA2 corresponds to position 116 in H3 HA2) that results in enhanced growth in

Vero cells relative to a corresponding virus with, for instance, an asparagine at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the recombinant influenza virus has an amino acid residue at position 117 in HA2 that results in fusion of the virus with membranes in endosomes, e.g., late endosomes, at a higher pH relative to a corresponding virus with, for instance, an asparagine at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the invention provides an isolated recombinant reassortant influenza virus having six “internal” gene segments from a vaccine influenza virus, a NA gene segment selected from a first influenza virus isolate, and a HA gene segment selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. For example, the NA and HA gene segments may be from a strain for a seasonal flu vaccine or from a pandemic strain, and in one embodiment, the HA2 sequence in the HA gene segment is mutated to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2.

As described herein, an influenza virus isolate useful as a vaccine virus (A/Puerto Rico/8/34 (PR8) to carry heterologous gene segments for NA and/or HA was serially passaged in Vero cells to obtain virus with enhanced replication in those cells. In one embodiment, viruses obtained after serial passage which have enhanced replication, have titers that are at least 2, 3, 4 or 5 logs higher than viruses that were not serially passaged. In one embodiment, viruses obtained after serial passage had substitutions in three gene segments, NA, HA and PB2, relative to the parent virus. It was determined that the substitution in HA2 was primarily associated with the enhanced growth phenotype. PR8 virus with HA2 N117D had at least a three log enhancement in titer in Vero cells. The HA2 N117D mutant fused cells at a higher pH than did wild-type HA. Three different recombinant (6:2 mutant reassortant) influenza viruses were prepared that had the same PR8 "internal" genes (i.e., those other than the HA and NA genes), and the NA and HA from a single isolate, and where the residue at position 117 (or position 116 in the H3 reassortant) in HA2 was altered to aspartic acid. All of the 6:2 mutant reassortants showed enhanced growth in Vero cells relative to the corresponding parent 6:2 reassortant. Thus, for vaccine viruses that are to be grown or passaged in cells in culture, e.g., Vero cells, replacement of the residue at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2, e.g., by mutation, or selection of a HA gene segment with a residue that confers enhanced growth of the virus in cultured cells, can result in significantly higher viral titers. Thus, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are rodent or primate, e.g., human, cells. Also provided is a method to identify a HA2 that confers altered growth of a recombinant influenza virus. The method includes introducing one or more substitutions in influenza virus HA2 into a HA gene segment to yield a mutant HA gene segment; and identifying whether the mutant HA gene segment, when present in a replication competent recombinant influenza virus, results in enhanced replication of the recombinant influenza virus in a cell relative to a corresponding replication competent influenza virus without the one or more substitutions in HA2. In one embodiment, at least one substitution is at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2, e.g., the at least one substitution is to aspartic acid or glutamic acid. In one embodiment,

the cells are rodent or primate cells. In one embodiment, the one or more substitutions are to an amino acid residue with an acidic side chain.

In one embodiment, the influenza virus of the invention is a recombinant influenza virus having a mutant HA2 protein with at least one substitution that replaces an amino acid residue with an aliphatic side chain, amide-containing side chain, basic side chain, or sulfur containing side chain with a residue with an aromatic side chain or acidic side chain (a nonconservative substitution), e.g., at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the influenza virus is a recombinant influenza virus having a HA2 protein with a residue with an aromatic side chain or acidic side chain at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the recombinant influenza virus has a mutant HA2 protein with at least one substitution that replaces a neutral or positively charged residue with a polar or negatively charged residue, e.g., at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the influenza virus is a recombinant influenza virus having a HA2 protein with a residue with a polar or negatively charged residue at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. The presence of the residue with the aromatic side chain or acidic side chain, or the polar or negatively charged residue, at position 117 in HA2 may alter the efficiency or rate of conformational change of HA or pH dependent membrane fusion. In one embodiment, the recombinant reassortant influenza virus comprises a HA gene segment selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein recombinant virus has enhanced replication in Vero cells relative to a corresponding virus that does not have aspartic acid or glutamic acid at position 117 in HA2, e.g., where the corresponding virus has an alanine, asparagine, arginine or lysine at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the recombinant virus has a NA gene segment with a tyrosine at position 255, wherein the numbering for NA residues is that for N1.

In one embodiment, the invention provides isolated influenza type A virus with a characteristic residue or substitution at position 117 of HA2, e.g., the residue at position 117 of HA2 is not asparagine, alanine, arginine or lysine, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the isolated influenza type A virus of the invention with a characteristic residue or substitution at position 117 of HA2, has an HA2 amino acid sequence with at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:16-20 or 22. In one embodiment, the isolated influenza type A virus of the invention with a characteristic residue or substitution at position 117 of HA2, has an HA1 from any one of subtypes 1-15 of HA. In one embodiment, an isolated influenza A virus of the invention has a nonconservative substitution at residue 117 of HA2, e.g., an asparagine to an asparatic acid substitution, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the isolated influenza virus of the invention has an aspartic acid or glutamic acid at position 117 of HA2, wherein the numbering for HA2 residues is that for H1 HA2. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side

chains is lysine, arginine and histidine; and a group of amino acids having sulfur-containing side chain is cysteine and methionine. In one embodiment, conservative amino acid substitution groups are: threonine-valine-leucine-isoleucine-alanine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine.

5 In one embodiment, a mutation is introduced into a HA gene segment of an influenza virus isolate, e.g., via recombinant DNA techniques including site-specific mutagenesis or replacing a portion of the HA coding sequence that includes residue 117 of HA2 with a portion that includes the characteristic residue(s), wherein the numbering for HA2 residues is that for H1 HA2.

In another embodiment, a HA gene segment with a residue that confers enhanced replication in Vero
10 cells is combined with a compatible NA segment, and internal gene segments of an influenza vaccine virus. In one embodiment, the substitution(s) in the HA2 protein, or the characteristic residue in the HA2 protein, that results in the enhanced replication, is/are at or within about 1 to 10 residues, or any integer in between, for instance, at or within 1 to 5, residues, of residue 117 of the HA2 protein of influenza A virus, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, a NA protein has at least one
15 substitution, or has the characteristic residue discussed herein, such as one that results in enhanced replication, at or within about 1 to 10 residues, or any integer in between, e.g., at or within 1 to 5 residues of the codon for residue 255 of the NA protein of influenza A virus, wherein the numbering for NA residues is that for N1.

The invention provides a plurality of influenza virus vectors of the invention, e.g., those useful to
20 prepare reassortant viruses including 6:1:1 reassortants, 6:2 reassortants and 7:1 reassortants. A 6:1:1 reassortant within the scope of the present invention is an influenza virus with 6 internal gene segments from a vaccine virus, a NA gene segment from a different (second) viral isolate, and a HA gene segment with a characteristic residue or substitution at position 117 of HA2 as described herein, where the HA gene segment is from a different viral source than the vaccine virus or the first viral isolate; a 6:2 reassortant within the scope
25 of the present invention is an influenza virus with 6 internal gene segments from a vaccine virus, and a NA gene segment and a HA gene segment from a different (second) viral isolate, where the HA gene segment has the characteristic residue or a substitution at position 117 of HA2 as described herein; and a 7:1 reassortant within the scope of the present invention is an influenza virus with 6 internal gene segments and a NA gene segment from a vaccine virus, and a HA gene segment with a characteristic residue or substitution
30 at position 117 of HA2 as described herein, where the HA gene segment is from a different viral source than the vaccine virus, or an influenza virus with 6 internal gene segments and a HA gene segment with the characteristic residue or substitution at position 117 of HA2 as described herein, and a NA gene segment is from a different viral source than the vaccine virus.

In one embodiment of the invention, the plurality includes vectors for vRNA production selected from
35 a vector comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector comprising a promoter

operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector comprising a operably linked to an influenza virus NS DNA linked to a transcription termination sequence. In one embodiment, the DNAs for vRNA production of PB1, PB2, PA, NP, M, and NS, have sequences from an influenza virus that replicates to high titers in cultured mammalian cells such as Vero cells or PER.C6® cells and also optionally embryonated eggs, and/or from a vaccine virus, e.g., one that does not cause significant disease in humans. The DNA for vRNA production of NA may be from any NA, e.g., any of N1-N9, and the DNA for vRNA production of HA may be from any HA, e.g., H1-H16. In one embodiment, the DNAs for vRNA production may be for an influenza B or C virus. For example, the DNAs for vRNA production include influenza B virus PA, PB1, PB2, NP, NS, and M or influenza B virus PA, PB1, PB2, NP, NS, M, and NA, wherein the vRNA for HA has a HA2 with a characteristic amino acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. The DNAs for vRNA production of NA and HA may be from different strains or isolates (6:1:1 reassortants) or from the same strain or isolate (6:2 reassortants), or the NA may be from the same strain or isolate as that for the internal genes (7:1 reassortant), where the HA2 sequence is selected to result in enhanced replication in Vero cells relative to a corresponding virus with, for example, an asparagine at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. The plurality also includes vectors for mRNA production selected from a vector encoding influenza virus PA, a vector encoding influenza virus PB1, a vector encoding influenza virus PB2, and a vector encoding influenza virus NP, and optionally one or more vectors encoding NP, NS, M, e.g., M1 and M2, HA or NA. The vectors encoding viral proteins may further include a transcription termination sequence.

Viruses that may provide the internal genes for reassortants within the scope of the invention include viruses that have high titers in Vero cells, e.g., titers of at least about 10^5 PFU/mL, e.g., at least 10^6 PFU/mL, 10^7 PFU/mL or 10^8 PFU/mL; high titers in embryonated eggs, e.g., titers of at least about 10^7 EID_{50}/mL, e.g., at least 10^8 EID_{50}/mL, 10^9 EID_{50}/mL or 10^{10} EID_{50}/mL; high titers in MDCK cells, e.g., titers of at least about 10^7 PFU/mL, e.g., at least 10^8 PFU/mL, or high titers in two of more of those host cells.

In one embodiment, the titers of the reassortant viruses of the invention in cells such as Vero cells may be over 1 log, 2 logs, 3 logs, or greater, than titers of the corresponding virus without a HA2 substitution or that lacks the selected residue at position 117 of HA2, wherein the numbering for HA2 residues is that for H1 HA2.

Other reassortants with internal genes from other PR8 isolates or vaccine viruses may be employed in recombinant reassortant viruses of the invention. In particular, 5:1:2 reassortants having PR8(UW) PB1, PB2, PA, NP, and M (“5”) and PR8(Cam) NS (“1”); 6:1:1 reassortants having PR8(UW) NA, PB1, PB2, PA, NP, and M (“6”) and PR8(Cam) NS (“1”); and 7:1 reassortants having PR8(UW) PB1, PB2, PA, NP, M, NA, and NS (“7”) may be employed.

In one embodiment, the DNAs for the internal genes for PB1, PB2, PA, NP, M, and NS encode proteins with substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. As used herein, "substantially the same activity" includes an activity that is about 0.1%,

1%, 10%, 30%, 50%, 90%, e.g., up to 100% or more, or detectable protein level that is about 80%, 90% or more, the activity or protein level, respectively, of the corresponding full-length polypeptide. In one embodiment, the nucleic acid a sequence encoding a polypeptide which is substantially the same as, e.g., having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. In one embodiment, the isolated and/or purified nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90%, including any integer between 50 and 100, or more contiguous nucleic acid sequence identity to one of SEQ ID NOs:1-6 or 33-38 and, in one embodiment, also encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, including any integer between 80 and 99, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 5, 10, 15, 20 or more, conservative amino acids substitutions, e.g., conservative substitutions of up to 10% or 20% of the residues, relative to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine; and a group of amino acids having sulfur-containing side chain is cysteine and methionine. In one embodiment, conservative amino acid substitution groups are: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine. In one embodiment, the influenza virus polypeptide has one or more, for instance, 2, 3 or 4, nonconservative amino acid substitutions, relative to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15.

The invention thus includes the use of isolated and purified vectors or plasmids, which express or encode influenza virus proteins, or express or encode influenza vRNA, both native and recombinant vRNA. The vectors comprise influenza cDNA, e.g., influenza A (e.g., any influenza A gene including any of the 16 HA or 9 NA subtypes), B or C DNA (see Fields Virology (Fields et al. (eds.), Lippincott, Williams and Wickens (2006), which is specifically incorporated by reference herein). Any suitable promoter or transcription termination sequence may be employed to express a protein or peptide, e.g., a viral protein or peptide, a protein or peptide of a nonviral pathogen, or a therapeutic protein or peptide.

A composition or plurality of vectors of the invention may also comprise a heterologous gene or open reading frame of interest, e.g., a foreign gene encoding an immunogenic peptide or protein useful as a vaccine or in gene replacement, fro instance imay encode an epitope useful in a cancer therapy or vaccine, or a peptide or polypeptide useful in gene therapy. When preparing virus, the vector or plasmid comprising the gene or cDNA of interest may substitute for a vector or plasmid for an influenza viral gene or may be in addition to vectors or plasmids for all influenza viral genes. Thus, another embodiment of the invention comprises a composition or plurality of vectors as described above in which one of the vectors is replaced with, or further comprises, 5' influenza virus sequences optionally including 5' influenza virus coding

sequences or a portion thereof, linked to a desired nucleic acid sequence, e.g., a desired cDNA, linked to 3' influenza virus sequences optionally including 3' influenza virus coding sequences or a portion thereof. In one embodiment, the desired nucleic acid sequence such as a cDNA is in an antisense (antigenomic) orientation. The introduction of such a vector in conjunction with the other vectors described above to a host
5 cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to the heterologous sequences of the vector.

The promoter in a vector for vRNA production may be a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T7 promoter, or a T3 promoter, and optionally the vector comprises a transcription termination sequence such as a RNA polymerase I transcription termination
10 sequence, a RNA polymerase II transcription termination sequence, a RNA polymerase III transcription termination sequence, or a ribozyme. Ribozymes within the scope of the invention include, but are not limited to, tetrahymena ribozymes, RNase P, hammerhead ribozymes, hairpin ribozymes, hepatitis ribozyme, as well as synthetic ribozymes. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter.

15 The promoter or transcription termination sequence in a vRNA or virus protein expression vector may be the same or different relative to the promoter or any other vector. In one embodiment, the vector or plasmid which expresses influenza vRNA comprises a promoter suitable for expression in at least one particular host cell, e.g., avian or mammalian host cells such as canine, feline, equine, bovine, ovine, or primate cells including human cells, or for expression in more than one host.

20 In one embodiment, at least one vector for vRNA comprises a RNA polymerase II promoter linked to a ribozyme sequence linked to viral coding sequences linked to another ribozyme sequences, optionally linked to a RNA polymerase II transcription termination sequence. In one embodiment, at least 2, e.g., 3, 4, 5, 6, 7 or 8, vectors for vRNA production comprise a RNA polymerase II promoter, a first ribozyme sequence, which is 5' to a sequence corresponding to viral sequences including viral coding sequences, which is 5' to a
25 second ribozyme sequence, which is 5' to a transcription termination sequence. Each RNA polymerase II promoter in each vRNA vector may be the same or different as the RNA polymerase II promoter in any other vRNA vector. Similarly, each ribozyme sequence in each vRNA vector may be the same or different as the ribozyme sequences in any other vRNA vector. In one embodiment, the ribozyme sequences in a single vector are not the same.

30 In one embodiment, the invention provides a plurality of influenza virus vectors for a reassortant, comprising a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription
35 termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an

influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the DNAs for PB1, PB2, PA, NP, NS, and M from one or more influenza vaccine seed viruses, wherein the DNA for NA has sequences for a heterologous NA, and wherein the DNA for HA selected
5 to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2; and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter
10 operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza
15 virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2. In one embodiment, at least one vector comprises sequences corresponding to those encoding PB1, PB2, PA, NP, M, or NS, or a portion thereof, having substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15, e.g., a sequence encoding a polypeptide with at least 80%, e.g., 85%, 90%, 92%, 95%, 98%, 99% or 100%, including any integer between
20 80 and 100, amino acid identity to a polypeptide encoded by one of SEQ ID NOs:1-6 or 10-15. Optionally, two vectors may be employed in place of the vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, e.g., a vector comprising a promoter operably linked to an influenza virus M1 cDNA linked to a transcription termination sequence and a vector comprising a promoter operably linked to an influenza virus M2 cDNA linked to a transcription termination sequence.
25 A plurality of the vectors of the invention may be physically linked or each vector may be present on an individual plasmid or other, e.g., linear, nucleic acid delivery vehicle. In one embodiment, each vRNA production vector is on a separate plasmid. In one embodiment, each mRNA production vector is on a separate plasmid.

The invention also provides a method to prepare influenza virus. The method comprises contacting a
30 cell with a plurality of the vectors of the invention, e.g., sequentially or simultaneously, in an amount effective to yield infectious influenza virus. The invention also includes isolating virus from a cell contacted with the plurality of vectors. Thus, the invention further provides isolated virus, as well as a host cell contacted with the plurality of vectors or virus of the invention. In another embodiment, the invention includes contacting the cell with one or more vectors, either vRNA or protein production vectors, prior to other vectors, either vRNA or
35 protein production vectors. In one embodiment, the promoter for vRNA vectors employed in the method is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter. In one embodiment, the RNA polymerase I promoter is a human RNA polymerase I promoter. In one embodiment, each vRNA vector employed in the method is on a separate plasmid. In one embodiment, the vRNA vectors employed in the method are on one plasmid or on two or three different

plasmids. In one embodiment, each mRNA vector employed in the method is on a separate plasmid. In one embodiment, the mRNA vectors for PA, PB1, PB2 and NP employed in the method are on one plasmid or on two or three different plasmids.

In one embodiment, the invention provides a method to select for influenza viruses with enhanced replication in cell culture. The method includes providing cells suitable for influenza vaccine production; serially culturing one or more influenza virus isolates in the cells; and isolating serially cultured virus with enhanced growth relative to the one or more isolates prior to serial culture. In one embodiment, the cells are rodent or primate cells.

Also provided is a method to identify a HA2 that confers altered growth of a recombinant influenza virus. The method includes introducing one or more substitutions in influenza virus HA2 into a HA gene segment to yield a mutant HA gene segment; and identifying whether the mutant HA gene segment, when present in a replication competent recombinant influenza virus, results in enhanced replication of the recombinant influenza virus in a cell relative to a corresponding replication competent influenza virus without the one or more substitutions in HA2. In one embodiment, at least one substitution is at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2, e.g., at least one substitution is to aspartic acid or glutamic acid. In one embodiment, the cell is a rodent or primate cell. In one embodiment, the one or more substitutions are to an amino acid residue with an acidic side chain.

In one embodiment, the invention provides a method to prepare a recombinant influenza virus with a HA gene segment having a mutant HA2. The method includes altering influenza virus HA nucleic acid at position 117 in HA2 to aspartic acid or glutamic acid; and expressing the altered nucleic acid in a cell having vectors for influenza vRNA production and viral protein production in an amount effective to yield recombinant influenza virus with a HA gene segment having the aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2. In one embodiment, the cell is a mammalian, e.g., a human cell, or avian cell.

The methods of producing virus described herein, which do not require helper virus infection, are useful in viral mutagenesis studies, and in the production of vaccines (e.g., for AIDS, influenza, hepatitis B, hepatitis C, rhinovirus, filoviruses, malaria, herpes, and foot and mouth disease) and gene therapy vectors (e.g., for cancer, AIDS, adenosine deaminase, muscular dystrophy, ornithine transcarbamylase deficiency and central nervous system tumors). Thus, a virus for use in medical therapy (e.g., for a vaccine or gene therapy) is provided.

The invention also provides isolated viral polypeptides, and methods of preparing and using recombinant virus of the invention. The methods include administering to a host organism, e.g., a mammal, an effective amount of the influenza virus of the invention, e.g., an inactivated virus preparation, optionally in combination with an adjuvant and/or a carrier, e.g., in an amount effective to prevent or ameliorate infection of an animal such as a mammal by that virus or an antigenically closely related virus. In one embodiment, the virus is administered intramuscularly while in another embodiment, the virus is administered intranasally. In some dosing protocols, all doses may be administered intramuscularly or intranasally, while in others a combination of intramuscular and intranasal administration is employed. The vaccine may further contain other isolates of influenza virus including recombinant influenza virus, other pathogen(s), additional biological

agents or microbial components, e.g., to form a multivalent vaccine. In one embodiment, intranasal vaccination, for instance containing with inactivated influenza virus, and a mucosal adjuvant may induce virus-specific IgA and neutralizing antibody in the nasopharynx as well as serum IgG.

