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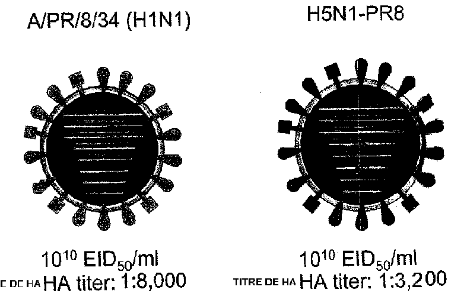
(54) Title: HIGH TITER RECOMBINANT INFLUENZA VIRUSES FOR VACCINES AND GENE THERAPY



(57) Abstract: The invention provides a composition useful to prepare high titer influenza viruses, e.g., in the absence of helper virus, which includes a sequence from a high titer influenza virus isolate.

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HIGH TITER RECOMBINANT INFLUENZA VIRUSES FOR VACCINES AND GENE THERAPY

Cross-Reference to Related Applications

The present application claims the benefit under 35 U.S.C. § 119(e) of the filing date of U.S. application Serial No. 60/473,798, filed May 28, 2003, the disclosure of which is incorporated by reference herein.

Statement of Government Rights

This invention was made with a grant from the Government of the United States of America (grant AI-47446 from the National Institute of Allergy and Infectious Diseases Public Health Service). The Government may have certain rights in the invention.

Background of the Invention

Negative-sense RNA viruses are classified into seven families (*Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae*, *Bornaviridae*, *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae*) which include common human pathogens, such as respiratory syncytial virus, influenza virus, measles virus, and Ebola virus, as well as animal viruses with major economic impact on the poultry and cattle industries (e.g., Newcastle disease virus and Rinderpest virus). The first four families are characterized by nonsegmented genomes, while the latter three have genomes comprised of six-to-eight, three, or two negative-sense RNA segments, respectively. The common feature of negative-sense RNA viruses is the negative polarity of their RNA genome; i.e., the viral RNA (vRNA) is complementary to mRNA and therefore is not infectious by itself. In order to initiate viral transcription and replication, the vRNA has to be transcribed into a plus-sense mRNA or cRNA, respectively, by the viral polymerase complex and the nucleoprotein; for influenza A viruses, the viral polymerase complex is comprised of the three polymerase proteins PB2, PB1, and PA. During viral replication, cRNA serves as a template for the synthesis of new vRNA molecules. For all negative-stranded RNA viruses, non-coding regions at both the 5' and 3' termini of the vRNA and cRNA are critical for transcription and replication of the viral genome. Unlike cellular or viral mRNA transcripts, both cRNA and vRNA are neither capped at the 5' end nor polyadenylated at the very 3' end.

The basic functions of many viral proteins have been elucidated biochemically and/or in the context of viral infection. However, reverse genetics systems have dramatically increased our knowledge of negative-stranded segmented and non-segmented RNA viruses with respect to their viral replication and pathogenicity, as well as to the development of live attenuated virus vaccines. Reverse genetics, as the term is used in molecular virology, is defined as the generation of virus possessing a genome derived from cloned cDNAs (for a review, see Neumann et al., 2002).

In order to initiate viral replication of negative-stranded RNA viruses, vRNA(s) or cRNA(s) must be coexpressed with the polymerase complex and the nucleoprotein. Rabies virus was the first non-segmented negative-sense RNA virus which was generated entirely from cloned cDNA: Schnell et al. (1994) generated recombinant rabies virus by cotransfection of a cDNA construct encoding the full-length cRNA and protein expression constructs for the L, P, and N proteins, all under control of the T7 RNA polymerase promoter. Infection with recombinant vaccinia virus, which provided T7 RNA polymerase, resulted in the generation of infectious rabies virus. In this T7 polymerase system, the primary transcription of the full length cRNA under control of the T7 RNA polymerase resulted in a non-capped cRNA transcript. However, three guanidine nucleotides, which form the optimal initiation sequence for T7 RNA polymerase, were attached to the 5' end. In order to create an authentic 3' end of the cRNA transcript which is essential for a productive infective cycle, the hepatitis delta ribozyme (HDVRz) sequence was used for exact autocatalytic cleavage at the 3' end of the cRNA transcript.