The influenza virus of the invention may employed with other anti-virals, e.g., amantadine, rimantadine, and/or neuraminidase inhibitors, e.g., may be administered separately in conjunction with those anti-virals, for instance, administered before, during and/or after.

The invention also provides a method in which the pH of media in which cells suitable for propagating influenza virus are cultured, is altered during virus propagation to allow for enhanced influenza virus replication in those cells. Thus, for cells with late endosomes having a pH that is higher than that in MDCK cells, altering media pH to maintain a higher pH during virus replication over time, may enhance virus production in the absence of a HA2 protein with a characteristic residue, such as aspartic acid, at position 117, wherein the numbering for HA2 residues is that for H1 HA2.

Brief Description of the Figures

Figure 1. Nucleotide sequence for PR8(Cambridge) genes (SEQ ID NOs:10-15).

Figure 2. Growth properties of Vero cell-adapted PR8 virus in Vero cells.

Figure 3. Comparison of amino acid sequence differences between PR8 and Vero cell-adapted PR8.

Figure 4. Growth properties of Vero cell-adapted PR8, non Vero cell-adapted “wild-type” PR8, and recombinant viruses with one or two substitutions relative to wild-type virus in Vero cells.

Figure 5. Growth properties of HA2 N117D virus and wild-type PR8 in MDCK cells.

Figure 6. Three dimensional structure of HA as a trimer (A), HA as a monomer (B) and HA2 (C).

Figure 7. Schematic of fusion assay which expresses full length HA.

Figure 8. Photomicrographs of Vero cells expressing wild-type PR8 HA or HA2 N117D virus at various pH conditions.

Figures 9A-B. pH sensitivity of Alexa647 and Oregon Green dyes. A) The fluorescence intensity of Oregon Green dye is sensitive to variations in pH while the fluorescence intensity of Alexa647 does not vary over pH 3 to 7. B) Schematic of assay to detect endosomal pH.

Figure 10. Comparison of endosomal pH in MDCK cells and Vero cells.

Figures 11A-C. HA2 N117D substitution mutants have enhanced infectivity titers in Vero cells. A) Vero cells were infected with A/Kawasaki/173/2001 (H1N1) and A/Kawasaki/173/2001 HA2 N117D and the titers over time determined. B) Vero cells were infected with A/Kawasaki/UTK-4/2009 (H1N1) and A/Kawasaki/UKT-4/2009 HA2 N117D and the titers over time determined. C) Vero cells were infected with A/Yokohama /2017/2003 (H3N2) and A/Yokohama/2017/2003 HA2 N116D and the titers over time determined.

Figure 12. A) Alignment of HA2 sequences from A/Aichi/2/68; A/Dk/Sing/97; A/HK/486/97; A/Sw/9/98; and A/HongKong/1073/99 (SEQ ID Nos.16-20). B) Amino acid sequence of HA sequence from A/California/08/2009 (SEQ ID NO:21). HA2 sequences correspond to residues 336-566 (SEQ ID NO:22)

Figure 13. HA2 sequences for A/Kawasaki/173/2001, A/Kawasaki/UKT-4/2009, and A/Yokohama/2017/2003. According to the NCBI database, influenza virus HA2 sequences for H1, H2, H3, H5, H7, and H9 HAs were generally conserved at position 116 or 117 (N116 or N117) (more than 99%).

5 **Detailed Description of the Invention**

Definitions

As used herein, the term "isolated" refers to *in vitro* preparation and/or isolation of a nucleic acid molecule, e.g., vector or plasmid, peptide or polypeptide (protein), or virus of the invention, so that it is not associated with *in vivo* substances, or is substantially purified from *in vitro* substances. An isolated virus
10 preparation is generally obtained by *in vitro* culture and propagation, and/or via passage in eggs, and is substantially free from other infectious agents.

As used herein, "substantially purified" means the object species is the predominant species, e.g., on a molar basis it is more abundant than any other individual species in a composition, and preferably is at least about 80% of the species present, and optionally 90% or greater, e.g., 95%, 98%, 99% or more, of the
15 species present in the composition.

As used herein, "substantially free" means below the level of detection for a particular infectious agent using standard detection methods for that agent.

A "recombinant" virus is one which has been manipulated *in vitro*, e.g., using recombinant DNA techniques, to introduce changes to the viral genome. Reassortant viruses can be prepared by recombinant
20 or nonrecombinant techniques.

As used herein, the term "recombinant nucleic acid" or "recombinant DNA sequence or segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from a source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in the native genome. An
25 example of DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

30 As used herein, a "heterologous" influenza virus gene or gene segment is from an influenza virus source that is different than a majority of the other influenza viral genes or gene segments in a recombinant, e.g., reassortant, influenza virus.

The terms "isolated polypeptide", "isolated peptide" or "isolated protein" include a polypeptide, peptide or protein encoded by cDNA or recombinant RNA including one of synthetic origin, or some
35 combination thereof.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule expressed from a recombinant DNA molecule. In contrast, the term "native protein" is used herein to indicate a protein isolated from a naturally occurring (i.e., a nonrecombinant) source. Molecular biological

techniques may be used to produce a recombinant form of a protein with identical properties as compared to the native form of the protein.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Alignments using these programs can be performed using the default parameters. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The algorithm may involve first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm may also perform a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm may be the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The BLASTN program (for nucleotide sequences) may use as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program may use as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. See http://www.ncbi.n1m.nih.gov. Alignment may also be performed manually by inspection.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

<u>Influenza Virus Structure and Propagation</u>

Influenza A viruses possess a genome of eight single-stranded negative-sense viral RNAs (vRNAs) that encode at least ten proteins. The influenza virus life cycle begins with binding of the hemagglutinin (HA) to sialic acid-containing receptors on the surface of the host cell, followed by receptor-mediated endocytosis. The low pH in late endosomes triggers a conformational shift in the HA, thereby exposing the N-terminus of the HA2 subunit (the so-called fusion peptide). The fusion peptide initiates the fusion of the viral and endosomal membrane, and the matrix protein (M1) and RNP complexes are released into the cytoplasm. RNPs consist of the nucleoprotein (NP), which encapsidates vRNA, and the viral polymerase complex, which is formed by the PA, PB1, and PB2 proteins. RNPs are transported into the nucleus, where transcription and replication take place. The RNA polymerase complex catalyzes three different reactions: synthesis of an mRNA with a 5' cap and 3' polyA structure, of a full-length complementary RNA (cRNA), and of genomic vRNA using the cRNA as a template. Newly synthesized vRNAs, NP, and polymerase proteins are then assembled into RNPs, exported from the nucleus, and transported to the plasma membrane, where budding of progeny virus particles occurs. The neuraminidase (NA) protein plays a crucial role late in infection by removing sialic acid from sialyloligosaccharides, thus releasing newly assembled virions from the cell surface and preventing the self aggregation of virus particles. Although virus assembly involves protein-protein and protein-vRNA interactions, the nature of these interactions is largely unknown.

Although influenza B and C viruses are structurally and functionally similar to influenza A virus, there are some differences. For example, influenza B virus does not have a M2 protein with ion channel activity but has BM2 and has a gene segment with both NA and NB sequences. Influenza C virus has only seven gene segments.

Cell Lines That Can Be Used in the Present Invention

Any cell, e.g., any avian or mammalian cell, such as a human, e.g., 293T or PER.C6® cells, or canine, bovine, equine, feline, swine, ovine, rodent, for instance mink, e.g., MvLu1 cells, or hamster, e.g., CHO cells, or non-human primate, e.g., Vero cells, including mutant cells, which supports efficient replication of influenza virus can be employed to isolate and/or propagate influenza viruses. Isolated viruses can be used to prepare a reassortant virus. In one embodiment, host cells for vaccine production are continuous mammalian or avian cell lines or cell strains. A complete characterization of the cells to be used, may be conducted so that appropriate tests for purity of the final product can be included. Data that can be used for the characterization of a cell includes (a) information on its origin, derivation, and passage history; (b) information on its growth and morphological characteristics; (c) results of tests of adventitious agents; (d) distinguishing features, such as biochemical, immunological, and cytogenetic patterns which allow the cells to be clearly recognized among other cell lines; and (e) results of tests for tumorigenicity. In one embodiment, the passage level, or population doubling, of the host cell used is as low as possible.

In one embodiment, the cells are WHO certified, or certifiable, continuous cell lines. The requirements for certifying such cell lines include characterization with respect to at least one of genealogy, growth characteristics, immunological markers, virus susceptibility tumorigenicity and storage conditions, as well as by testing in animals, eggs, and cell culture. Such characterization is used to confirm that the cells are free from detectable adventitious agents. In some countries, karyology may also be required. In addition, tumorigenicity may be tested in cells that are at the same passage level as those used for vaccine production.

The virus may be purified by a process that has been shown to give consistent results, before vaccine production (see, e.g., World Health Organization, 1982).

Virus produced by the host cell may be highly purified prior to vaccine or gene therapy formulation. Generally, the purification procedures result in extensive removal of cellular DNA and other cellular 5 compoenents, and adventitious agents. Procedures that extensively degrade or denature DNA may also be used.

Influenza Vaccines

A vaccine of the invention includes an isolated recombinant influenza virus of the invention, and optionally one or more other isolated viruses including other isolated influenza viruses, one or more 10 immunogenic proteins or glycoproteins of one or more isolated influenza viruses or one or more other pathogens, e.g., an immunogenic protein from one or more bacteria, non-influenza viruses, yeast or fungi, or isolated nucleic acid encoding one or more viral proteins (e.g., DNA vaccines) including one or more immunogenic proteins of the isolated influenza virus of the invention. In one embodiment, the influenza viruses of the invention may be vaccine vectors for influenza virus or other pathogens.

15 A complete virion vaccine may be concentrated by ultrafiltration and then purified by zonal centrifugation or by chromatography. Viruses other than the virus of the invention, such as those included in a multivalent vaccine, may be inactivated before or after purification using formalin or beta-propiolactone, for instance.

A subunit vaccine comprises purified glycoproteins. Such a vaccine may be prepared as follows: 20 using viral suspensions fragmented by treatment with detergent, the surface antigens are purified, by ultracentrifugation for example. The subunit vaccines thus contain mainly HA protein, and also NA. The detergent used may be cationic detergent for example, such as hexadecyl trimethyl ammonium bromide (Bachmeyer, 1975), an anionic detergent such as ammonium deoxycholate (Laver & Webster, 1976); or a nonionic detergent such as that commercialized under the name TRITON X100. The hemagglutinin may also 25 be isolated after treatment of the virions with a protease such as bromelin, and then purified. The subunit vaccine may be combined with an attenuated virus of the invention in a multivalent vaccine.

A split vaccine comprises virions which have been subjected to treatment with agents that dissolve lipids. A split vaccine can be prepared as follows: an aqueous suspension of the purified virus obtained as above, inactivated or not, is treated, under stirring, by lipid solvents such as ethyl ether or chloroform, 30 associated with detergents. The dissolution of the viral envelope lipids results in fragmentation of the viral particles. The aqueous phase is recuperated containing the split vaccine, constituted mainly of hemagglutinin and neuraminidase with their original lipid environment removed, and the core or its degradation products. Then the residual infectious particles are inactivated if this has not already been done. The split vaccine may be combined with an attenuated virus of the invention in a multivalent vaccine.

35 Inactivated Vaccines. Inactivated influenza virus vaccines are provided by inactivating replicated virus using known methods, such as, but not limited to, formalin or β-propiolactone treatment. Inactivated vaccine types that can be used in the invention can include whole-virus (WV) vaccines or subvirion (SV) (split) vaccines. The WV vaccine contains intact, inactivated virus, while the SV vaccine contains purified virus

disrupted with detergents that solubilize the lipid-containing viral envelope, followed by chemical inactivation of residual virus.

In addition, vaccines that can be used include those containing the isolated HA and NA surface proteins, which are referred to as surface antigen or subunit vaccines.

5 Live Attenuated Virus Vaccines. Live, attenuated influenza virus vaccines, such as those including a recombinant virus of the invention can be used for preventing or treating influenza virus infection. Attenuation may be achieved in a single step by transfer of attenuated genes from an attenuated donor virus to a replicated isolate or reassorted virus according to known methods. Since resistance to influenza A virus is mediated primarily by the development of an immune response to the HA and/or NA glycoproteins, the genes 10 coding for these surface antigens come from the reassorted viruses or clinical isolates. The attenuated genes are derived from an attenuated parent. In this approach, genes that confer attenuation generally do not code for the HA and NA glycoproteins.

Viruses (donor influenza viruses) are available that are capable of reproducibly attenuating influenza viruses, e.g., a cold adapted (ca) donor virus can be used for attenuated vaccine production. Live, attenuated 15 reassortant virus vaccines can be generated by mating the ca donor virus with a virulent replicated virus. Reassortant progeny are then selected at 25°C (restrictive for replication of virulent virus), in the presence of an appropriate antiserum, which inhibits replication of the viruses bearing the surface antigens of the attenuated ca donor virus. Useful reassortants are: (a) infectious, (b) attenuated for seronegative non-adult mammals and immunologically primed adult mammals, (c) immunogenic and (d) genetically stable. The 20 immunogenicity of the ca reassortants parallels their level of replication. Thus, the acquisition of the six transferable genes of the ca donor virus by new wild-type viruses has reproducibly attenuated these viruses for use in vaccinating susceptible mammals both adults and non-adult.

Other attenuating mutations can be introduced into influenza virus genes by site-directed mutagenesis to rescue infectious viruses bearing these mutant genes. Attenuating mutations can be 25 introduced into non-coding regions of the genome, as well as into coding regions. Such attenuating mutations can also be introduced into genes other than the HA or NA, e.g., the PB2 polymerase gene. Thus, new donor viruses can also be generated bearing attenuating mutations introduced by site-directed mutagenesis, and such new donor viruses can be used in the production of live attenuated reassortants vaccine candidates in a manner analogous to that described above for the ca donor virus. Similarly, other known and suitable 30 attenuated donor strains can be reassorted with influenza virus to obtain attenuated vaccines suitable for use in the vaccination of mammals.

In one embodiment, such attenuated viruses maintain the genes from the virus that encode antigenic determinants substantially similar to those of the original clinical isolates. This is because the purpose of the attenuated vaccine is to provide substantially the same antigenicity as the original clinical isolate of the virus, 35 while at the same time lacking pathogenicity to the degree that the vaccine causes minimal chance of inducing a serious disease condition in the vaccinated mammal.

The viruses in a multivalent vaccine can thus be attenuated or inactivated, formulated and administered, according to known methods, as a vaccine to induce an immune response in an animal, e.g., a mammal. Methods are well-known in the art for determining whether such attenuated or inactivated vaccines

have maintained similar antigenicity to that of the clinical isolate or high growth strain derived therefrom. Such known methods include the use of antisera or antibodies to eliminate viruses expressing antigenic determinants of the donor virus; chemical selection (e.g., amantadine or rimantidine); HA and NA activity and inhibition; and nucleic acid screening (such as probe hybridization or PCR) to confirm that donor genes encoding the antigenic determinants (e.g., HA or NA genes) are not present in the attenuated viruses.

Pharmaceutical Compositions

Pharmaceutical compositions of the present invention, suitable for inoculation, e.g., nasal, parenteral or oral administration, comprise one or more influenza virus isolates, e.g., one or more attenuated or inactivated influenza viruses, a subunit thereof, isolated protein(s) thereof, and/or isolated nucleic acid encoding one or more proteins thereof, optionally further comprising sterile aqueous or non-aqueous solutions, suspensions, and emulsions. The compositions can further comprise auxiliary agents or excipients, as known in the art. The composition of the invention is generally presented in the form of individual doses (unit doses).

Conventional vaccines generally contain about 0.1 to 200 µg, e.g., 30 to 100 µg, of HA from each of the strains entering into their composition. The vaccine forming the main constituent of the vaccine composition of the invention may comprise a single influenza virus, or a combination of influenza viruses, for example, at least two or three influenza viruses, including one or more reassortant(s).

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and/or emulsions, which may contain auxiliary agents or excipients known in the art. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include adjuvants, wetting agents, emulsifying and suspending agents, or sweetening, flavoring, or perfuming agents.

When a composition of the present invention is used for administration to an individual, it can further comprise salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. For vaccines, adjuvants, substances which can augment a specific immune response, can be used. Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately, but into the same site of the organism being immunized.

Heterogeneity in a vaccine may be provided by mixing replicated influenza viruses for at least two influenza virus strains, such as 2-20 strains or any range or value therein. Vaccines can be provided for variations in a single strain of an influenza virus, using techniques known in the art.

A pharmaceutical composition according to the present invention may further or additionally comprise at least one chemotherapeutic compound, for example, for gene therapy, immunosuppressants, anti-inflammatory agents or immune enhancers, and for vaccines, chemotherapeutics including, but not limited to, gamma globulin, amantadine, guanidine, hydroxybenzimidazole, interferon-α, interferon-β, interferon-γ, tumor

necrosis factor-alpha, thiosemicarbarzones, methisazone, rifampin, ribavirin, a pyrimidine analog, a purine analog, foscarnet, phosphonoacetic acid, acyclovir, dideoxynucleosides, a protease inhibitor, or ganciclovir.

The composition can also contain variable but small quantities of endotoxin-free formaldehyde, and preservatives, which have been found safe and not contributing to undesirable effects in the organism to which the composition is administered.

Pharmaceutical Purposes

The administration of the composition (or the antisera that it elicits) may be for either a “prophylactic” or “therapeutic” purpose. When provided prophylactically, the compositions of the invention which are vaccines are provided before any symptom or clinical sign of a pathogen infection becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate any subsequent infection. When provided prophylactically, the gene therapy compositions of the invention, are provided before any symptom or clinical sign of a disease becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate one or more symptoms or clinical signs associated with the disease.

When provided therapeutically, a viral vaccine is provided upon the detection of a symptom or clinical sign of actual infection. The therapeutic administration of the compound(s) serves to attenuate any actual infection. When provided therapeutically, a gene therapy composition is provided upon the detection of a symptom or clinical sign of the disease. The therapeutic administration of the compound(s) serves to attenuate a symptom or clinical sign of that disease.

Thus, a vaccine composition of the present invention may be provided either before the onset of infection (so as to prevent or attenuate an anticipated infection) or after the initiation of an actual infection. Similarly, for gene therapy, the composition may be provided before any symptom or clinical sign of a disorder or disease is manifested or after one or more symptoms are detected.

A composition is said to be “pharmacologically acceptable” if its administration can be tolerated by a recipient mammal. Such an agent is said to be administered in a “therapeutically effective amount” if the amount administered is physiologically significant. A composition of the present invention is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient, e.g., enhances at least one primary or secondary humoral or cellular immune response against at least one strain of an infectious influenza virus.

The “protection” provided need not be absolute, i.e., the influenza infection need not be totally prevented or eradicated, if there is a statistically significant improvement compared with a control population or set of mammals. Protection may be limited to mitigating the severity or rapidity of onset of symptoms or clinical signs of the influenza virus infection.

Pharmaceutical Administration

A composition of the present invention may confer resistance to one or more pathogens, e.g., one or more influenza virus strains, by either passive immunization or active immunization. In active immunization, an attenuated live vaccine composition is administered prophylactically to a host (e.g., a mammal), and the host’s immune response to the administration protects against infection and/or disease. For passive immunization, the elicited antisera can be recovered and administered to a recipient suspected of having an

infection caused by at least one influenza virus strain. A gene therapy composition of the present invention may yield prophylactic or therapeutic levels of the desired gene product by active immunization.

In one embodiment, the vaccine is provided to a mammalian female (at or prior to pregnancy or parturition), under conditions of time and amount sufficient to cause the production of an immune response which serves to protect both the female and the fetus or newborn (via passive incorporation of the antibodies across the placenta or in the mother's milk).

The present invention thus includes methods for preventing or attenuating a disorder or disease, e.g., an infection by at least one strain of pathogen. As used herein, a vaccine is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (i.e., suppression) of a clinical sign or condition of the disease, or in the total or partial immunity of the individual to the disease. As used herein, a gene therapy composition is said to prevent or attenuate a disease if its administration results either in the total or partial attenuation (i.e., suppression) of a clinical sign or condition of the disease, or in the total or partial immunity of the individual to the disease.