Since the initial report by Schnell et al. (1994), reverse genetics systems using similar techniques led to the generation of many non-segmented negative strand RNA viruses (Conzelmann, 1996; Conzelmann, 1998; Conzelmann et al., 1996; Marriott et al., 1999; Munoz et al., 2000; Nagai, 1999; Neumann et al., 2002; Roberts et al., 1998; Rose, 1996). Refinements of the original rescue procedure included the expression of T7 RNA polymerase from stably transfected cell lines (Radecke et al., 1996) or from protein expression plasmids

(Lawson et al., 1995), or heat shock procedures to increase rescue efficiencies (Parks et al., 1999). Based on the T7 polymerase system, Bridgen and Elliott (1996) created Bunyamwera virus (family *Bunyaviridae)* from cloned cDNAs and demonstrated the feasibility of artificially generating a segmented negative-sense RNA virus by the T7 polymerase system.

In 1999, a plasmid-based reverse genetics technique was generated based on the cellular RNA polymerase I for the generation of segmented influenza A virus entirely from cloned cDNAs (Fodor et al., 1999; Neumann and Kawaoka, 1999). RNA polymerase I, a nucleolar enzyme, synthesizes ribosomal RNA which, like influenza virus RNA, does not contain 5' cap or 3' polyA structures. The RNA polymerase I transcription of a construct containing an influenza viral cDNA, flanked by RNA polymerase I promoter and terminator sequences, resulted in influenza vRNA synthesis (Fodor et al., 1999; Neumann and Kawaoka, 1999; Neumann and Kawaoka, 2001; Pekosz et al., 1999). The system was highly efficient, producing more than 10^8 infectious virus particles per ml of supernatant of plasmid-transfected cells 48 hours post-transfection.

What is needed is a method to prepare high titer orthomyxoviruses such as influenza A virus, entirely from cloned cDNAs.

All references, including any patents or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in Australia or in any other country.

In the claims of this application and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Summary of the Invention

The inventors disclose an isolated and/or purified nucleic acid molecule (polynucleotide) encoding at least one of the proteins of a high titer, e.g., titers greater than 10^9/ml, e.g., greater than 10^{10}/ml, influenza virus, or a portion thereof, or the complement of the nucleic acid molecule. The isolated and/or purified nucleic acid molecule may encode HA, NA, 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:1-8. 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. The isolated and/or purified nucleic acid molecule may encode a polypeptide which is substantially the same as, e.g., having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOs:1-8. The isolated and/or purified nucleic acid molecule may comprise a nucleotide sequence which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90% or more contiguous nucleic acid sequence identity to, one of SEQ ID NOs:1-8, or the complement thereof, and, in one embodiment, also encodes a polypeptide having at least 80%, e.g., 90%, 92%, 95%, 97% or 99%, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:1-8. The isolated and/or purified nucleic acid molecule may encode a polypeptide with 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:1-8. "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. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine.

The isolated and/or purified nucleic acid molecule or the complement thereof, may hybridize to one of SEQ ID NOs:1-8, or the complement thereof, under low stringency, moderate stringency or stringent conditions. For example, the following conditions may be employed: 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C (low stringency), more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C (moderate stringency), more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C (stringent), preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C (more stringent), more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C (very stringent). The nucleic acid molecule may encode a polypeptide which is substantially the same as, e.g., having at least 50%, e.g., 60%, 70%, 80% or 90% or more contiguous nucleic acid sequence identity to, one of SEQ ID NOs:1-8, and preferably has substantially the same activity as a corresponding full-length polypeptide encoded by one of SEQ ID NOs:1-8.

The nucleic acid molecule may be employed to express influenza proteins, to prepare chimeric genes, e.g., with other viral genes including other influenza virus genes, and/or to prepare recombinant virus. Thus, the inventors also disclose isolated polypeptides, recombinant virus, and host cells contacted with the nucleic acid molecules or recombinant virus of the invention.

The inventors also disclose at least one of the following isolated and/or purified vectors: a vector comprising a promoter operably linked to an influenza virus PA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein at least one vector comprises sequences encoding HA, NA, PB1, PB2, PA, NP, M, NS, or a portion thereof, having substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:1-8, e.g., a sequence encoding a polypeptide with at least 80% amino acid identity to a polypeptide encoded by one of SEQ ID NOs:1-8. 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.

The inventors disclose isolated and purified vectors or plasmids, which express or encode influenza virus proteins, or express or encode influenza vRNA, both native and recombinant vRNA. Preferably, the vectors comprise influenza cDNA, e.g., influenza A (e.g., any influenza A gene including any of the 15 HA or 9 NA subtypes), B or C DNA (see Chapters 45 and 46 of Fields Virology (Fields et al. (eds.), Lippincott-Raven Publ., Philadelphia, PA (1996), which are specifically incorporated by reference herein), although it is envisioned that the gene(s) of any organism may be employed in the vectors or methods of the invention. The cDNA may be in the sense or antisense orientation relative to the promoter. Thus, a vector of the invention may encode an influenza virus protein (sense) or vRNA (antisense). 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.

The invention provides a composition comprising a plurality of influenza virus vectors. In one embodiment of the invention, the composition comprises: a) at least two vectors selected from a vector comprising a promoter operably linked to an influenza virus PA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus

NA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, and a vector comprising a operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein at least one vector comprises a promoter operably linked to a nucleic acid molecule of the invention linked to a transcription termination sequence; and b) at least two vectors 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. Optionally, the vectors of b) include one or more vectors encoding NP, NS, M, e.g., M1 and M2, HA or NA. Preferably, the vectors encoding viral proteins further comprise a transcription termination sequence.

In another embodiment, the composition comprises: a) at least two vectors selected from a vector comprising a promoter operably linked to an influenza virus PA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA and NB cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, a vector comprising a operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, and a vector comprising a promoter operably linked to an influenza virus BM2 cDNA operably linked to a transcription sequence, wherein at least one vector comprises a promoter operably linked to a nucleic acid molecule of the invention linked to a transcription termination sequence; and b) at least two vectors 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. Optionally, the vectors of

b) include one or more vectors encoding NP, NS, M, HA or NA. Preferably, the vectors encoding viral proteins further comprise a transcription termination sequence.

A composition of the invention may also comprise a gene or open reading frame of interest, e.g., a foreign gene encoding an immunogenic peptide or protein useful as a vaccine. Thus, another embodiment of the invention comprises a composition of the invention as described above in which one of the vectors is replaced with, or the composition further comprises, a vector comprising a promoter linked to 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, linked to a transcription termination sequence. Preferably, the desired nucleic acid sequence such as a cDNA is in an antisense orientation. The introduction of such a composition to a host cell permissive for influenza virus replication results in recombinant virus comprising vRNA corresponding to sequences of the vector. The promoter in such 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, and a T3 promoter, and optionally the vector comprises a transcription termination sequence such as a RNA polymerase I transcription termination sequence, a RNA polymerase II transcription termination sequence, a RNA polymerase III transcription termination sequence, or a ribozyme. In one embodiment, the vector comprising the desired nucleic acid sequence comprises a cDNA of interest. The cDNA of interest, whether in a vector for vRNA or protein production, may encode an immunogenic epitope, such as 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.

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.

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. Preferably, 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 preferably, for expression in more than one host.

In one embodiment, one or more vectors capable of producing vRNA comprise a promoter including, but not limited to, a RNA polymerase I promoter, e.g., a human RNA polymerase I promoter, a RNA polymerase II promoter, a RNA polymerase III promoter, a T7 promoter, or a T3 promoter. Preferred transcription termination sequences for the vRNA vectors include, but are not limited to, a RNA polymerase I transcription termination 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, at least one vector capable of producing 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 and preferably more, 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 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.

The invention also provides a method to prepare influenza virus. The method comprises contacting a cell with a plurality of the vectors of the invention, e.g., sequentially or simultaneously, for example, employing a composition of the invention, in an amount effective to yield infectious influenza virus. The invention also includes isolating virus from a cell contacted with the composition. Thus, the inventors further disclose isolated virus, as well as a host cell contacted with the composition or virus of the invention. The inventors disclose contacting the cell with one or more vectors, either vRNA or protein production vectors, prior to other vectors, either vRNA or protein production vectors.

The method disclosed allows easy manipulation of influenza viruses, e.g., by the introduction of attenuating mutations into the viral genome. Further, because influenza viruses induce strong humoral and cellular immunity, the invention greatly enhances these viruses as vaccine vectors, particularly in view of the availability of natural variants of the virus, which may be employed sequentially, allowing repetitive use for gene therapy.

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 disclosed.

The inventors also disclose a method to immunize an individual against a pathogen, e.g., a bacteria, virus, or parasite, or a malignant tumor. The method comprises administering to the individual an amount of at least one isolated virus of the invention, optionally in combination with an adjuvant, effective to immunize the individual. The virus comprises vRNA comprising a polypeptide encoded by the pathogen or a tumor-specific polypeptide.