A composition having at least one influenza virus of the present invention, including one which is attenuated and one or more other isolated viruses, one or more isolated viral proteins thereof, one or more isolated nucleic acid molecules encoding one or more viral proteins thereof, or a combination thereof, may be administered by any means that achieve the intended purposes.

For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, oral or transdermal routes. Parenteral administration can be accomplished by bolus injection or by gradual perfusion over time.

A typical regimen for preventing, suppressing, or treating an influenza virus related pathology, comprises administration of an effective amount of a vaccine composition as described herein, administered as a single treatment, or repeated as enhancing or booster dosages, over a period up to and including between one week and about 24 months, or any range or value therein.

According to the present invention, an "effective amount" of a composition is one that is sufficient to achieve a desired effect. It is understood that the effective dosage may be dependent upon the species, age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect wanted. The ranges of effective doses provided below are not intended to limit the invention and represent dose ranges.

The dosage of a live, attenuated or killed virus vaccine for an animal such as a mammalian adult organism may be from about 10^2-10^{15}, e.g., 10^3-10^{12}, plaque forming units (PFU)/kg, or any range or value therein. The dose of inactivated vaccine may range from about 0.1 to 1000, e.g., 30 to 100 µg, of HA protein. However, the dosage should be a safe and effective amount as determined by conventional methods, using existing vaccines as a starting point.

The dosage of immunoreactive HA in each dose of replicated virus vaccine may be standardized to contain a suitable amount, e.g., 30 to 100 µg or any range or value therein, or the amount recommended by government agencies or recognized professional organizations. The quantity of NA can also be standardized, however, this glycoprotein may be labile during purification and storage.

The dosage of immunoreactive HA in each dose of replicated virus vaccine can be standardized to contain a suitable amount, e.g., 1-50 µg or any range or value therein, or the amount recommended by the U.S. Public Heath Service (PHS), which is usually 15 µg, per component for older children 3 years of age, and 7.5 µg per component for older children <3 years of age. The quantity of NA can also be standardized, however, this glycoprotein can be labile during the processor purification and storage (Kendal et al., 1980; Kerr et al., 1975). Each 0.5-ml dose of vaccine may contains approximately 1-50 billion virus particles, and preferably 10 billion particles.

The invention will be described by the following nonlimiting examples.

Example 1

Methods

Cells and viruses

293T human embryonic kidney cells are maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells are grown in MEM with 5% newborn calf serum and antibiotics. African green monkey Vero WCB cells, which had been established after biosafety tests for use in human vaccine production (Sugawara et al., 2002), are maintained in serum-free VP-SFM medium (GIBCO-BRL) with antibiotics. Cells are maintained at 37°C in 5% CO_2. A WHO-recommended vaccine seed virus is NIBRG-14.

Construction of plasmids and reverse genetics

To generate reassortants of influenza A viruses, a plasmid-based reverse genetics (Neumann et al., 1999) is used. The full-length cDNAs were cloned into a plasmid under control of the human polymerase I promoter and the mouse RNA polymerase I terminator (PolI plasmids).

A previously produced series of PolI constructs, derived from A/WSN/33 (H5N1; WSN) or PR8 strains is used, for reverse genetics (Horimoto et al., 2006; Neumann et al., 1999). The World Health Organization (WHO) recommends A/Puerto Rico/8/34 (H1N1; PR8) as a donor virus, because of its safety in humans (Wood & Robertson, 2004; Webby & Webster, 2003).

Plasmids expressing WSN or PR8 NP, PA, PB1, or PB2 under control of the chicken □actin promoter are used for all reverse genetics experiments (Horimoto et al., 2006; Neumann et al., 1999). Briefly, PolI plasmids and protein expression plasmids are mixed with a transfection reagent, Trans-IT 293T (Panvera), incubated at room temperature for 15 minutes, and then added to 293T cells. Transfected cells are incubated in Opti-MEM I (GIBCO-BRL) for 48 hours. For reverse genetics in Vero WCB cells, an electroporator (Amaxa) is used to transfect the plasmid mixtures according to the manufacturer's instructions. Sixteen hours after transfection, freshly prepared Vero WCB cells were added onto the transfected cells and TPCK-trypsin (1 □g/mL) is added to the culture 6 hours later. Transfected cells are incubated in serum-free VP-SFM for a total of 4 days. Supernatants containing infectious viruses are harvested, and may bebiologically cloned by limiting dilution.

A recombinant virus having the HA and NA genes from A/Hong Kong/213/2003 (H5N1) and the remainder of the type A influenza virus genes from PR8(UW) was prepared. The titer of the recombinant virus was $10^{10.67}$ EID_{50}/mL, and the HA titer was 1:1600

Table 1

Virus possessing PR8 genes together with the following HA and NA genes	HA titer (HAU/mL) in each dilition						
	10-2	10-3	10-4	10-5	10-6	10-7	10-8
WSN-HA NA	160	40	40	320	40	640	<1
HK-HAavir NA	400	800	400	400	400	800	<1

5 The sequences of PR8 (UW) genes are as follows:

PA

AGCGAAAGCA GGTACTGATC CAAAATGGAA GATTTTGTGC GACAATGCTT
10 CAATCCGATG ATTGTCGAGC TTGCGGAAAA AACAATGAAA GAGTATGGGG
AGGACCTGAA AATCGAAACA AACAAATTTG CAGCAATATG CACTCACTTG
GAAGTATGCT TCATGTATTC AGATTTTCAC TTCATCAATG AGCAAGGCGA
GTCAATAATC GTAGAACTTG GTGATCCAAA TGCACTTTTG AAGCACAGAT
TTGAAATAAT CGAGGGAAGA GATCGCACAA TGGCCTGGAC AGTAGTAAAC
15 AGTATTTGCA ACACTACAGG GGCTGAGAAA CCAAAGTTTC TACCAGATTT
GTATGATTAC AAGGAGAATA GATTCATCGA AATTGGAGTA ACAAGGAGAG
AAGTTCACAT ATACTATCTG GAAAAGGCCA ATAAAATTAA ATCTGAGAAA
ACACACATCC ACATTTTCTC GTTCACTGGG GAAGAAATGG CCACAAAGGC
AGACTACACT CTCGATGAAG AAAGCAGGGC TAGGATCAAA ACCAGACTAT
20 TCACCATAAG ACAAGAAATG GCCAGCAGAG GCCTCTGGGA TTCCTTTCGT
CAGTCCGAGA GAGGAGAAGA GACAATTGAA GAAAGGTTTG AAATCACAGG
AACAATGCGC AAGCTTGCCG ACCAAAGTCT CCCGCCGAAC TTCTCCAGCC
TTGAAAATTT TAGAGCCTAT GTGGATGGAT TCGAACCGAA CGGCTACATT
GAGGGCAAGC TGTCTCAAAT GTCCAAAGAA GTAAATGCTA GAATTGAACC
25 TTTTTTGAAA ACAACACCAC GACCACTTAG ACTTCCGAAT GGGCCTCCCT
GTTCTCAGCG GTCCAAATTC CTGCTGATGG ATGCCTTAAA ATTAAGCATT
GAGGACCCAA GTCATGAAGG AGAGGGAATA CCGCTATATG ATGCAATCAA
ATGCATGAGA ACATTCTTTG GATGGAAGGA ACCCAATGTT GTTAAACCAC
ACGAAAAGGG AATAAATCCA AATTATCTTC TGTCATGGAA GCAAGTACTG
30 GCAGAACTGC AGGACATTGA GAATGAGGAG AAAATTCCAA AGACTAAAAA
TATGAAGAAA ACAAGTCAGC TAAAGTGGGC ACTTGGTGAG AACATGGCAC
CAGAAAAGGT AGACTTTGAC GACTGTAAAG ATGTAGGTGA TTTGAAGCAA
TATGATAGTG ATGAACCAGA ATTGAGGTCG CTTGCAAGTT GGATTCAGAA
TGAGTTTAAC AAGGCATGCG AACTGACAGA TTCAAGCTGG ATAGAGCTCG
35 ATGAGATTGG AGAAGATGTG GCTCCAATTG AACACATTGC AAGCATGAGA
AGGAATTATT TCACATCAGA GGTGTCTCAC TGCAGAGCCA CAGAATACAT
AATGAAGGGA GTGTACATCA ATACTGCCTT GCTTAATGCA TCTTGTGCAG
CAATGGATGA TTTCCAATTA ATTCCAATGA TAAGCAAGTG TAGAACTAAG
GAGGGAAGGC GAAAGACCAA CTTGTATGGT TTCATCATAA AAGGAAGATC
40 CCACTTAAGG AATGACACCG ACGTGGTAAA CTTTGTGAGC ATGGAGTTTT
CTCTCACTGA CCCAAGACTT GAACCACATA AATGGGAGAA GTACTGTGTT
CTTGAGATAG GAGATATGCT TATAAGAAGT GCCATAGGCC AGGTTTCAAG
GCCCATGTTC TTGTATGTGA GAACAAATGG AACCTCAAAA ATTAAAATGA
AATGGGGAAT GGAGATGAGG CGTTGCCTCC TCCAGTCACT TCAACAAATT
45 GAGAGTATGA TTGAAGCTGA GTCCTCTGTC AAAGAGAAAG ACATGACCAA
AGAGTTCTTT GAGAACAAAT CAGAAACATG GCCCATTGGA GAGTCCCCCA

AAGGAGTGGA GGAAAGTTCC ATTGGGAAGG TCTGCAGGAC TTTATTAGCA
AAGTCGGTAT TCAACAGCTT GTATGCATCT CCACAACTAG AAGGATTTTC
AGCTGAATCA AGAAAACTGC TTCTTATCGT TCAGGCTCTT AGGGACAACC
TGGAACCTGG GACCTTTGAT CTTGGGGGGC TATATGAAGC AATTGAGGAG
5 TGCCTGATTA ATGATCCCTG GGTTTTGCTT AATGCTTCTT GGTTCAACTC
CTTCCTTACA CATGCATTGA GTTAGTTGTG GCAGTGCTAC TATTTGCTAT
CCATACTGTC CAAAAAAGTA CCTTGTTTCT ACT
(SEQ ID NO:1)

10 **PB1**

AGCGAAAGCA GGCAAACCAT TTGAATGGAT GTCAATCCGA
CCTTACTTTT CTTAAAAGTG CCAGCACAAA ATGCTATAAG CACAACTTTC
CCTTATACTG GAGACCCTCC TTACAGCCAT GGGACAGGAA CAGGATACAC
15 CATGGATACT GTCAACAGGA CACATCAGTA CTCAGAAAAG GGAAGATGGA
CAACAAACAC CGAAACTGGA GCACCGCAAC TCAACCCGAT TGATGGGCCA
CTGCCAGAAG ACAATGAACC AAGTGGTTAT GCCCAAACAG ATTGTGTATT
GGAGGCGATG GCTTTCCTTG AGGAATCCCA TCCTGGTATT TTTGAAAACT
CGTGTATTGA AACGATGGAG GTTGTTCAGC AAACACGAGT AGACAAGCTG
20 ACACAAGGCC GACAGACCTA TGACTGGACT CTAAATAGAA ACCAACCTGC
TGCAACAGCA TTGGCCAACA CAATAGAAGT GTTCAGATCA AATGGCCTCA
CGGCCAATGA GTCTGGAAGG CTCATAGACT TCCTTAAGGA TGTAATGGAG
TCAATGAACA AAGAAGAAAT GGGGATCACA ACTCATTTTC AGAGAAAGAG
ACGGGTGAGA GACAATATGA CTAAGAAAAT GATAACACAG AGAACAATGG
25 GTAAAAAGAA GCAGAGATTG AACAAAAGGA GTTATCTAAT TAGAGCATTG
ACCCTGAACA CAATGACCAA AGATGCTGAG AGAGGGAAGC TAAAACGGAG
AGCAATTGCA ACCCCAGGGA TGCAAATAAG GGGGTTTGTA TACTTTGTTG
AGACACTGGC AAGGAGTATA TGTGAGAAAC TTGAACAATC AGGGTTGCCA
GTTGGAGGCA ATGAGAAGAA AGCAAAGTTG GCAAATGTTG TAAGGAAGAT
30 GATGACCAAT TCTCAGGACA CCGAACTTTC TTTCACCATC ACTGGAGATA
ACACCAAATG GAACGAAAAT CAGAATCCTC GGATGTTTTT GGCCATGATC
ACATATATGA CCAGAAATCA GCCCGAATGG TTCAGAAATG TTCTAAGTAT
TGCTCCAATA ATGTTCTCAA ACAAAATGGC GAGACTGGGA AAAGGGTATA
TGTTTGAGAG CAAGAGTATG AAACTTAGAA CTCAAATACC TGCAGAAATG
35 CTAGCAAGCA TCGATTTGAA ATATTTCAAT GATTCAACAA GAAAGAAGAT
TGAAAAAATC CGACCGCTCT TAATAGAGGG GACTGCATCA TTGAGCCCTG
GAATGATGAT GGGCATGTTC AATATGTTAA GCACTGTATT AGGCGTCTCC
ATCCTGAATC TTGGACAAAA GAGATACACC AAGACTACTT ACTGGTGGGA
TGGTCTTCAA TCCTCTGACG ATTTTGCTCT GATTGTGAAT GCACCCAATC
40 ATGAAGGGAT TCAAGCCGGA GTCGACAGGT TTTATCGAAC CTGTAAGCTA
CTTGGAATCA ATATGAGCAA GAAAAAGTCT TACATAAACA GAACAGGTAC
ATTTGAATTC ACAAGTTTTT TCTATCGTTA TGGGTTTGTT GCCAATTTCA
GCATGGAGCT TCCCAGTTTT GGGGTGTCTG GGATCAACGA GTCAGCGGAC
ATGAGTATTG GAGTTACTGT CATCAAAAAC AATATGATAA ACAATGATCT
45 TGGTCCAGCA ACAGCTCAAA TGGCCCTTCA GTTGTTCATC AAAGATTACA
GGTACACGTA CCGATGCCAT ATAGGTGACA CACAAATACA AACCCGAAGA
TCATTTGAAA TAAAGAAACT GTGGGAGCAA ACCCGTTCCA AAGCTGGACT
GCTGGTCTCC GACGGAGGCC CAAATTTATA CAACATTAGA AATCTCCACA
TTCCTGAAGT CTGCCTAAAA TGGGAATTGA TGGATGAGGA TTACCAGGGG
50 CGTTTATGCA ACCCACTGAA CCCATTTGTC AGCCATAAAG AAATTGAATC
AATGAACAAT GCAGTGATGA TGCCAGCACA TGGTCCAGCC AAAAACATGG
AGTATGATGC TGTTGCAACA ACACACTCCT GGATCCCCAA AAGAAATCGA
TCCATCTTGA ATACAAGTCA AAGAGGAGTA CTTGAGGATG AACAAATGTA
CCAAAGGTGC TGCAATTTAT TTGAAAAATT CTTCCCCAGC AGTTCATACA
55 GAAGACCAGT CGGGATATCC AGTATGGTGG AGGCTATGGT TTCCAGAGCC
CGAATTGATG CACGGATTGA TTTCGAATCT GGAAGGATAA AGAAAGAAGA
GTTCACTGAG ATCATGAAGA TCTGTTCCAC CATTGAAGAG CTCAGACGGC
AAAAATAGTG AATTTAGCTT GTCCTTCATG AAAAAATGCC TTGTTTCTAC
T

(SEQ ID NO:2)

PB2

AGCGAAAGCA GGTCAATTAT ATTCAATATG GAAAGAATAA AAGAACTACG
AAATCTAATG TCGCAGTCTC GCACCCGCGA GATACTCACA AAAACCACCG
TGGACCATAT GGCCATAATC AAGAAGTACA CATCAGGAAG ACAGGAGAAG
AACCCAGCAC TTAGGATGAA ATGGATGATG GCAATGAAAT ATCCAATTAC
AGCAGACAAG AGGATAACGG AAATGATTCC TGAGAGAAAT GAGCAAGGAC
AAACTTTATG GAGTAAAATG AATGATGCCG GATCAGACCG AGTGATGGTA
TCACCTCTGG CTGTGACATG GTGGAATAGG AATGGACCAA TAACAAATAC
AGTTCATTAT CCAAAAATCT ACAAAACTTA TTTTGAAAGA GTCGAAAGGC
TAAAGCATGG AACCTTTGGC CCTGTCCATT TTAGAAACCA AGTCAAAATA
CGTCGGAGAG TTGACATAAA TCCTGGTCAT GCAGATCTCA GTGCCAAGGA
GGCACAGGAT GTAATCATGG AAGTTGTTTT CCCTAACGAA GTGGGAGCCA
GGATACTAAC ATCGGAATCG CAACTAACGA TAACCAAAGA GAAGAAAGAA
GAACTCCAGG ATTGCAAAAT TTCTCCTTTG ATGGTTGCAT ACATGTTGGA
GAGAGAACTG GTCCGCAAAA CGAGATTCCT CCCAGTGGCT GGTGGAACAA
GCAGTGTGTA CATTGAAGTG TTGCATTTGA CTCAAGGAAC ATGCTGGGAA
CAGATGTATA CTCCAGGAGG GGAAGTGAGG AATGATGATG TTGATCAAAG
CTTGATTATT GCTGCTAGGA ACATAGTGAG AAGAGCTGCA GTATCAGCAG
ATCCACTAGC ATCTTTATTG GAGATGTGCC ACAGCACACA GATTGGTGGA
ATTAGGATGG TAGACATCCT TAGGCAGAAC CCAACAGAAG AGCAAGCCGT
GGATATATGC AAGGCTGCAA TGGGACTGAG AATTAGCTCA TCCTTCAGTT
TTGGTGGATT CACATTTAAG AGAACAAGCG GATCATCAGT CAAGAGAGAG
GAAGAGGTGC TTACGGGCAA TCTTCAAACA TTGAAGATAA GAGTGCATGA
GGGATATGAA GAGTTCACAA TGGTTGGGAG AAGAGCAACA GCCATACTCA
GAAAAGCAAC CAGGAGATTG ATTCAGCTGA TAGTGAGTGG GAGAGACGAA
CAGTCGATTG CCGAAGCAAT AATTGTGGCC ATGGTATTTT CACAAGAGGA
TTGTATGATA AAAGCAGTCA GAGGTGATCT GAATTTCGTC AATAGGGCGA
ATCAACGATT GAATCCTATG CATCAACTTT TAAGACATTT TCAGAAGGAT
GCGAAAGTGC TTTTTCAAAA TTGGGGAGTT GAACCTATCG ACAATGTGAT
GGGAATGATT GGGATATTGC CCGACATGAC TCCAAGCATC GAGATGTCAA
TGAGAGGAGT GAGAATCAGC AAAATGGGTG TAGATGAGTA CTCCAGCACG
GAGAGGGTAG TGGTGAGCAT TGACCGTTTT TTGAGAATCC GGGACCAACG
AGGAAATGTA CTACTGTCTC CCGAGGAGGT CAGTGAAACA CAGGGAACAG
AGAAACTGAC AATAACTTAC TCATCGTCAA TGATGTGGGA GATTAATGGT
CCTGAATCAG TGTTGGTCAA TACCTATCAA TGGATCATCA GAAACTGGGA
AACTGTTAAA ATTCAGTGGT CCCAGAACCC TACAATGCTA TACAATAAAA
TGGAATTTGA ACCATTTCAG TCTTTAGTAC CTAAGGCCAT TAGAGGCCAA
TACAGTGGGT TTGTAAGAAC TCTGTTCCAA CAAATGAGGG ATGTGCTTGG
GACATTTGAT ACCGCACAGA TAATAAAACT TCTTCCCTTC GCAGCCGCTC
CACCAAAGCA AAGTAGAATG CAGTTCTCCT CATTTACTGT GAATGTGAGG
GGATCAGGAA TGAGAATACT TGTAAGGGGC AATTCTCCTG TATTCAACTA
TAACAAGGCC ACGAAGAGAC TCACAGTTCT CGGAAAGGAT GCTGGCACTT
TAACTGAAGA CCCAGATGAA GGCACAGCTG GAGTGGAGTC CGCTGTTCTG
AGGGGATTCC TCATTCTGGG CAAAGAAGAC AAGAGATATG GGCCAGCACT
AAGCATCAAT GAACTGAGCA ACCTTGCGAA AGGAGAGAAG GCTAATGTGC
TAATTGGGCA AGGAGACGTG GTGTTGGTAA TGAAACGGAA ACGGGACTCT
AGCATACTTA CTGACAGCCA GACAGCGACC AAAAGAATTC GGATGGCCAT
CAATTAGTGT CGAATAGTTT AAAAACGACC TTGTTTCTAC T
(SEQ ID NO:3)

NP

AGCAAAAGCA GGGTAGATAA TCACTCACTG AGTGACATCA
AAATCATGGC GTCTCAAGGC ACCAAACGAT CTTACGAACA GATGGAGACT

GATGGAGAAC GCCAGAATGC CACTGAAATC AGAGCATCCG TCGGAAAAAT
GATTGGTGGA ATTGGACGAT TCTACATCCA AATGTGCACC GAACTCAAAC
TCAGTGATTA TGAGGGACGG TTGATCCAAA ACAGCTTAAC AATAGAGAGA
ATGGTGCTCT CTGCTTTTGA CGAAAGGAGA AATAAATACC TTGAAGAACA
5 TCCCAGTGCG GGGAAAGATC CTAAGAAAAC TGGAGGACCT ATATACAGGA
GAGTAAACGG AAAGTGGATG AGAGAACTCA TCCTTTATGA CAAAGAAGAA
ATAAGGCGAA TCTGGCGCCA AGCTAATAAT GGTGACGATG CAACGGCTGG
TCTGACTCAC ATGATGATCT GGCATTCCAA TTTGAATGAT GCAACTTATC
AGAGGACAAG AGCTCTTGTT CGCACCGGAA TGGATCCCAG GATGTGCTCT
10 CTGATGCAAG GTTCAACTCT CCCTAGGAGG TCTGGAGCCG CAGGTGCTGC
AGTCAAAGGA GTTGGAACAA TGGTGATGGA ATTGGTCAGA ATGATCAAAC
GTGGGATCAA TGATCGGAAC TTCTGGAGGG GTGAGAATGG ACGAAAAACA
AGAATTGCTT ATGAAAGAAT GTGCAACATT CTCAAAGGGA AATTTCAAAC
TGCTGCACAA AAAGCAATGA TGGATCAAGT GAGAGAGAGC CGGAACCCAG
15 GGAATGCTGA GTTCGAAGAT CTCACTTTTC TAGCACGGTC TGCACTCATA
TTGAGAGGGT CGGTTGCTCA CAAGTCCTGC CTGCCTGCCT GTGTGTATGG
ACCTGCCGTA GCCAGTGGGT ACGACTTTGA AAGGGAGGGA TACTCTCTAG
TCGGAATAGA CCCTTTCAGA CTGCTTCAAA ACAGCCAAGT GTACAGCCTA
ATCAGACCAA ATGAGAATCC AGCACACAAG AGTCAACTGG TGTGGATGGC
20 ATGCCATTCT GCCGCATTTG AAGATCTAAG AGTATTAAGC TTCATCAAAG
GGACGAAGGT GCTCCCAAGA GGGAAGCTTT CCACTAGAGG AGTTCAAATT
GCTTCCAATG AAAATATGGA GACTATGGAA TCAAGTACAC TTGAACTGAG
AAGCAGGTAC TGGGCCATAA GGACCAGAAG TGGAGGAAAC ACCAATCAAC
AGAGGGCATC TGCGGGCCAA ATCAGCATAC AACCTACGTT CTCAGTACAG
25 AGAAATCTCC CTTTTGACAG AACAACCATT ATGGCAGCAT TCAATGGGAA
TACAGAGGGG AGAACATCTG ACATGAGGAC CGAAATCATA AGGATGATGG
AAAGTGCAAG ACCAGAAGAT GTGTCTTTCC AGGGGCGGGG AGTCTTCGAG
CTCTCGGACG AAAAGGCAGC GAGCCCGATC GTGCCTTCCT TTGACATGAG
TAATGAAGGA TCTTATTTCT TCGGAGACAA TGCAGAGGAG TACGACAATT
30 AAAGAAAAAT ACCCTTGTTT CTACT
(SEQ ID NO:4)

M

35 AGCAAAAGCA GGTAGATATT GAAAGATGAG TCTTCTAACC GAGGTCGAAA
CGTACGTACT CTCTATCATC CCGTCAGGCC CCCTCAAAGC CGAGATCGCA
CAGAGACTTG AAGATGTCTT TGCAGGGAAG AACACCGATC TTGAGGTTCT
CATGGAATGG CTAAAGACAA GACCAATCCT GTCACCTCTG ACTAAGGGGA
TTTTAGGATT TGTGTTCACG CTCACCGTGC CCAGTGAGCG AGGACTGCAG
40 CGTAGACGCT TTGTCCAAAA TGCCCTTAAT GGGAACGGGG ATCCAAATAA
CATGGACAAA GCAGTTAAAC TGTATAGGAA GCTCAAGAGG GAGATAACAT
TCCATGGGGC CAAAGAAATC TCACTCAGTT ATTCTGCTGG TGCACTTGCC
AGTTGTATGG GCCTCATATA CAACAGGATG GGGGCTGTGA CCACTGAAGT
GGCATTTGGC CTGGTATGTG CAACCTGTGA ACAGATTGCT GACTCCCAGC
45 ATCGGTCTCA TAGGCAAATG GTGACAACAA CCAATCCACT AATCAGACAT
GAGAACAGAA TGGTTTTAGC CAGCACTACA GCTAAGGCTA TGGAGCAAAT
GGCTGGATCG AGTGAGCAAG CAGCAGAGGC CATGGAGGTT GCTAGTCAGG
CTAGACAAAT GGTGCAAGCG ATGAGAACCA TTGGGACTCA TCCTAGCTCC
AGTGCTGGTC TGAAAAATGA TCTTCTTGAA AATTTGCAGG CCTATCAGAA
50 ACGAATGGGG GTGCAGATGC AACGGTTCAA GTGATCCTCT CACTATTGCC
GCAAATATCA TTGGGATCTT GCACTTGACA TTGTGGATTC TTGATCGTCT
TTTTTTCAAA TGCATTTACC GTCGCTTTAA ATACGGACTG AAAGGAGGGC
CTTCTACGGA AGGAGTGCCA AAGTCTATGA GGGAAGAATA TCGAAAGGAA
CAGCAGAGTG CTGTGGATGC TGACGATGGT CATTTTGTCA GCATAGAGCT
55 GGAGTAAAAA ACTACCTTGT TTCTACT
(SEQ ID NO:5)

NS

AGCAAAAGCA GGGTGACAAA AACATAATGG ATCCAAACAC TGTGTCAAGC
TTTCAGGTAG ATTGCTTTCT TTGGCATGTC CGCAAACGAG TTGCAGACCA
AGAACTAGGC GATGCCCCAT TCCTTGATCG GCTTCGCCGA GATCAGAAAT
CCCTAAGAGG AAGGGGCAGT ACTCTCGGTC TGGACATCAA GACAGCCACA
5 CGTGCTGGAA AGCAGATAGT GGAGCGGATT CTGAAAGAAG AATCCGATGA
GGCACTTAAA ATGACCATGG CCTCTGTACC TGCGTCGCGT TACCTAACTG
ACATGACTCT TGAGGAAATG TCAAGGGACT GGTCCATGCT CATACCCAAG
CAGAAAGTGG CAGGCCCTCT TTGTATCAGA ATGGACCAGG CGATCATGGA
TAAGAACATC ATACTGAAAG CGAACTTCAG TGTGATTTTT GACCGGCTGG
10 AGACTCTAAT ATTGCTAAGG GCTTTCACCG AAGAGGGAGC AATTGTTGGC
GAAATTTCAC CATTGCCTTC TCTTCCAGGA CATACTGCTG AGGATGTCAA
AAATGCAGTT GGAGTCCTCA TCGGAGGACT TGAATGGAAT GATAACACAG
TTCGAGTCTC TGAAACTCTA CAGAGATTCG CTTGGAGAAG CAGTAATGAG
AATGGGAGAC CTCCACTCAC TCCAAAACAG AAACGAGAAA TGGCGGGAAC
15 AATTAGGTCA GAAGTTTGAA GAAATAAGAT GGTTGATTGA AGAAGTGAGA
CACAAACTGA AGATAACAGA GAATAGTTTT GAGCAAATAA CATTTATGCA
AGCCTTACAT CTATTGCTTG AAGTGGAGCA AGAGATAAGA ACTTTCTCGT
TTCAGCTTAT TTAGTACTAA AAAACACCCT TGTTTCTACT
(SEQ ID NO:6)
20

HA

AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGAAGGCAAACCTACTGGTCCTGTTATGTGCACTTGC
AGCTGCAGAT
25 GCAGACACAATATGTATAGGCTACCATGCGAACAATTCAACCGACACTGTTGACACAGTACTCGAGAAGA
ATGTGACAGT
GACACACTCTGTTAACCTGCTCGAAGACAGCCACAACGGAAAACTATGTAGATTAAAAGGAATAGCCCCA
CTACAATTGG
GGAAATGTAACATCGCCGGATGGCTCTTGGGAAACCCAGAATGCGACCCACTGCTTCCAGTGAGATCAT
30 GGTCCTACATT
GTAGAAACACCAAACTCTGAGAATGGAATATGTTATCCAGGAGATTTCATCGACTATGAGGAGCTGAGGG
AGCAATTGAG
CTCAGTGTCATCATTCGAAAGATTCGAAATATTTCCCAAAGAAAGCTCATGGCCCAACCACAACACAAAC
GGAGTAACGG
35 CAGCATGCTCCCATGAGGGGAAAAGCAGTTTTTACAGAAATTTGCTATGGCTGACGGAGAAGGAGGGCT
CATACCCAAAG
CTGAAAAATTCTTATGTGAACAAAAAAGGGAAAGAAGTCCTTGTACTGTGGGGTATTCATCACCCGCCTA
ACAGTAAGGA
ACAACAGAATCTCTATCAGAATGAAAATGCTTATGTCTCTGTAGTGACTTCAAATTATAACAGGAGATTTA
40 CCCCGGAAA
TAGCAGAAAGACCCAAAGTAAGAGATCAAGCTGGGAGGATGAACTATTACTGGACCTTGCTAAAACCCG
GAGACACAATA
ATATTTGAGGCAAATGGAAATCTAATAGCACCAATGTATGCTTTCGCACTGAGTAGAGGCTTTGGGTCCG
GCATCATCAC
45 CTCAAACGCATCAATGCATGAGTGTAACACGAAGTGTCAAACACCCCTGGGAGCTATAAACAGCAGTCTC
CCTTACCAGA
ATATACACCCAGTCACAATAGGAGAGTGCCCAAAATACGTCAGGAGTGCCAAATTGAGGATGGTTACAG
GACTAAGGAAC
ATTCCGTCCATTCAATCCAGAGGTCTATTTGGAGCCATTGCCGGTTTTATTGAAGGGGGATGGACTGGAA
50 TGATAGATGG
ATGGTATGGTTATCATCATCAGAATGAACAGGGATCAGGCTATGCAGCGGATCAAAAAAGCACACAAAAT
GCCATTAACG
GGATTACAAACAAGGTGAACACTGTTATCGAGAAAATGAACATTCAATTCACAGCTGTGGGTAAAGAATT
CAACAAATTA
55 GAAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGGACATTTGGACATATAATGCAGA
ATTGTTAGT
TCTACTGGAAAATGAAAGGACTCTGGATTTCCATGACTCAAATGTGAAGAATCTGTATGAGAAAGTAAAAA
GCCAATTAA
AGAATAATGCCAAAGAAATCGGAAATGGATGTTTTGAGTTCTACCACAAGTGTGACAATGAATGCATGGA

AAGTGTAAGA
AATGGGACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACAGGGAAAAGGTAGATGGAGTGA
AATTGGAATC
AATGGGGATCTATCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTTTGGTCTCCCTG
GGGGCAATCA
GTTTCTGGATGTGTTCTAATGGATCTTTGCAGTGCAGAATATGCATCTGAGATTAGAATTTCAGAGATATG
AGGAAAAAC
ACCCTTGTTTCTACT (SEQ ID NO:7)

NA

AGCAAAAGCAGGGGTTTAAAATGAATCCAAATCAGAAAATAATAACCATTGGATCAATCTGTCTGGTAGTC
GGACTAATT
AGCCTAATATTGCAAATAGGGAATATAATCTCAATATGGATTAGCCATTCAATTCAAACTGGAAGTCAAAA
CCATACTGG
AATATGCAACCAAAACATCATTACCTATAAAAATAGCACCTGGGTAAAGGACACAACTTCAGTGATATTAA
CCGGCAATT
CATCTCTTTGTCCCATCCGTGGGTGGGCTATATACAGCAAAGACAATAGCATAAGAATTGGTTCCAAAGG
AGACGTTTTT
GTCATAAGAGAGCCCTTTATTTCATGTTCTCACTTGGAATGCAGGACCTTTTTTCTGACCCAAGGTGCCTT
ACTGAATGA
CAAGCATTCAAGTGGGACTGTTAAGGACAGAAGCCCTTATAGGGCCTTAATGAGCTGCCCTGTCGGTGA
AGCTCCGTCCC
CGTACAATTCAAGATTTGAATCGGTTGCTTGGTCAGCAAGTGCATGTCATGATGGCATGGGCTGGCTAAC
AATCGGAATT
TCAGGTCCAGATAATGGAGCAGTGGCTGTATTAAAATACAACGGCATAATAACTGAAACCATAAAAAGTT
GGAGGAAGAA
AATATTGAGGACACAAGAGTCTGAATGTGCCTGTGTAAATGGTTCATGTTTTACTATAATGACTGATGGCC
CGAGTGATG
GGCTGGCCTCGTACAAAATTTTCAAGATCGAAAAGGGGAAGGTTACTAAATCAATAGAGTTGAATGCACC
TAATTCTCAC
TATGAGGAATGTTCCTGTTACCCTGATACCGGCAAAGTGATGTGTGTGTGCAGAGACAATTGGCATGGTT
CGAACCGGCC
ATGGGTGTCTTTCGATCAAAACCTGGATTATCAAATAGGATACATCTGCAGTGGGGTTTTCGGTGACAAC
CCGCGTCCCG
AAGATGGAACAGGCAGCTGTGGTCCAGTGTATGTTGATGGAGCAAACGGAGTAAAGGGATTTTCATATA
GGTATGGTAAT
GGTGTTTGGATAGGAAGGACCAAAAGTCACAGTTCCAGACATGGGTTTGAGATGATTTGGGATCCTAATG
GATGGACAGA
GACTGATAGTAAGTTCTCTGTGAGGCAAGATGTTGTGGCAATGACTGATTGGTCAGGGTATAGCGGAAG
TTTCGTTCAAC
ATCCTGAGCTGACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTTGAATTAATCAGGGGACGACCTA
AAGAAAAAACA
ATCTGGACTAGTGCGAGCAGCATTTCTTTTTGTGGCGTGAATAGTGATACTGTAGATTGGTCTTGGCCAG
ACGGTGCTGA
GTTGCCATTCAGCATTGACAAGTAGTCTGTTCAAAAAACTCCTTGTTTCTACT (SEQ ID NO:8)

High-titer A/PR/8/34 (H1N1, PR8(UW)) virus grows 10 times better than other A/PR/8/34 PR8 strains in eggs (10^{10} EID_{50}/mL; HA titer:1:8,000). Thus, replacement of the HA and NA genes of PR8(UW) with those of a currently circulating strain of influenza virus results in a vaccine strain that can be safely produced, and validates the use of PR8(UW) as a master vaccine strain.

Genes that contribute to different growth properties between PR8(UW) and PR8 (Cambridge), which provides the non-HA and -NA genes of the NIBRG-14 vaccine strain (Figure 1), were determined. Higher titers in eggs were obtained when the majority of internal genes were from PR8(UW). Highest titers were with

the M gene segment of PR8(UW) and the NS gene of PR8 (Cambridge). The NS gene in PR8(UW) has a K (lysine) at residue 55 while the NS gene in PR8(Cam) has a E (glutamic acid). The polymerase subunit (PA, PB1, and PB2) and NP genes of PR8(UW) enhanced the growth of an H5N1 vaccine seed virus in chicken embryonated eggs, and the NS gene of PR8(Cambridge) enhanced the growth of an H5N1 vaccine seed virus in chicken embryonated eggs. A tyrosine (Y) at position 360 in PB2 of PR8(UW) likely contributes to the high growth rate of that virus in MDCK cells.

Example 2

To establish robust systems for influenza vaccine production, egg-free, cell culture-based systems are needed. Vero cells are approved for human use and so are candidate hosts for influenza virus vaccine production. To elucidate the molecular basis for efficient growth of influenza vaccine seed virus in Vero cells, A/Puerto Rico/8/34 (PR8) virus was passaged through Vero cells 12 times and the infectivity titer of the resulting virus was determined. Vero cell-adapted PR8 had over a 4 log increase in infectivity titers relative to non Vero cell-adapted PR8 (Figure 2).

To determine the molecular basis for that growth difference, the genomes of both isolates were sequenced. Three amino acid differences were found: one in HA2, one in NA and one in PB2 (Figure 3). To identify the contribution of each individual substitution, and of a combination of two of the substitutions, recombinant viruses with the individual substitution(s) were prepared and the growth of those recombinant viruses was compared to Vero cell-adapted PR8 and non Vero cell-adapted PR8 (Figure 4). The results indicated that the substitution in HA2 was primarily responsible for the enhanced growth in Vero cells. The substitution in HA2 (N117D) did not enhance growth in MDCK cells (Figure 5).

Because HA2 has a fusion domain that is exposed after infection, a fusion assay was employed to compare the properties of wild-type PR8 HA2 and HA2 N117D (Figures 7-8). The HA2 N117D mutant fused Vero cells at a higher pH than wild-type PR8. The endosomal pH in Vero cells and MDCK cells was determined using pH sensitive and insensitive dyes (Figures 9-10). The endosomes of Vero cells likely have a higher pH than those from MDCK cells. Thus, the HA2 N117D mutation may elevate the optimal pH for membrane fusion mediated by HA2, thereby enhancing virus replication efficiency in Vero cells.

To determine if the HA2 N117D mutation alone could enhance virus replication efficiency in different viruses in Vero cells, that substitution was introduced into two different H1N1 viruses (a AAT to GAT mutation) and one H3N2 virus (a AAC to GAC mutation) in a PR8 background (six gene segments were from Vero cell-adapted PR8; PA, PB1, PB2, M, NS and NP) (Figure 11). The HA2 N117D mutation enhanced the replication efficiency of all three tested viruses in Vero cells. Such a strategy may be employed to prepare vaccine viruses with enhanced replication in Vero cells.

References

Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, Ltd., Williams and Wilkins, Baltimore, MD (1987).

Aymard-Henry et al., Virology: A Practical Approach, Oxford IRL Press, Oxford, 119-150 (1985).

Bachmeyer, Intervirology, 5:260 (1975).

Berkow et al., eds., The Merck Manual, 16th edition, Merck & Co., Rahway, NJ (1992).
Hatta et al., Science, 293:1840 (2001).
Horimoto et al., J. Virol., 68:3120 (1994).
Horimoto et al., Vaccine, 24:3669 (2006).
5 Keitel et al., in Textbook of Influenza, eds. Nickolson, K. G., Webster, R. G., and Hay, A. (Blackwell, Oxford), pp. 373-390 (1998).
Laver & Webster, Virology, 69:511 (1976).
Neumann et al., Adv. Virus Res., 53:265 (1999).
Neumann et al., J. Gen. Virol., 83:2635 (2002).
10 Neumann et al., J. Virol., 71:9690 (1997).
Neumann et al., Proc. Natl. Acad. Sci. USA, 96:9345 (1999).
Neumann et al., Virology, 287:243 (2001).
Osol (ed.), Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA 1324-1341 (1980).
15 Sugawara et al., Biologicals, 30:303 (2002).
Webby & Webster et al., Science, 302:1519 (2003).
Wood & Robertson, Nat. Rev. Microbiol., 2:842 (2004).
World Health Organization TSR No. 673 (1982).
World Health Organization. Confirmed human cases of avian influenza A (H5N1).
20 http://www.who.int/csr/disease/avian_influenza/country/en/index.html

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art
25 that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. An isolated recombinant reassortant influenza virus having PA, PB1, PB2, NP, NS, and M gene segments from a first influenza vaccine virus isolate, a heterologous influenza virus NA gene segment, and an influenza virus HA gene segment selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2.