Also disclosed is a method to augment or increase the expression of an endogenous protein in a mammal having an indication or disease characterized by a decreased amount or a lack of the endogenous protein. The method comprises administering to the mammal an amount of an isolated virus of the invention effective to augment or increase the amount of the endogenous protein in the mammal. Preferably, the mammal is a human.

Brief Description of the Drawings

Figure 1. Schematic diagram of established reverse genetics systems. In the RNP transfection method (A), purified NP and polymerase proteins are assembled into RNPs with use of *in vitro*-synthesized vRNA. Cells are transfected with RNPs, followed by helper virus infection. In the RNA polymerase I method (B), a plasmid containing the RNA polymerase I promoter, a cDNA encoding the vRNA to be rescued, and the RNA polymerase I terminator is transfected into cells. Intracellular transcription by RNA polymerase I yields synthetic vRNA, which is packaged into progeny virus particles upon infection with helper virus. With both methods, transfectant viruses (i.e., those containing RNA derived from cloned cDNA), are selected from the helper virus population.

Figure 2. Schematic diagram of the generation of RNA polymerase I constructs. cDNAs derived from influenza virus were amplified by PCR, digested with *Bsm*BI and cloned into the *Bsm*BI sites of the pHH21 vector (E. Hoffmann, Ph.D. thesis, Justus, Liebig-University, Giessen, Germany), which contains the human RNA polymerase I promoter (P) and the mouse RNA polymerase I terminator (T). The thymidine nucleotide upstream of the terminator sequence (*T) represents the 3' end of the influenza viral RNA. Influenza A virus sequences are shown in bold face letters. (SEQ ID NOs:29-40)

Figure 3. Proposed reverse genetics method for generating segmented negative-sense RNA viruses. Plasmids containing the RNA polymerase I promoter a cDNA for each of the eight viral RNA segments, and the RNA polymerase I terminator are transfected into cells together with protein expression plasmids. Although infectious viruses can be generated with plasmids expressing PA, PB1, PB2, and NP, expression of all remaining structural proteins (shown in brackets) increases the efficiency of virus production depending on the virus generated.

Figure 4. Titer of various influenza viruses.

Detailed Description of the Invention

Definitions

As used herein, the terms "isolated and/or purified" refer to *in vitro* preparation, isolation and/or purification of a vector, plasmid 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 preparation is generally obtained by *in vitro* culture and propagation and is substantially free from other infectious agents.

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.

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 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.

Influenza virus replication

Influenza A viruses possess a genome of eight single-stranded negative-sense viral RNAs (vRNAs) that encode a total of 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 cDNA 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. Similarly, influenza C virus does not have a M2 protein with ion channel activity. However, the CM1 protein is likely to have this activity. The activity of an ion channel protein may be measured by methods well-known to the art, see, e.g., Holsinger et al. (1994) and WO 01/79273.

Cell Lines and Influenza Viruses That Can Be Used in the Present Invention

According to the present invention, any cell which supports efficient replication of influenza virus can be employed in the invention, including mutant cells which express reduced or decreased levels of one or more sialic acids which are receptors for influenza virus. Viruses obtained by the methods can be made into a reassortant virus.

Preferably, 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 is preferably tested in cells that are at the same passage level as those used for vaccine production. The virus is preferably purified by a process that has been shown to give consistent results, before being inactivated or attenuated for vaccine production (see, e.g., World Health Organization, 1982).

It is preferred to establish a complete characterization of the cell lines to be used, so that appropriate tests for purity of the final product can be included. Data that can be used for the characterization of a cell to be used in the present invention 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. Preferably, the passage level, or population doubling, of the host cell used is as low as possible.

It is preferred that the virus produced in the cell is highly purified prior to vaccine or gene therapy formulation. Generally, the purification procedures will result in the extensive removal of cellular DNA, other cellular components, and adventitious agents. Procedures that extensively degrade or denature DNA can also be used. See, e.g., Mizrahi, 1990.

Vaccines

A vaccine of the invention may comprise immunogenic proteins including glycoproteins of any pathogen, e.g., an immunogenic protein from one or more bacteria, viruses, yeast or fungi. Thus, in one embodiment, the influenza viruses of the invention may be vaccine vectors for influenza virus or other viral pathogens including but not limited to lentiviruses such as HIV, hepatitis B virus, hepatitis C virus, herpes viruses such as CMV or HSV or foot and mouth disease virus.

A complete virion vaccine is concentrated by ultrafiltration and then purified by zonal centrifugation or by chromatography. It is 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: 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 be isolated after treatment of the virions with a protease such as bromelin, then purified by a method such as that described by Grand and Skehel (1972).

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, 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.

Inactivated Vaccines. Inactivated influenza virus vaccines of the invention are provided by inactivating replicated virus of the invention 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. In general, the responses to SV and surface antigen (i.e., purified HA or NA) vaccines are similar. An experimental inactivated WV vaccine containing an NA antigen immunologically related to the epidemic virus and an unrelated HA appears to be less effective than conventional vaccines (Ogra et al., 1977). Inactivated vaccines containing both relevant surface antigens are preferred.

Live Attenuated Virus Vaccines. Live, attenuated influenza virus vaccines, can also be used for preventing or treating influenza virus infection, according to known method steps. Attenuation is preferably 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 (see, e.g., Murphy, 1993). Since resistance to influenza A virus is mediated by the development of an immune response to the HA and NA glycoproteins, the genes coding for these surface antigens must come from the reassorted viruses or high growth clinical isolates. The attenuated genes are derived from the attenuated parent. In this approach, genes that confer attenuation preferably do not code for the HA and NA glycoproteins. Otherwise, these genes could not be transferred to reassortants bearing the surface antigens of the clinical virus isolate.

Many donor viruses have been evaluated for their ability to reproducibly attenuate influenza viruses. As a non-limiting example, the A/Ann Arbor(AA)/6/60 (H2N2) cold adapted (ca) donor virus can be used for attenuated vaccine production (see, e.g., Edwards, 1994; Murphy, 1993). Additionally, live, attenuated reassortant virus vaccines can be generated by mating the ca donor virus with a virulent replicated virus of the invention. Reassortant progeny are then selected at 25°C, (restrictive for replication of virulent virus), in the presence of an H2N2 antiserum, which inhibits replication of the viruses bearing the surface antigens of the attenuated A/AA/6/60 (H2N2) ca donor virus.

A large series of H1N1 and H3N2 reassortants have been evaluated in humans and found to be satisfactorily: (a) infectious, (b) attenuated for seronegative children and immunologically primed adults, (c) immunogenic and (d) genetically stable. The 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 adults and children.

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 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 (Subbarao et al., 1993). 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 reduction of live attenuated reassortants H1N1 and H3N2 vaccine candidates in a manner analogous to that described above for the A/AA/6/60 ca donor virus. Similarly, other known and suitable attenuated donor strains can be reassorted with influenza virus of the invention to obtain attenuated vaccines suitable for use in the vaccination of mammals (Enami et al., 1990; Muster et al., 1991; Subbarao et al., 1993).

It is preferred that 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, while at the same time lacking infectivity to the degree that the vaccine causes minimal change of inducing a serious pathogenic condition in the vaccinated mammal.

The virus 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 DNA 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. See, e.g., Robertson et al., 1988; Kilbourne, 1969; Aymard-Henry et al., 1985; Robertson et al., 1992.

Pharmaceutical Compositions

Pharmaceutical compositions of the present invention, suitable for inoculation or for parenteral or oral administration, comprise attenuated or inactivated influenza viruses, 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. See, e.g., Berkow et al., 1987; Avery's Drug Treatment, 1987; Osol, 1980; Katzung, 1992. 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, preferably 10 to 15 μg, of hemagglutinin 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 virus of type A, B or C, or any combination thereof, for example, at least two of the three types, at least two of different subtypes, at least two of the same type, at least two of the same subtype, or a different isolate(s) or reassortant(s). Human influenza virus type A includes H1N1, H2N2 and H3N2 subtypes.

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. See, e.g., Berkow et al., 1992; Avery's, 1987; Osol, 1980; and Katzung, 1992.

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. Examples of materials suitable for use in vaccine compositions are provided in Osol (1980).

Heterogeneity in a vaccine may be provided by mixing replicated influenza viruses for at least two influenza virus strains, such as 2-50 strains or any range or value therein. Influenza A or B virus strains having a modern antigenic composition are preferred. According to the present invention, 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. See, e.g., Katzung (1992), and the references cited therein on pages 798-800 and 680-681, respectively.

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 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 of a disease becomes manifest. The prophylactic administration of the composition serves to prevent or attenuate one or more symptoms associated with the disease.