2. The isolated virus of claim 1 wherein the NA gene segment and the HA gene segment are from the same influenza virus isolate.

3. The isolated virus of claim 1 or 2 wherein the HA gene segment is mutated to encode the aspartic acid or glutamic acid at position 117.

4. The isolated virus of any one of claims 1 to 3 wherein the PA, PB1, PB2, NP, NS, and M gene segments are from the same influenza virus isolate.

5. The isolated virus of any one of claims 1 to 4 wherein the PA, PB1, PB2, NP, NS, and M gene segments comprise sequences for at least one of the following: a PB1 having the amino acid sequence encoded by SEQ ID NO:2 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:2; a PB2 having the amino acid sequence encoded by SEQ ID NO:3 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO:3; a PA having the amino acid sequence encoded by SEQ ID NO:1 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO:1; a NP having the amino acid sequence encoded by SEQ ID NO:4 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:4; a M having the amino acid sequence encoded by SEQ ID NO:5 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO:5; or a NS having the amino acid sequence encoded by SEQ ID NO:6 or NS with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:6.

6. The isolated virus of any one of claims 1 to 4 wherein the PA, PB1, PB2, NP, NS, and M gene segments comprise sequences for at least one of the following: a PB1 having the amino acid sequence encoded by SEQ ID NO:10 or PB1 with at least 95% amino acid sequence identity to the PB1 encoded by SEQ ID NO:10; a PB2 having the amino acid sequence encoded by SEQ ID NO:11 or PB2 with at least 95% amino acid sequence identity to the PB2 encoded by SEQ ID NO:11; a PA having the amino acid sequence encoded by SEQ ID NO:12 or PA with at least 95% amino acid sequence identity to the PA encoded by SEQ ID NO:12; a NP having the amino acid sequence encoded by SEQ ID NO:13 or NP with at least 95% amino acid sequence identity to the NP encoded by SEQ ID NO:13; a M having the amino acid sequence encoded by SEQ ID NO:14 or M with at least 95% amino acid sequence identity to the M encoded by SEQ ID NO:14; or a NS having the amino acid sequence encoded by SEQ ID NO:15 or NS with with at least 95% amino acid sequence identity to the NS encoded by SEQ ID NO:15.

7. A vaccine having the isolated recombinant virus of any one of claims 1 to 6.

8. A plurality of influenza virus vectors for preparing a reassortant, comprising

5 a) a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza 10 virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production 15 comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production of NA has sequences for a heterologous NA, and wherein the HA DNA in the vector for vRNA production of HA is selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 20 residues is that for H1 HA2; and

b) a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably 25 linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza 30 virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2.

9. The vectors of claim 8 wherein the vaccine virus isolate is selected as one that replicates to high titers in embryonated eggs, MDCK cells, Vero cells, or a combination thereof.

35

10. The vectors of claim 8 or 9 wherein the vaccine virus isolate is selected as one that replicates to high titers in mammalian cells.

11. The vectors of claim 10 wherein the mammalian cells are human, Vero or CHO cells.

12. The vectors of any one of claims 8 to 11 wherein the wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production have a sequence corresponding to one that encodes a polypeptide having at least 95% amino acid sequence identity to a corresponding polypeptide encoded by SEQ ID NOs:1-
5 6 or 10-15.

13. The vectors of any one of claims 8 to 11 wherein the promoter for vRNA vectors is a RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T3 promoter or a T7 promoter.

10 14. The vectors of any one of claims 8 to 13 wherein the NA is N1.

15. A method to prepare influenza virus, comprising: contacting a cell with:

a vector for vRNA production comprising a promoter operably linked to an influenza virus PA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably
15 linked to an influenza virus PB1 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus PB2 DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus HA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus NP DNA linked to a transcription termination sequence, a
20 vector for vRNA production comprising a promoter operably linked to an influenza virus NA DNA linked to a transcription termination sequence, a vector for vRNA production comprising a promoter operably linked to an influenza virus M DNA linked to a transcription termination sequence, and a vector for vRNA production comprising a promoter operably linked to an influenza virus NS DNA linked to a transcription termination sequence, wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA production are from one
25 or more influenza vaccine virus isolates, wherein the NA DNA in the vector for vRNA production of NA has sequences for a heterologous NA, and wherein the HA DNA in the vector for vRNA production of HA is selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2; and

a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding
30 influenza virus PA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NP, and optionally a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector for mRNA
35 production comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector for mRNA production comprising a promoter operably linked to a DNA segment encoding influenza virus NS2;

in an amount effective to yield infectious influenza virus.

16. The method of claim 15 wherein the cell is an avian cell.

5 17. The method of claim 15 wherein the cell is a mammalian cell.

18. The method of claim 17 wherein the cell is a Vero cell, a human cell or a hamster cell.

19. The method of any one of claims 15 to 18 wherein the influenza vaccine virus isolate is selected as
10 one that replicates to high titers in embryonated eggs, human cells, CHO cells, MDCK cells, Vero cells, or a combination thereof.

20. The method of any one of claims 15 to 19 wherein the wherein the PB1, PB2, PA, NP, NS, and M DNAs in the vectors for vRNA productions have a sequence that corresponds to one that encodes a
15 polypeptide having at least 95% amino acid sequence identity to a corresponding polypeptide encoded by SEQ ID NOs:1-6 or 10-15.

21. The method of any one of claims 15 to 20 further comprising isolating the virus.

20 22. Virus obtained by the method of claim 21.

23. An isolated recombinant reassortant influenza virus comprising a HA gene segment selected to encode an aspartic acid or glutamic acid at position 117 in HA2, wherein recombinant virus has enhanced replication in Vero cells relative to a corresponding virus that does not have aspartic acid or glutamic acid at
25 position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2.

24. The isolated recombinant virus of claim 23 wherein the NA gene segment has a tyrosine at position 255, wherein the numbering for NA residues is that for N1.

30 25. The isolated recombinant virus of claim 23 or 24 wherein the corresponding virus has an alanine, asparagine, arginine or lysine at position 117 in HA2.

26. A method to prepare a recombinant influenza virus with a HA gene segment having a mutant HA2, comprising: altering influenza virus HA nucleic acid at position 117 in HA2 to aspartic acid or glutamic acid;
35 and expressing the altered nucleic acid in a cell having vectors for influenza vRNA production and viral protein production in an amount effective to yield recombinant influenza virus with a HA gene segment having the aspartic acid or glutamic acid at position 117 in HA2, wherein the numbering for HA2 residues is that for H1 HA2.

27. The method of claim 26 wherein the cell is a mammalian or avian cell.

28. The method of claim 26 wherein the cell is a human cell.

PR8(Cambridge)

PB2

AGCGAAAGCAGGTCAATTATATTCAATATGGAAAGAATAAAAGAACTAAGAAATCTAATGTCGCAGTCTCGCACCCGCGAGATA
CTCACAAAAACCACCGTGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCACTTAGGATG
AAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATAACGGAAATGATTCCTGAGAGAAATGAGCAAGGACAA
ACTTTATGGAGTAAAATGAATGATGCCGGATCAGACCGAGTGATGGTATCACCTCTGGCTGTGACATGGTGGAATAGGAATGGA
CCAATGACAAATACAGTTCATTATCCAAAAATCTACAAAACTTATTTTGAAAGAGTCGAAAGGCTAAAGCATGGAACCTTTGGC
CCTGTCCATTTTAGAAACCAAGTCAAAATACGTCGGAGAGTTGACATAAATCCTGGTCATGCAGATCTCAGTGCCAAGGAGGCA
CAGGATGTAATCATGGAAGTTGTTTTCCCTAACGAAGTGGGAGCCAGGATACTAACATCGGAATCGCAACTAACGATAACCAAA
GAGAAGAAAGAAGAACTCCAGGATTGCAAAATTTCTCCTTTGATGGTTGCATACATGTTGGAGAGAGAACTGGTCCGCAAAACG
AGATTCCTCCCAGTGGCTGGTGGAACAAGCAGTGTGTACATTGAAGTGTTGCATTTGACTCAAGGAACATGCTGGGAACAGATG
TATACTCCAGGAGGGGAAGTGAAGAATGATGATGTTGATCAAAGCTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCA
GTATCAGCAGACCCACTAGCATCTTTATTGGAGATGTGCCACAGCACACAGATTGGTGGAATTAGGATGGTAGACATCCTTAAG
CAGAACCCAACAGAAGAGCAAGCCGTGGATATATGCAAGGCTGCAATGGGACTGAGAATTAGCTCATCCTTCAGTTTTGGTGGA
TTCACATTTAAGAGAACAAGCGGATCATCAGTCAAGAGAGAGGAAGAGGTGCTTACGGGCAATCTTCAAACATTGAAGATAAGA
GTGCATGAGGGATCTGAAGAGTTCACAATGGTTGGGAGAAGAGCAACAGCCATACTCAGAAAAGCAACCAGGAGATTGATTCAG
CTGATAGTGAGTGGGAGAGACGAACAGTCGATTGCCGAAGCAATAATTGTGGCCATGGTATTTTCACAAGAGGATTGTATGATA
AAAGCAGTTAGAGGTGATCTGAATTTCGTCAATAGGGCGAATCAGCGACTGAATCCTATGCATCAACTTTTAAGACATTTTCAG
AAGGATGCGAAAGTGCTTTTTCAAAATTGGGGAGTTGAACCTATCGACAATGTGATGGGAATGATTGGGATATTGCCCGACATG
ACTCCAAGCATCGAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTAGATGAGTACTCCAGCACGGAGAGGGTAGTG
GTGAGCATTGACCGGTTCTTGAGAGTCAGGGACCAACGAGGAAATGTACTACTGTCTCCCGAGGAGGTCAGTGAAACACAGGGA
ACAGAGAAACTGACAATAACTTACTCATCGTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGTTGGTCAATACCTATCAA
TGGATCATCAGAAACTGGGAAACTGTTAAAATTCAGTGGTCCCAGAACCCTACAATGCTATACAATAAAATGGAATTTGAACCA
TTTCAGTCTTTAGTACCTAAGGCCATTAGAGGCCAATACAGTGGGTTTGTAAGAACTCTGTTCCAACAAATGAGGGATGTGCTT
GGGACATTTGATACCGCACAGATAATAAAACTTCTTCCCTTCGCAGCCGCTCCACCAAAGCAAAGTAGAATGCAGTTCTCCTCA
TTTACTGTGAATGTGAGGGGATCAGGAATGAGAATACTTGTAAGGGGCAATTCTCCTGTATTCAACTACAACAAGGCCACGAAG
AGACTCACAGTTCTCGGAAAGGATGCTGGCACTTTAACCGAAGACCCAGATGAAGGCACAGCTGGAGTGGAGTCCGCTGTTCTG
AGGGGATTCCTCATTCTGGGCAAAGAAGACAGGAGATATGGGCCAGCATTAAGCATCAATGAACTGAGCAACCTTGCGAAAGGA
GAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTTGGTAATGAAACGAAAACGGGACTCTAGCATACTTACTGACAGC
CAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCGAATAGTTTAAAAACGACCTTGTTTCTACT

SEQ ID NO:11

PB1

AGCGAAAGCAGGCAAACCATTTGAATGGATGTCAATCCGACCTTACTTTTCTTAAAAGTGCCAGCACAAAATGCTATAAGCACA
ACTTTCCCTTATACCGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGACACATCAG
TACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCAACCCGATTGATGGGCCACTGCCAGAAGAC
AATGAACCAAGTGGTTATGCCCAAACAGATTGTGTATTGGAAGCAATGGCTTTCCTTGAGGAATCCCATCCTGGTATTTTTGAA
AACTCGTGTATTGAAACGATGGAGGTTGTTCAGCAAACACGAGTAGACAAGCTGACACAAGGCCGACAGACCTATGACTGGACT
TTAAATAGAAACCAGCCTGCTGCAACAGCATTGGCCAACACAATAGAAGTGTTCAGATCAAATGGCCTCACGGCCAATGAGTCA
GGAAGGCTCATAGACTTCCTTAAGGATGTAATGGAGTCAATGAAAAAAGAAGAAATGGGGATCACAACTCATTTTCAGAGAAAG
AGACGGGTGAGAGACAATATGACTAAGAAAATGATAACACAGAGAACAATAGGTAAAAGGAAACAGAGATTGAACAAAAGGGGT
TATCTAATTAGAGCATTGACCCTGAACACAATGACCAAAGATGCTGAGAGAGGGAAGCTAAAACGGAGAGCAATTGCAACCCCA
GGGATGCAAATAAGGGGGTTTGTATACTTTGTTGAGACACTGGCAAGGAGTATATGTGAGAAACTTGAACAATCAGGGTTGCCA
GTTGGAGGCAATGAGAAGAAAGCAAAGTTGGCAAATGTTGTAAGGAAGATGATGACCAATTCTCAGGACACCGAACTTTCTTTC
ACCATCACTGGAGATAACACCAAATGGAACGAAAATCAGAATCCTCGGATGTTTTTGGCCATGATCACATATATGACCAGAAAT
CAGCCCGAATGGTTCAGAAATGTTCTAAGTATTGCTCCAATAATGTTCTCAAACAAAATGGCGAGACTGGGAAAAGGGTATATG
TTTGAGAGCAAGAGTATGAAACTTAGAACTCAAATACCTGCAGAAATGCTAGCAAGCATTGATTTGAAATATTTCAATGATTCA
ACAAGAAAGAAGATTGAAAAAATCCGACCGCTCTTAATAGAGGGGACTGCATCATTGAGCCCTGGAATGATGATGGGCATGTTC
AATATGTTAAGCACTGTATTAGGCGTCTCCATCCTGAATCTTGGACAAAAGAGATACACCAAGACTACTTACTGGTGGGATGGT
CTTCAATCCTCTGACGATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGTTTTATCGA
ACCTGTAAGCTACTTGGAATCAATATGAGCAAGAAAAAGTCTTACATAAACAGAACAGGTACATTTGAATTCACAAGTTTTTTC
TATCGTTATGGGTTTGTTGCCAATTTCAGCATGGAGCTTCCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGT
ATTGGAGTTACTGTCATCAAAAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAATGGCCCTTCAGTTGTTCATC
AAAGATTACAGGTACACGTACCGATGCCATAGAGGTGACACACAAATACAAACCCGAAGATCATTTGAAATAAAGAAACTGTGG
GAGCAAACCCGTTCCAAAGCTGGACTGCTGGTCTCCGACGGAGGCCCAAATTTATACAACATTAGAAATCTCCACATTCCTGAA
GTCTGCCTAAAATGGGAATTGATGGATGAGGATTACCAGGGGCGTTTATGCAACCCACTGAACCCATTTGTCAGCCATAAAGAA
ATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACATGGAGTATGATGCTGTTGCAACAACACAC
TCCTGGATCCCCAAAAGAAATCGATCCATCTTGAATACAAGTCAAAGAGGAGTACTTGAAGATGAACAAATGTACCAAAGGTGC
TGCAATTTATTTGAAAAATTCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCCAGTATGGTGGAGGCTATGGTTTCC
AGAGCCCGAATTGATGCACGGATTGATTTCGAATCTGGAAGGATAAAGAAAGAAGAGTTCACTGAGATCATGAAGATCTGTTCC
ACCATTGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCCTTCATGAAAAAATGCCTTGTTTCTACT

SEQ ID NO:10

Fig. 1A

PR8(Cambridge)

PA

AGCGAAAGCAGGTACTGATTCAAAATGGAAGATTTTGTGCGACAATGCTTCAATCCGATGATTGTCGAGCTTGCGGAAAAAACA
ATGAAAGAGTATGGGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCTTCATGTAT
TCAGATTTCCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTGATCCTAATGCACTTTTGAAGCACAGATTT
GAAATAATCGAGGGAAGAGATCGCACAATGGCCTGGACAGTAGTAAACAGTATTTGCAACACTACAGGGGCTGAGAAACCAAAG
TTTCTACCAGATTTGTATGATTACAAGGAAAATAGATTCATCGAAATTGGAGTAACAAGGAGAGAAGTTCACATATACTATCTG
GAAAAGGCCAATAAAATTAAATCTGAGAAAACACACATCCACATTTTCTCGTTCACTGGGGAAGAAATGGCCACAAGGGCCGAC
TACACTCTCGATGAAGAAAGCAGGGCTAGGATCAAAACCAGGCTATTCACCATAAGACAAGAAATGGCCAGCAGAGGCCTCTGG
GATTCCTTTCGTCAGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTGAAATCACAGGAACAATGCGCAAGCTTGCCGAC
CAAAGTCTCCCGCCGAACTTCTCCAGCCTTGAAAATTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTACATTGAGGGC
AAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTAGAATTGAACCTTTTTTGAAAACAACACCACGACCACTTAGACTTCCGAAT
GGGCCTCCCTGTTCTCAGCGGTCCAAATTCCTGCTGATGGATGCCTTAAAATTAAGCATTGAGGACCCAAGTCATGAAGGAGAG
GGAATACCGCTATATGATGCAATCAAATGCATGAGAACATTCTTTGGATGGAAGGAACCCAATGTTGTTAAACCACACGAAAAG
GGAATAAATCCAAATTATCTTCTGTCATGGAAGCAAGTACTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCCAAAG
ACTAAAAATATGAAAAAAACAAGTCAGCTAAAGTGGGCACTTGGTGAGAACATGGCACCAGAAAAGGTAGACTTTGACGACTGT
AAAGATGTAGGTGATTTGAAGCAATATGATAGTGATGAACCAGAATTGAGGTCGCTTGCAAGTTGGATTCAGAATGAGTTCAAC
AAGGCATGCGAACTGACAGATTCAAGCTGGATAGAGCTTGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGC
ATGAGAAGGAATTATTTCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATAATGAAGGGGGTGTACATCAATACTGCC
TTACTTAATGCATCTTGTGCAGCAATGGATGATTTCCAATTAATTCCAATGATAAGCAAGTGTAGAACTAAGGAGGGAAGGCGA
AAGACCAACTTGTATGGTTTCATCATAAAAGGAAGATCCCACTTAAGGAATGACACCGACGTGGTAAACTTTGTGAGCATGGAG
TTTTCTCTCACTGACCCAAGACTTGAACCACACAAATGGGAGAAGTACTGTGTTCTTGAGATAGGAGATATGCTTCTAAGAAGT
GCCATAGGCCAGGTTTCAAGGCCCATGTTCTTGTATGTGAGGACAAATGGAACCTCAAAAATTAAAATGAAATGGGGAATGGAG
ATGAGGCGTTGTCTCCTCCAGTCACTTCAACAAATTGAGAGTATGATTGAAGCTGAGTCCTCTGTCAAAGAGAAAGACATGACC
AAAGAGTTCTTTGAGAACAAATCAGAAACATGGCCCATTGGAGAGTCTCCCAAAGGAGTGGAGGAAAGTTCCATTGGGAAGGTC
TGCAGGACTTTATTAGCAAAGTCGGTATTTAACAGCTTGTATGCATCTCCACAACTAGAAGGATTTTCAGCTGAATCAAGAAAA
CTGCTTCTTATCGTTCAGGCTCTTAGGGACAATCTGGAACCTGGGACCTTTGATCTTGGGGGGCTATATGAAGCAATTGAGGAG
TGCCTAATTAATGATCCCTGGGTTTTGCTTAATGCTTCTTGGTTCAACTCCTTCCTTACACATGCATTGAGTTAGTTGTGGCAG
TGCTACTATTTGCTATCCATACTGTCCAAAAAAGTACCTTGTTTCTACT