When provided therapeutically, an attenuated or inactivated viral vaccine is provided upon the detection of a symptom of actual infection. The therapeutic administration of the compound(s) serves to attenuate any actual infection. See, e.g., Berkow et al., 1992; Avery, 1987; and Katzung, 1992. When provided therapeutically, a gene therapy composition is provided upon the detection of a symptom or indication of the disease. The therapeutic administration of the compound(s) serves to attenuate a symptom or indication of that disease.

Thus, an attenuated or inactivated vaccine composition of the present invention may thus 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 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 patient. 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 patients. Protection may be limited to mitigating the severity or rapidity of onset of symptoms 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 inactivated or 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 symptom 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 symptom or condition of the disease, or in the total or partial immunity of the individual to the disease.

At least one inactivated or attenuated influenza virus, or composition thereof, of the present invention may be administered by any means that achieve the intended purposes, using a pharmaceutical composition as previously described.

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 by bolus injection or by gradual perfusion over time. A preferred mode of using a pharmaceutical composition of the present invention is by intramuscular or subcutaneous application. See, e.g., Berkow et al., 1992; Avery, 1987; and Katzung, 1992.

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 biological effect. It is understood that the effective dosage will be dependent upon the 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 preferred dose ranges. However, the most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. See, e.g., Berkow et al., 1992; Avery's, 1987; and Katsung, 1992.

The dosage of an attenuated virus vaccine for a mammalian (e.g., human) or avian adult organism can be from about 10^3-10^7 plaque forming units (PFU)/kg, or any range or value therein. The dose of inactivated vaccine can range from about 0.1 to 200, e.g., 50 μg of hemagglutinin 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 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). Each 0.5-ml dose of vaccine preferably contains approximately 1-50 billion virus particles, and preferably 10 billion particles.

The invention will be further described by the following examples.

Example 1

Materials and Methods

Cells and viruses. 293T human embryonic kidney cells and Madin-Darby canine kidney cells (MDCK) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and in modified Eagle's medium (MEM) containing 5% newborn calf serum, respectively. All cells were maintained at 37°C in 5% CO_2. Influenza viruses A/WSN/33 (H1N1) and A/PR/8/34 (H1N1) were propagated in 10-day-old eggs.

Construction of plasmids. To generate RNA polymerase I constructs, cloned cDNAs derived from A/WSN/33 or A/PR/8/34 viral RNA were introduced between the promoter and terminator sequences of RNA polymerase I. Briefly, the cloned cDNAs were amplified by PCR with primers containing *Bsm*BI sites, digested with *Bsm*BI, and cloned into the *Bsm*BI sites of the pHH21 vector which contains the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, separated by *Bsm*BI sites (Figure 2). The PB2, PB1, PA, HA, NP, NA, M, and NS genes of the A/WSN/33 strain were PCR-amplified by use of the following plasmids: pSCWPB2, pGW-PB1, and pSCWPA (all obtained from Dr. Debi Nayak at the University of California Los Angeles), and pWH17, pWNP152, pT3WNA15 (Castrucci et al., 1992), pGT3WM, and pWNS1, respectively. The PB1 gene of influenza A/PR/8/34 virus was amplified by using pcDNA774 (PB1) (Perez et al., 1998) as a template. See Figure 6 for the sequences of the primers. To ensure that the genes were free of unwanted mutations, PCR-derived fragments were sequences with an autosequencer (Applied Biosystem Inc., CA, USA) according to the protocol recommended by the manufacturer. The cDNAs encoding the HA, NP, NA, and M1 genes of A/WSN/33 virus were cloned as described (Huddleston et al., 1982) and subcloned into the eukaryotic expression vector pCAGGS/MCS (controlled by the chicken *β*-actin promoter) (Niwa et al., 1991), resulting in pEWSN-HA, pCAGGS-WSN-NP0-14, pCAGGS-WNA15, and pCAGGS-WSN-M1-2/1, respectively. The M2 and NS2 genes from the A/PR/8/34 virus were amplified by PCR and cloned into pCAGGS/MCS, yielding pEP24c and pCA-NS2. Finally, pcDNA774(PB1), pcDNA762(PB2), and pcDNA787(PA) were used to express the PB2, PB1, and PA proteins under control of the cytomegalovirus promoter (Perez et al., 1998).

Generation of infectious influenza particles. 293T cells (1 × 10^6) were transfected with a maximum of 17 plasmids in different amounts with use of Trans IT LT-1 (Panvera, Madison, Wisconsin) according to the manufacturer's instructions. Briefly, DNA and transfection reagent were mixed (2 μl Trans IT-LT-1 per μg of DNA), incubated at room temperature for 45 minutes and added to the cells. Six hours later, the DNA-transfection reagent mixture was replaced by Opti-MEM (Gibco/BRL, Gaithersburg, Maryland) containing 0.3% bovine serum albumin and 0.01% fetal calf serum. At different times after transfection, viruses were harvested from the supernatant and titrated on MDCK cells. Since helper virus was not required by this procedure, the recovered transfectant viruses were analyzed without plaque purification.

Determination of the percentage of plasmid-transfected cells producing viruses. Twenty-four hours after transfection, 293T cells were dispersed with 0.02% EDTA into single cells. The cell suspension was then diluted 10-fold and transferred to confluent monolayers of MDCK cells in 24-well plates. Viruses were detected by the hemagglutination assay.

Immunostaining assay. Nine hours after infection with influenza virus, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (in PBS) for 20 minutes at room temperature. Next, they were treated with 0.1% Triton X-100 and processed as described by Neumann et al. (1997).

Results

Generation of infectious virus by plasmid-driven expression of viral RNA segments, three polymerase subunits and NP protein. Although transfection of cells with a mixture of RNPs extracted from purified virions results in infectious influenza particles, this strategy is not likely to be efficient when used with eight different *in vitro* generated RNPs. To produce infectious influenza viruses entirely from cDNAs, eight viral RNPs were generated *in vivo*. Thus, plasmids were prepared that contain cDNAs for the full-length viral RNAs of the A/WSN/33 virus, flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator. In principle, transfection of these eight plasmids into eukaryotic cells should result in the synthesis of all eight influenza vRNAs. The PB2, PB1, PA and NP proteins, generated by cotransfection of protein expression plasmids, should then assemble the vRNAs into functional vRNPs that are replicated and transcribed, ultimately forming infectious influenza viruses (Figure 3). 1 × 106 293T cells were transfected with protein expression plasmids (1 μg of pcDNA762(PB2), 1 μg of pcDNA774(PB1), 0.1 μg of pcDNA787(PA), and 1 μg of pCAGGS-WSN-NP0/14) and 1 μg of each of the following RNA polymerase I plasmids (pPolI-WSN-PB2, pPolI-WSN-PB1, pPolI-WSN-PA, pPolI-WSN-HA, pPolI-WSN-NP, pPolI-WSN-NA, pPolI-WSN-M, and pPolI-WSN-NS). The decision to use a reduced amount of pcDNA787(PA) was based on previous observations (Mena et al., 1996), and data on the optimal conditions for generation of virus-like particles (VLPs) (data not shown). Twenty-four hours after transfection of 293T cells, 7 × 103 pfu of virus per ml was found in the supernatant (Experiment 1, Table 1), demonstrating for the first time the capacity of reverse genetics to produce influenza A virus entirely from plasmids.

Table 1. Plasmid sets used to produce influenza virus from cloned cDNA*

		Experiment							
RNA polymerase I plasmids for:†		1	2	3	4	5	6	7	8
	PB1	+	+	-	-	-	-	-	-
	PR8-PB1	-	-	+	+	+	+	+	+
	PB2	+	+	+	+	+	+	+	+
	PA	+	+	+	+	+	+	+	+
	HA	+	+	+	+	+	+	+	+
	NP	+	+	+	+	+	+	+	+
	NA	+	+	+	+	+	+	+	+
	M	+	+	+	+	+	+	+	+
	NS	+	+	+	+	+	+	+	+
Protein expression plasmids for:									
	PB1	+	+	+	+	+		+	+
	PB2	+	+	+	+	+	+	-	+
	PA	+	+	+	+	+	+	-	+
	NP	+	+	+	+	+	+	+	-
	HA	-	+	-	+	+	+	+	+
	NA	-	+	-	+	+	+	+	+
	M1	-	+	-	+	+	+	+	+
	M2	-	+	-	+	+	+	+	+
	NS2	-	+	-	+	+	+	+	+
Virus titer (pfu/ml)		7 × 103	7 × 103	1 × 103	3 × 104	0	0	0	0

* 293T cells were transfected with the indicated plasmids. Twenty-four (Experiments 1 and 2) or forty-eight hours (Experiments 3-8) later, the virus titer in the supernatant was determined in MDCK cells.

† Unless otherwise indicated, plasmids were constructed with cDNAs representing the RNAs of A/WSN/33 virus.