SEQ ID NO:12

NP

AGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGCGTCCCAAGGCACCAAACGGTCTTACGAACAGATG
GAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAAATGATTGGTGGAATTGGACGATTCTACATC
CAAATGTGCACAGAACTTAAACTCAGTGATTATGAGGGACGGTTGATCCAAAACAGCTTAACAATAGAGAGAATGGTGCTCTCT
GCTTTTGACGAAAGGAGAAATAAATACCTGGAAGAACATCCCAGTGCGGGGAAAGATCCTAAGAAAACTGGAGGACCTATATAC
AGAAGAGTAAACGGAAAGTGGATGAGAGAACTCATCCTTTATGACAAAGAAGAAATAAGGCGAATCTGGCGCCAAGCTAATAAT
GGTGACGATGCAACGGCTGGTCTGACTCACATGATGATCTGGCATTCCAATTTGAATGATGCAACTTATCAGAGGACAAGGGCT
CTTGTTCGCACCGGAATGGATCCCAGGATGTGCTCTCTGATGCAAGGTTCAACTCTCCCTAGGAGGTCTGGAGCCGCAGGTGCT
GCAGTCAAAGGAGTTGGAACAATGGTGATGGAATTGGTCAGGATGATCAAACGTGGGATCAATGATCGGAACTTCTGGAGGGGT
GAGAATGGACGAAAAACAAGAATTGCTTATGAAAGAATGTGCAACATTCTCAAAGGGAAATTTCAAACTGCTGCACAAAAAGCA
ATGATGGATCAAGTGAGAGAGAGCCGGAACCCAGGGAATGCTGAGTTCGAAGATCTCACTTTTCTAGCACGGTCTGCACTCATA
TTGAGAGGGTCGGTTGCTCACAAGTCCTGCCTGCCTGCCTGTGTGTATGGACCTGCCGTAGCCAGTGGGTACGACTTTGAAAGA
GAGGGATACTCTCTAGTCGGAATAGACCCTTTCAGACTGCTTCAAAACAGCCAAGTGTACAGCCTAATCAGACCAAATGAGAAT
CCAGCACACAAGAGTCAACTGGTGTGGATGGCATGCCATTCTGCCGCATTTGAAGATCTAAGAGTATTGAGCTTCATCAAAGGG
ACGAAGGTGGTCCCAAGAGGGAAGCTTTCCACTAGAGGAGTTCAAATTGCTTCCAATGAAAATATGGAGACTATGGAATCAAGT
ACACTTGAACTGAGAAGCAGGTACTGGGCCATAAGGACCAGAAGTGGAGGAAACACCAATCAACAGAGGGCATCTGCGGGCCAA
ATCAGCATACAACCTACGTTCTCAGTACAGAGAAATCTCCCTTTTGACAGAACAACCGTTATGGCAGCATTCACTGGGAATACA
GAGGGGAGAACATCTGACATGAGGACCGAAATCATAAGGATGATGGAAAGTGCAAGACCAGAAGATGTGTCTTTCCAGGGGCGG
GGAGTCTTCGAGCTCTCGGACGAAAAGGCAGCGAGCCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTC
GGAGACAATGCAGAGGAGTACGACAATTAAAGAAAAATACCCTTGTTTCTACT

SEQ ID NO:13

M

AGCAAAAGCAGGTAGATATTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCT
CAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTGCAGGGAAGAACACCGATCTTGAGGTTCTCATGGAATGGCTAAAGAC
AAGACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCG
TAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGATCCAAATAACATGGACAAAGCAGTTAAACTGTATAGGAAGCTCAA
GAGGGAGATAACATTCCATGGGGCCAAAGAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGTATGGGCCTCATATA
CAACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGCCTGGTATGTGCAACCTGTGAACAGATTGCTGACTCCCAGCATCG

Fig. 1B

PR8(Cambridge)

GTCTCATAGGCAAATGGTGACAACAACCAACCCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAGCACTACAGCTAAGGC
TATGGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGGCCATGGAGGTTGCTAGTCAGGCTAGGCAAATGGTGCAAGCGAT
GAGAACCATTGGGACTCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGGCCTATCAGAAACGAAT
GGGGGTGCAGATGCAACGGTTCAAGTGATCCTCTCGCTATTGCCGCAAATATCATTGGGATCTTGCACTTGATATTGTGGATTC
TTGATCGTCTTTTTTTCAAATGCATTTACCGTCGCTTTAAATACGGACTGAAAGGAGGGCCTTCTACGGAAGGAGTGCCAAAGT
CTATGAGGGAAGAATATCGAAAGGAACAGCAGAGTGCTGTGGATGCTGACGATGGTCATTTTGTCAGCATAGAGCTGGAGTAAA
AAACTACCTTGTTTCTACT

SEQ ID NO:14

NS

AGCAAAAGCAGGGTGACAAAGACATAATGGATCCAAACACTGTGTCAAGCTTTCAGGTAGATTGCTTTCTTTGGCATGTCCGCA
AACGAGTTGCAGACCAAGAACTAGGTGATGCCCCATTCCTTGATCGGCTTCGCCGAGATCAGAAATCCCTAAGAGGAAGGGGCA
GCACTCTTGGTCTGGACATCGAGACAGCCACACGTGCTGGAAAGCAGATAGTGGAGCGGATTCTGAAAGAAGAATCCGATGAGG
CACTTAAAATGACCATGGCCTCTGTACCTGCGTCGCGTTACCTAACCGACATGACTCTTGAGGAAATGTCAAGGGAATGGTCCA
TGCTCATACCCAAGCAGAAAGTGGCAGGCCCTCTTTGTATCAGAATGGACCAGGCGATCATGGATAAAAACATCATACTGAAAG
CGAACTTCAGTGTGATTTTTGACCGGCTGGAGACTCTAATATTGCTAAGGGCTTTCACCGAAGAGGGAGCAATTGTTGGCGAAA
TTTCACCATTGCCTTCTCTTCCAGGACATACTGCTGAGGATGTCAAAAATGCAGTTGGAGTCCTCATCGGAGGACTTGAATGGA
ATGATAACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAATGGGAGACCTCCACTCACTC
CAAAACAGAAACGAGAAATGGCGGGAACAATTAGGTCAGAAGTTTGAAGAAATAAGATGGTTGATTGAAGAAGTGAGACACAAA
CTGAAGGTAACAGAGAATAGTTTTGAGCAAATAACATTTATGCAAGCCTTACATCTATTGCTTGAAGTGGAGCAAGAGATAAGA
ACTTTCTCATTTCAGCTTATTTAATAATAAAAAACACCCTTGTTTCTACT

SEQ ID NO:15

Fig. 1C

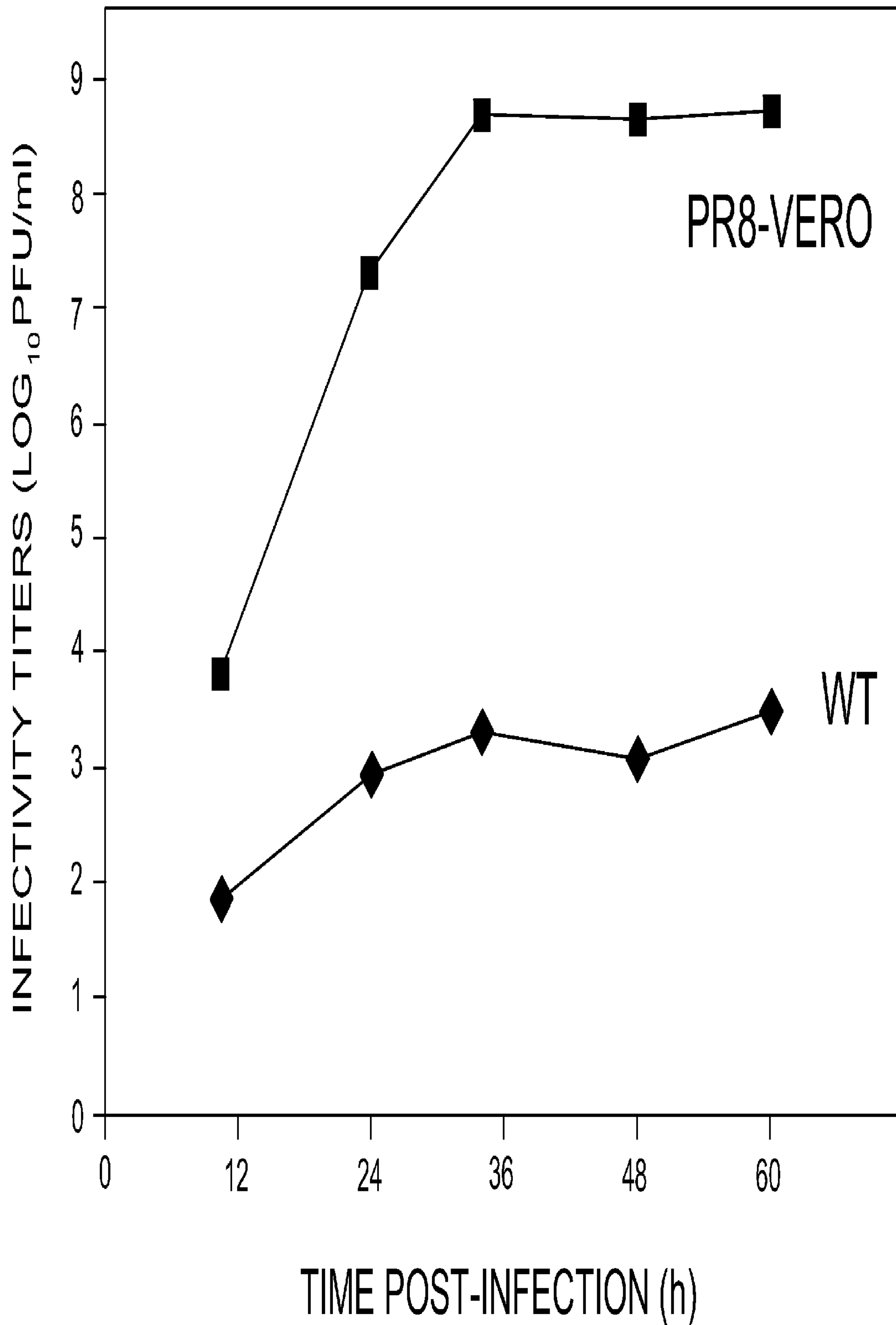
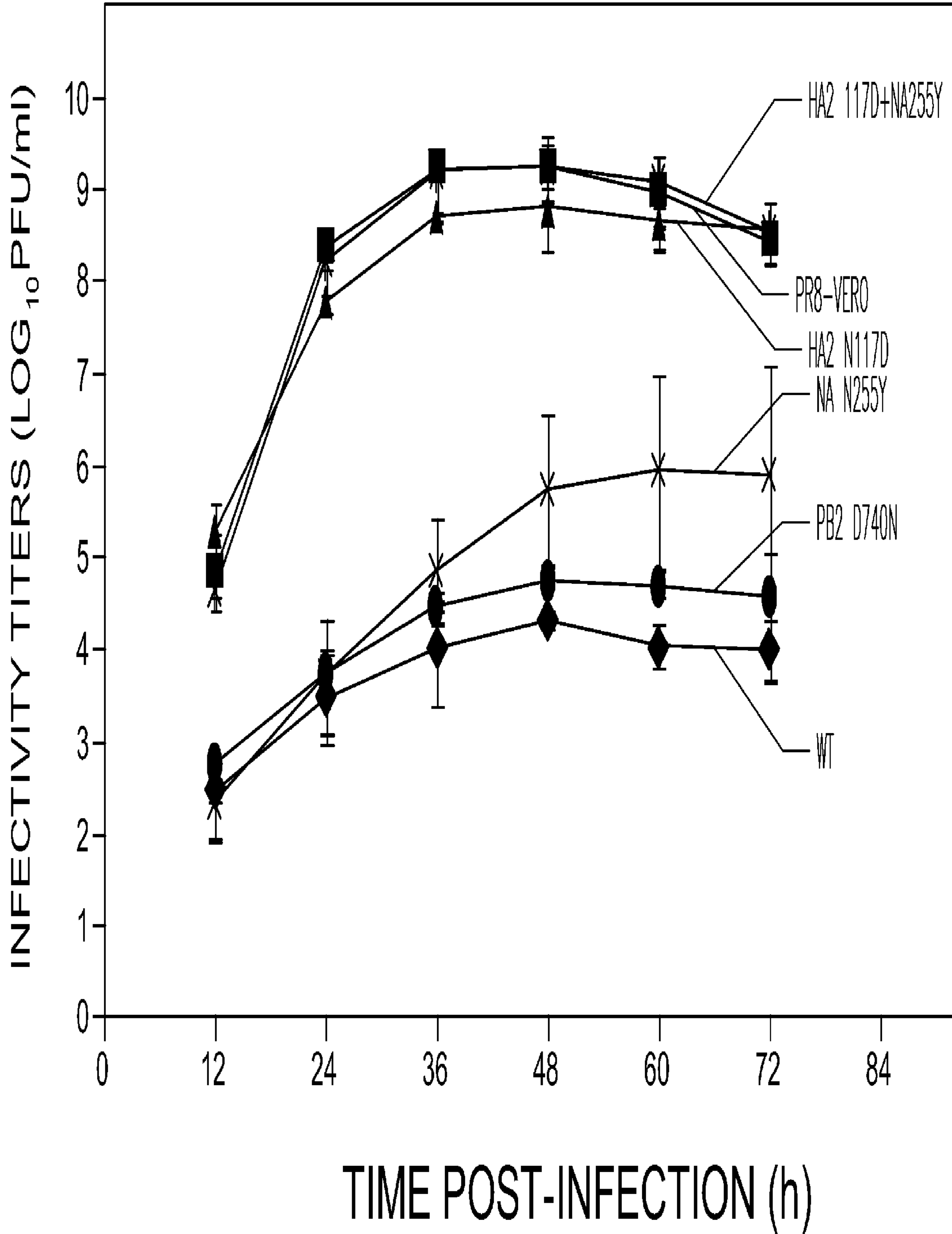


Fig.2

COMPARISON OF AMINO ACID SEQUENCES BETWEEN WT AND PR8-VERO

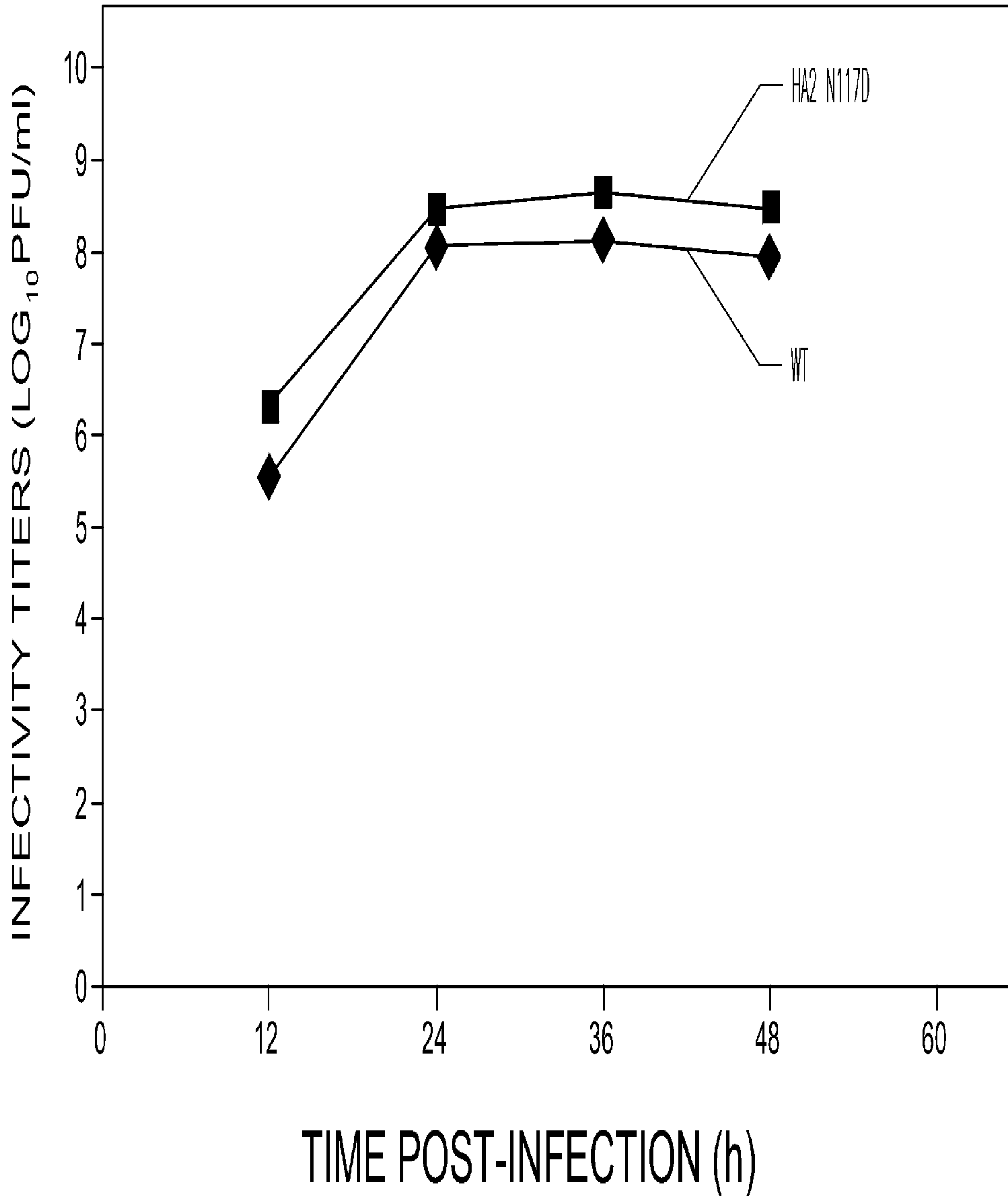
	POSITION	WT	PR8-VERO
HA2	117	N	D
NA	255	N	Y
PB2	740	D	N(2/4)

Fig.3



THE HA2 N117D MUTATION WAS MAINLY RESPONSIBLE FOR THE HIGH GROWTH PROPERTIES IN VERO CELLS.

Fig.4



REPLICATION EFFICIENCY WAS COMPARABLE BETWEEN THE WT AND THE MUTANT.

Fig.5

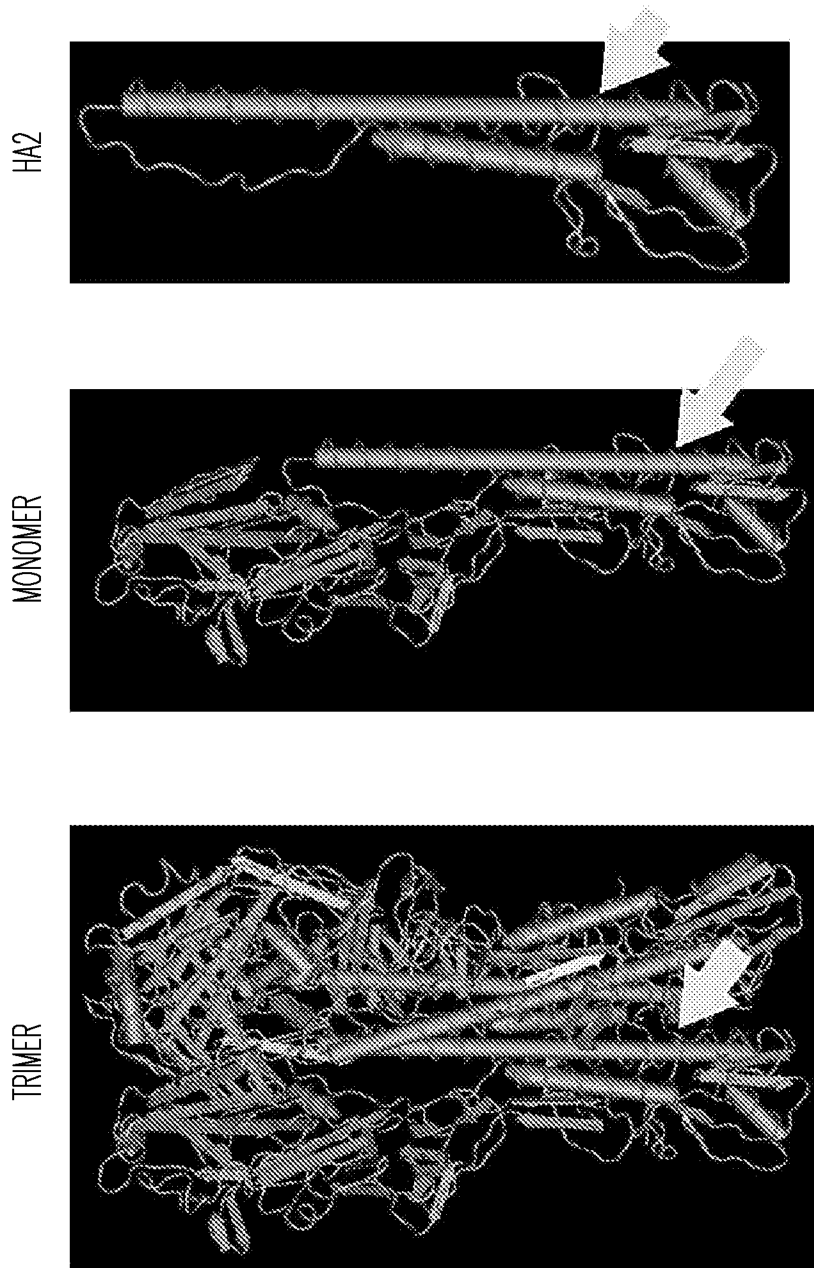


Fig. 6

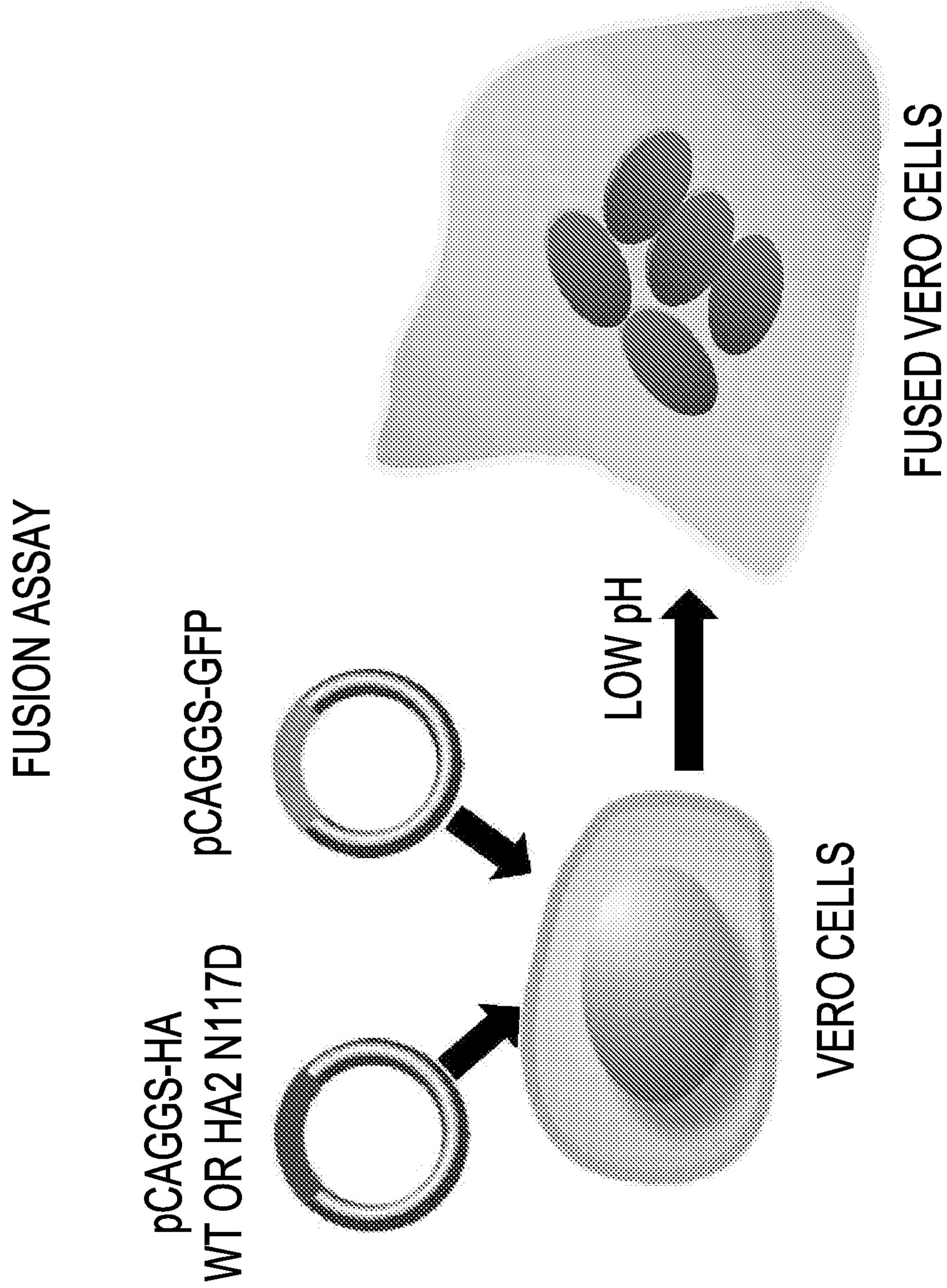
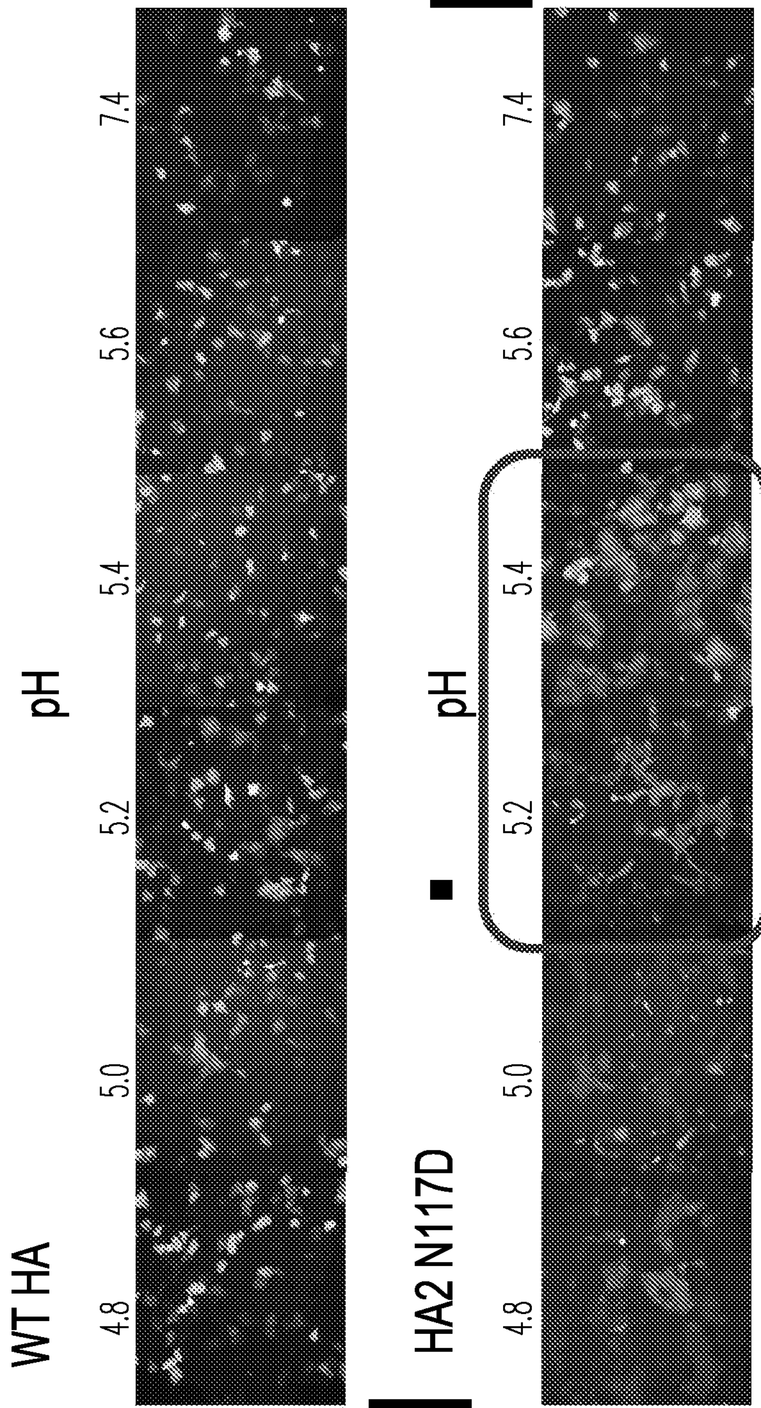


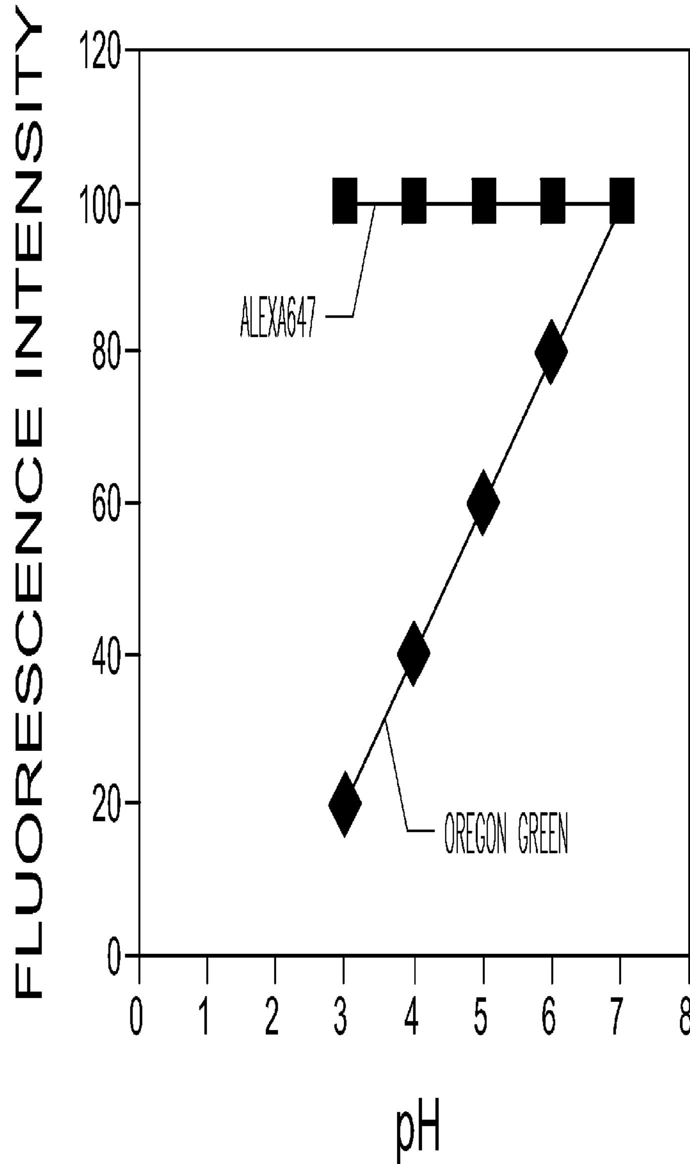
Fig. 7



THE HA2 N117D MUTANT FUSED CELLS AT A HIGHER PH THAN DID WT.

Fig. 8

THE PRINCIPAL OF THE METHOD OF COMPARISON OF ENDOSOMAL pH BETWEEN TWO DIFFERENT CELLS (MDCK VS. VERO CELLS)



FLUORESCENCE INTENSITY OF OREGON GREEN IS SENSITIVE TO LOW PH ALTHOUGH INTENSITY OF ALEXA647 IS NOT SENSITIVE TO PH VALUE.

Fig.9A

PH CAN BE COMPARED BY MEASURING THE INTENSITY AND CALCULATING THE RATIO BETWEEN ALEXA647 AND OREGON GREEN.

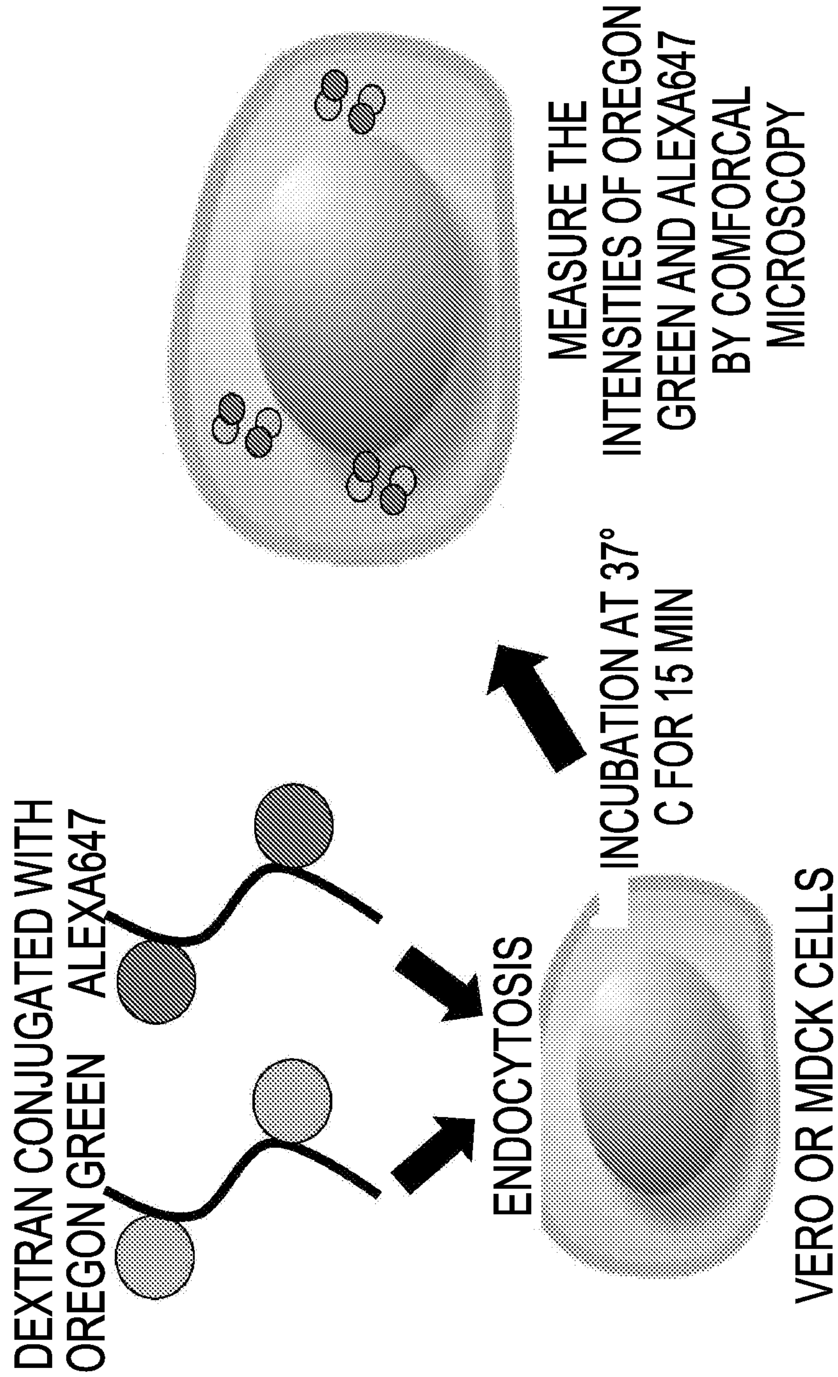


Fig. 9B

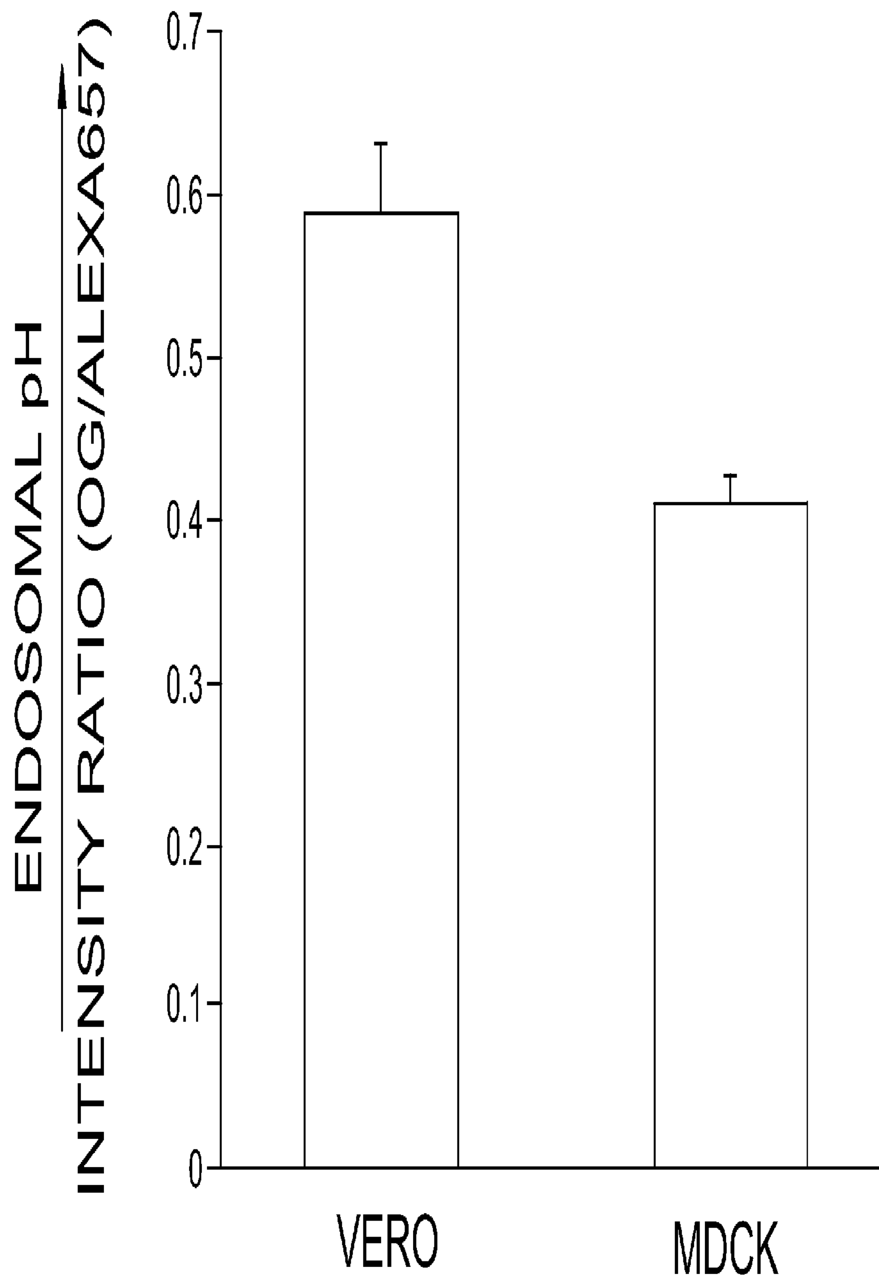


Fig.10

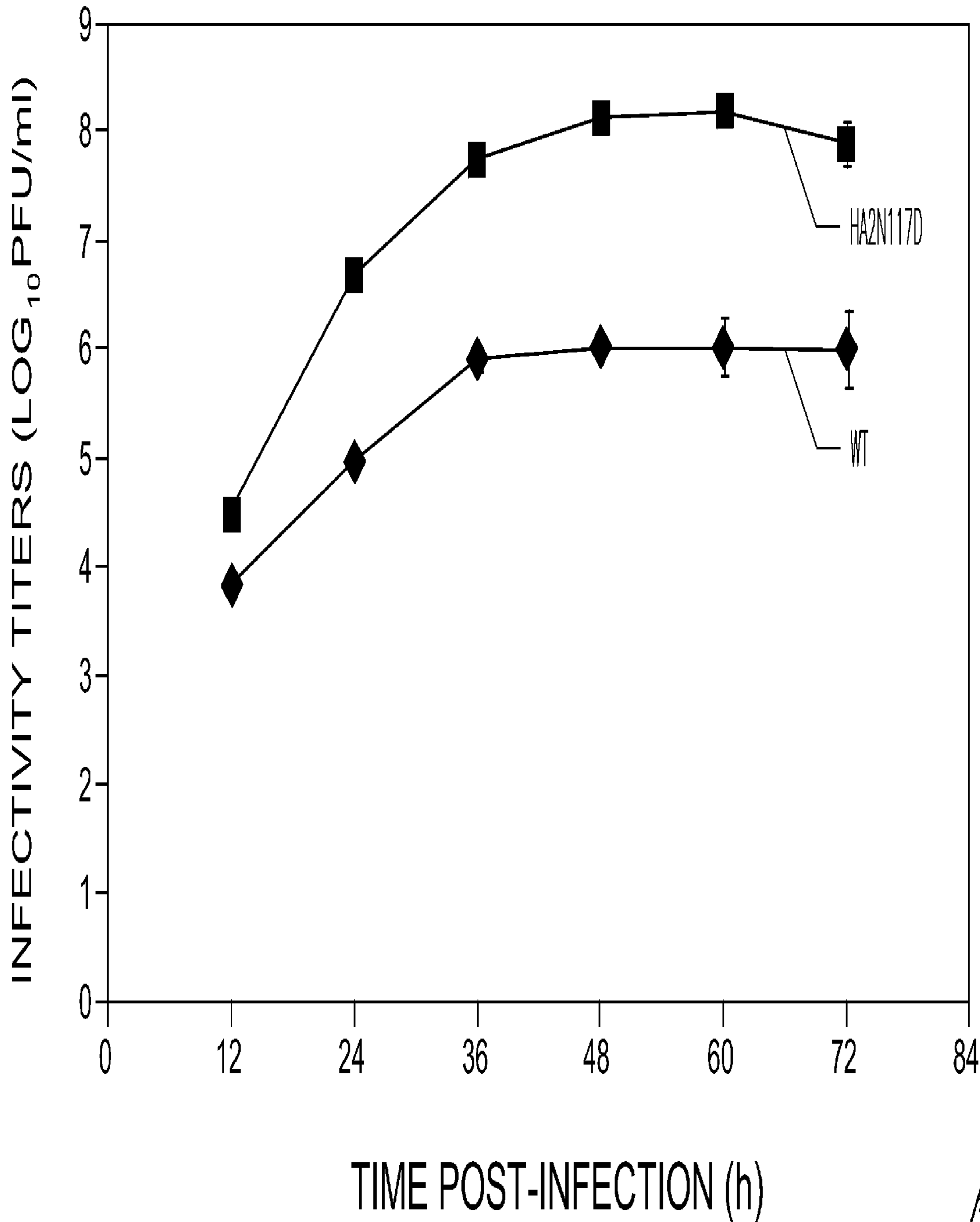
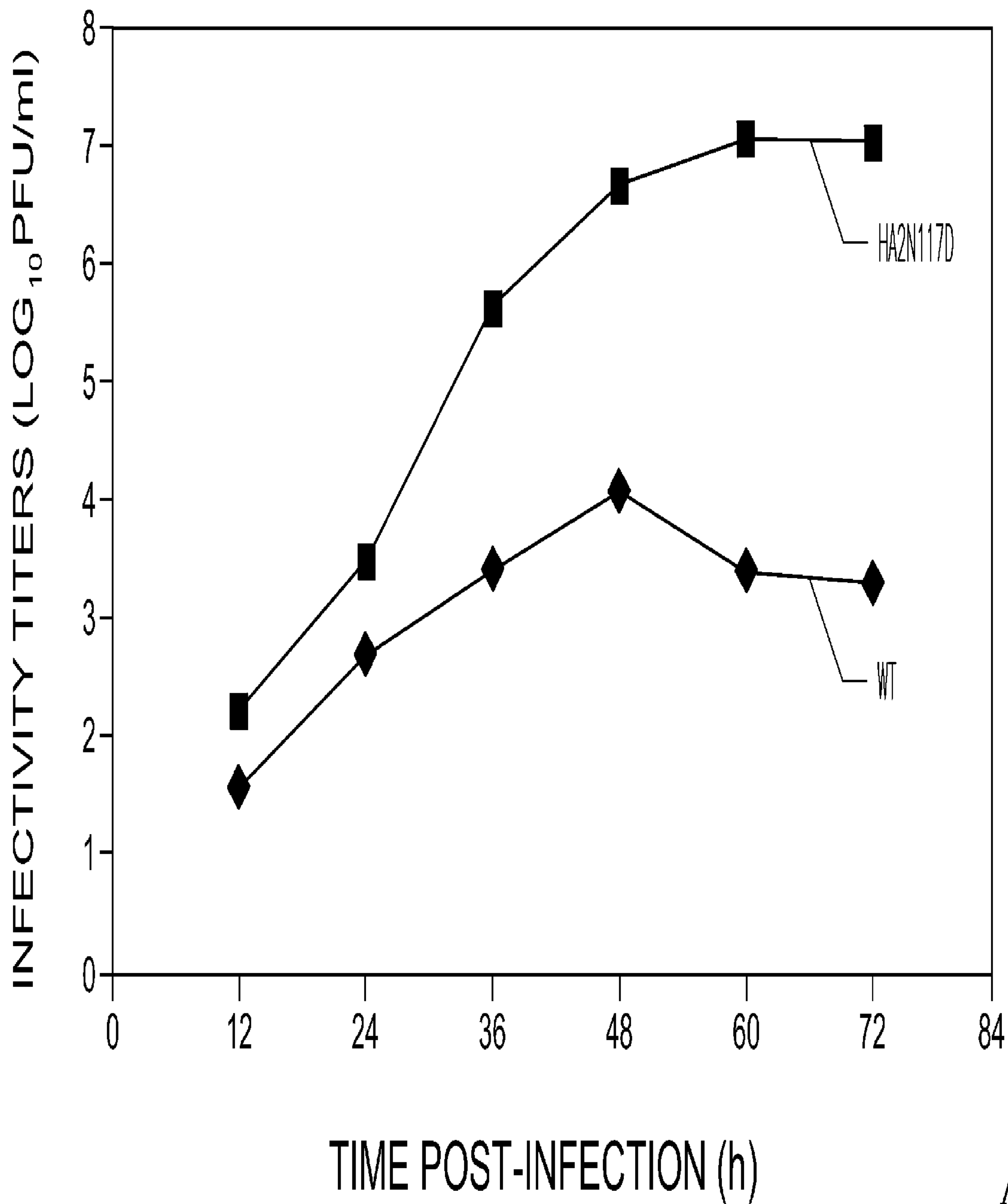


Fig.11A

THE HA2 N117D MUTATION ENHANCES THE REPLICATION EFFICIENCY OF THE A/KAWASAKI/UTK-4/2009 (H1N1) 6:2 REASSORTANT WITH A PR8 DONOR IN VERO CELLS.



TIME POST-INFECTION (h)

Fig.11B

THE HA2 N117D MUTATION ENHANCES THE REPLICATION EFFICIENCY OF THE A/YOKOHAMA/2017/2003 (H3N2) 6:2 REASSORTANT WITH A PR8 DONOR IN VERO CELLS.

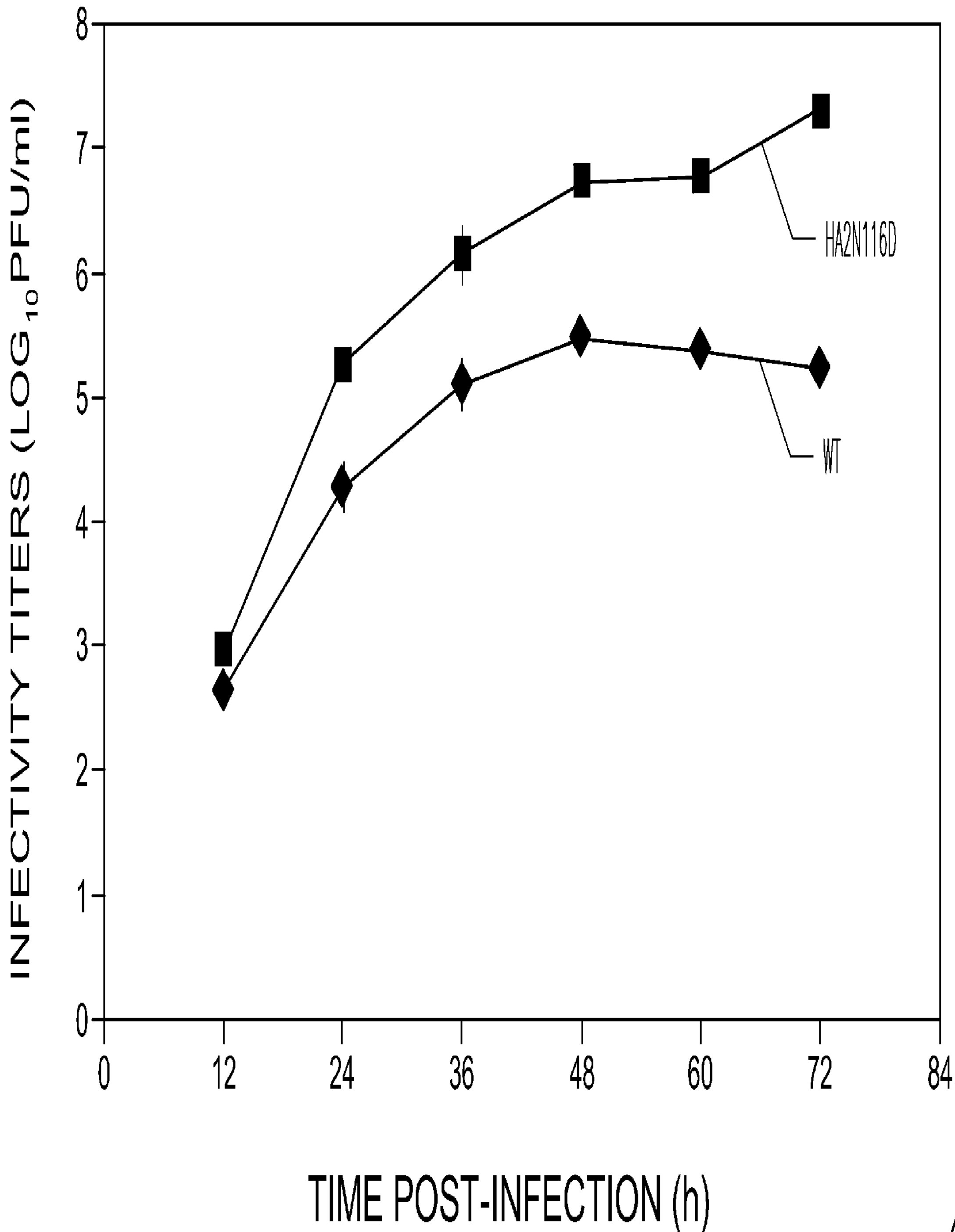


Fig.11C

```
HA1
      11                                                                                         107
H3HU  ATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQSSSTGKICN.NPHRILDGIDCTLIDALLGDPHCDVFQN.ETWDLFVERSKAFS.NCYPYDVPDYAS
H5AV  DQI I Y  NNSTEQ D  MEKN T  H QDILEKTHN  L DL GVKP ILR  SVAGW   N M  E L VPE SYI  KDNPVNGL   ENFN  EE
H5HU  DQI I Y  NNSTEQ D  MEKN T  H QDILERTHN  L DL GVKP ILR  SVAGW   N M  E I VPE SYI  KASPANDL   GNFN  EE
H9SW  DKI I YQSTNSTET D L ETN P  H K  LHTEHN ML AT LGHP ILDT  IEGLIY N S  LLLGGRE SYI   PS VNGM   GN ENLEE
H9HU  DKI I  QSTNSTET D L ETN P  H K  LHTEHN ML ATSLGHP ILDT  IEGLVY N S  LLLGGRE SYI    S VNGT   GN ENLEE

      108                                                                                        203
H3HU  LRSLVASSGTLEFITEGF...TWTGVTQN.GGSNACKRGPGSGFFSRLNWLTKSGSTYPVLNVTMPNNDNFDKLYIWGIHHPSTNQEQTSLYVQASGRVT
H5AV   KH LS TNHF K RI.IPRSS SNHDASS V S  PYNGR S  RNVV  I KNNA  TIKRSYN TNQE L IL      NDAA   K  QNPTTY S
H5HU   KH LSRINHF K QI.IPKSS SNHDASS V S  PYLGR S  RNVV  I KN A  TIKRSYN TNQE L VL      NDAA   K  QNPTTY S
H9SW      FS ASSYQR QI. PDTI .N SYS. T K  S....DS  RSMR   QKNNA   QDAQYT  RGKSI  M   N  P DTV   N  TRTDTTTS
H9HU    T FS ASSYQR QI. PDT  .N  YT. T R  S....GS  RSMR   QKSGF   QDAQYT  RGKSI         P YT    N   RNDTTTS

      204                                                                                        302
H3HU  VSTRRSQQTIIPNIGSRPWVRGLSSRISIYWTIVKPGDVLVINSNGNLIAPR.GYFKMRTGKSSIMRSDAPIDTCISECITPNGSIPNDKPFQNVNKITY
H5AV   G STLN RS  E AT  K N Q G MEF    L  N AINFE    F    EYA KIVKK G A  K GLEYGN NTK Q  M A NSSM  H  HPL I
H5HU   G STLN RL  E AT  K N Q G MEF    L  N AINFE    F    EYA KIVKK D T  K ELEYGN NTK Q  M A NSSM  H  HPL I
H9SW   T EDINR FK V  P  L N  HG  DY  S L   QT R R          WY HILSGESHGR LKT LNSGN  VQ Q ER GLNTTL  H  S YA
H9HU   T EDLNR FK V  P  L N  QG  DY  S L   QT R R          WY HVLSGGSHGR LKT LKGGN  VQ Q EK GLNSTL  H  S YA

      303                       328    *
H3HU  GACPKYVKQNTLKLATGMRNVPEKQT....R   SEQ ID NO:16
H5AV   E      SGR V    L    QRE ....   SEQ ID NO:17
H5HU   E       S R V    L   T QRERRRKK   SEQ ID NO:18
H9SW   N     GVKS     V L    ARSS....   SEQ ID NO:19
H9HU   T     RV S     V L    ARSS....   SEQ ID NO:20
```

Fig. 12A

```
HA2
      1                                                                                                  100
H3HU  GLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLV
H5AV             G  Q         H S EQ S Y   KE   K   GTTN V S  D M TQ EA G   NNL R  EN N KM  GFL V T
H5HU             G  Q         H S EQ S Y   KE   K   G TN V S  N M TQ EA GR  NNL R  EN N KM  GFL V T
H9SW             G  P L A     Q S DQ V M   RD   K   K TS V N  D M KQ GI DH      T LNMINNK D QIQ I T
H9HU             G  P L A     Q S DQ V M   RD   K   K TS V N  D M KQ EI DH      T LNMINNK D QIQ V A

      101                                                                                                199
H3HU  ALENQHTIDLTDSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWI.SFAISCFLLC
H5AV  LM  ER L FH  NVKN  D V L   D  K L     EF      E M   K     YPQ SE  RL  EE S  K E MGIYQ  S Y TVA SLA A
H5HU  LM  ER L FH  NVKN  D V L   D  K L     EF      E M   K     YPQ SE  RL  EE S  K E MGTYQ  S Y TVA SLA A
H9SW  L    K L EH ANV N  N VK A GS  M D K   EL     DQ M T      NRRK KE SKLE QK E  K E EGTYK  T Y TVA SLVIA
H9HU  L    K L EH ANV N  N VK A GS  M D K   EL     DQ M T      NRRK  E SRLE QK E -------------------------

      200                 221
H3HU  VVLLGFIMWACQRGNIRCNICI   SEQ ID NO:23
H5AV   MIA LSL M SN SLQ R     SEQ ID NO:24
H5HU   MVA LSL M SN SLQ R     SEQ ID NO:25
H9SW  MGFAA LF  MS----------   SEQ ID NO:26
H9HU  ----------------------   SEQ ID NO:27
```

Fig.12A CONT'D

```
  1 MKAILVVLLY TFATANADTL CIGYHANNST DTVDTVLEKN VTVTHSVNLL EDKHNGKLCK
 61 LRGVAPLHLG KCNIAGWILG NPECESLSTA SSWSYIVETP SSDNGTCYPG DFIDYEELRE
121 QLSSVSSFER FEIFPKTSSW PNHDSNKGVT AACPHAGAKS FYKNLIWLVK KGNSYPKLSK
181 SYINDKGKEV LVLWGIHHPS TSADQQSLYQ NADAYVFVGS SRYSKKFKPE IAIRPKVRDQ
241 EGRMNYYWTL VEPGDKITFE ATGNLVVPRY AFAMERNAGS GIIISDTPVH DCNTTCQTPK
301 GAINTSLPFQ NIHPITIGKC PKYVKSTKLR LATGLRNIPS IQSRGLFGAI AGFIEGGWTG
361 MVDGWYGYHH QNEQGSGYAA DLKSTQNAID EITNKVNSVI EKMNTQFTAV GKEFNHLEKR
421 IENLNKKVDD GFLDIWTYNA ELLVLLENER TLDYHDSNVK NLYEKVRSQL KNNAKEIGNG
481 CFEFYHKCDN TCMESVKNGT YDYPKYSEEA KLNREEIDGV KLESTRIYQI LAIYSTVASS
541 LVLVVSLGAI SFWMCSNGSL QCRICI SEQ ID NO:21
```

Fig.12B

A/Kawasaki/173/2001 (H1N1)
GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVG
KEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKDLYEKVKSQLKNNAKEIGNGCF
EFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM
CSNGSLQCRICI

SEQ ID NO:28

A/Kawasaki/UTK-4/2009 (H1N1)
GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVG
KEFNKLERRMENLNKKVDDGFIDIWTYNAELLVLLENERTLDFHDSNVKDLYEKVKSQLKNNAKEIGNGCFE
FYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMC
SNGSLQCRICI

SEQ ID NO:29

A/Yokohama/2017/2003 (H3N2)
GIFGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAINQINGKLNRLIGKTNEKFHQIEKEF
SEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMDKLFERTKKQLRENAEDMGNGCFKIYH
KCDNACIESIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN
IRCNICI

SEQ ID NO:30

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/054128

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/11 C12N7/08 C12N7/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, Sequence Search, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUNHAM ELECA J ET AL: "Different Evolutionary Trajectories of European Avian-Like and Classical Swine H1N1 Influenza A Viruses", June 2009 (2009-06), JOURNAL OF VIROLOGY, VOL. 83, NR. 11, PAGE(S) 5485-5494, XP002620796, ISSN: 0022-538X	1-7,22, 23,25
Y	sequence CY037929	8-21, 26-28

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 2011	23/02/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2, NL - 2280 HV Rijswijk, Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schulz, Regine

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE MIN-SHIUH ET AL: "Genetic and pathogenic characterization of H6N1 avian influenza viruses isolated in Taiwan between 1972 and 2005.", AVIAN DISEASES DEC 2006 LNKD- PUBMED:17274295, vol. 50, no. 4, December 2006 (2006-12), pages 561-571, XP002620797, ISSN: 0005-2086	1-7,22, 23,25
Y	sequence ABD355630	8-21, 26-28
Y	NEUMANN G ET AL: "An improved reverse genetics system for influenza A virus generation and its implications for vaccine production", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES (PNAS), NATIONAL ACADEMY OF SCIENCE, US, vol. 102, no. 46, 15 November 2005 (2005-11-15), pages 16825-16829, XP002471231, ISSN: 0027-8424, DOI: DOI:10.1073/PNAS.0505587102 page 16825 - page 16825; figure 1	8-21, 26-28
Y	WO 2007/126810 A2 (WARF WISCONSIN ALUMNI RES FOUN [US]; KAWAOKA YOSHIHIRO [US]; HORIMOTO) 8 November 2007 (2007-11-08) page 3, line 17 - page 13, line 32; claims 1-36; examples 1-4	8-21, 26-28
T	NEUMANN GABRIELE ET AL: "Reverse Genetics of Influenza Viruses - Applications in Research and Vaccine Design", MONOGRAPHS IN VIROLOGY KARGER, POSTFACH, CH-4009 BASEL, SWITZERLAND SERIES : MONOGRAPHS IN VIROLOGY (ISSN 0077-0965(PRINT)), 2008, pages 118-133, XP008132725, the whole document	8-28
T	NEUMANN GABRIELE ET AL: "Emergence and pandemic potential of swine-origin H1N1 influenza virus", NATURE (LONDON), vol. 459, no. 7249, June 2009 (2009-06), pages 931-939, XP002620795, ISSN: 0028-0836 page 931 - page 937; figures 3, 4	1-7,22, 23

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	LI K S ET AL: "Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia", NATURE, NATURE PUBLISHING GROUP, LONDON, GB, vol. 430, no. 6996, 8 July 2004 (2004-07-08), pages 209-213, XP002544219, ISSN: 0028-0836, DOI: DOI:10.1038/NATURE02746 the whole document -----	1-7,22, 23

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/054128

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007126810 A2	08-11-2007	AU 2007245192 A1	08-11-2007
		CA 2647985 A1	08-11-2007
		CN 101472941 A	01-07-2009
		EP 2010557 A2	07-01-2009
		JP 2009532352 T	10-09-2009