Efficiency of influenza virus production with coexpression of all viral structural proteins. Although expression of the viral NP and polymerase proteins is sufficient for the plasmid-driven generation of influenza viruses, it was possible that the efficiency could be improved. In previous studies, the expression of all influenza virus structural proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, and NS2) resulted in VLPs that contained an artificial vRNA encoding a reporter chloramphenicol-acetyltransferase gene (Mena et al., 1996). Thus, the availability of the entire complement of structural proteins, instead of only those required for viral RNA replication and transcription, might improve the efficiency of virus production. To this end, 293T cells were transfected with optimal amounts of viral protein expression plasmids (as judged by VLP production; unpublished data): 1 μg of pcDNA762(PB2) and pcDNA774(PB1); 0.1 μg of pcDNA787(PA); 1 μg of pEWSN-HA, pCAGGS-WSN-NP0/14, and pCAGGS-WNA15; 2 μg of pCAGGS-WSN-M1-2/1; 0.3 μg of pCA-NS2; and 0.03 μg of pEP24c (for M2), together with 1 μg of each RNA polymerase I plasmid (Experiment 2, Table 1). A second set of cells was transfected with the same set of RNA polymerase I plasmids, with the exception of the PB1 gene, for which pPolI-PR/8/34-PB1 was substituted in an effort to generate a reassortant virus, together with plasmids expressing only PA, PB1, PB2, and NP (Experiment 3, Table 1) or those expressing all the influenza structural proteins (Experiment 4, Table 1). Yields of WSN virus did not appreciably differ at 24 hours (Experiments 1 and 2, Table 1) or at 36 hours (data not shown) post-transfection. However, more than a 10-fold increase in yields of the virus with PR/8/34-PB1 was found when all the influenza viral structural proteins were provided (Experiments 3 and 4, Table 1). Negative controls, which lacked one of the plasmids for the expression of PA, PB1, PB2, of NP proteins, did not yield any virus (Experiments 5-8, Table 1). Thus, depending on the virus generated, expression of all influenza A virus structural proteins appreciably improved the efficiency of the reverse genetics method.

Next, the kinetics of virus production after transfection of cells was determined using the set of plasmids used to generate a virus with the A/PR/8/34-PB1 gene. In two of three experiments, virus was first detected at 24 hours after transfection. The titer measured at that time, >103 pfu/ml, had increased to >106 pfu/ml by 48 hours after transfection (Table 2). To estimate the percentage of plasmid-transfected cells that were producing viruses, 293T cells were treated with EDTA (0.02%) at 24 hours after transfection to disperse the cells, and then performed limiting dilution studies. In this experiment, no free virus was found in the culture supernatant at this time point. The results indicated that 1 in 103.3 cells was generating infectious virus particles.

Table 2. Kinetics of virus production after plasmid transfection into 293T cells*

Hours after plasmid transfection	Virus titers in culture supernatant (pfu/ml)		
	Experiment		
	1	2	2
6	0	ND	ND

12	0	ND	0
18	0	ND	0
24	0	2×10^3	6×10^3
30	ND	5×10^4	9×10^4
36	6×10^2	$> 1 \times 10^5$	7×10^5
42	ND	$> 1 \times 10^6$	5×10^6
48	8×10^4	$> 1 \times 10^6$	1×10^7

* 293T cells were transfected with eight RNA polymerase I plasmids encoding A/WSN/33 virus genes with the exception of PB1 gene, which is derived from A/PR/8/34 virus, and nine protein expression plasmids as described in the text. At different time points, we titrated virus in the culture supernatant in MDCK cells. ND = not done.

Recovery of influenza virus containing the FLAG epitope in the NA protein. To verify that the new reverse genetics system allowed the introduction of mutations into the genome of influenza A viruses, a virus containing a FLAG epitope (Castrucci et al., 1992) in the NA protein was generated. 293T cells were transfected with an RNA polymerase I plasmid (pPolI-WSN-NA/FL79) that contained a cDNA encoding both the NA protein and a FLAG epitope at the bottom of the protein's head, together with the required RNA polymerase I and protein expression plasmids. To confirm that the recovered virus (PR8-WSN-FL79) did in fact express the NA-FLAG protein, immunostaining assays of cells infected with PR8-WSN-FL79 or A/WSN/33 wild-type virus was performed. A monoclonal antibody to the FLAG epitope detected cells infected with PR8-WSN-FL79, but not those infected with wild-type virus. Recovery of the PR8-WSN-FL79 virus was as efficient as that for the untagged wild-type virus (data not shown). These results indicate that the new reverse genetics system allows one to introduce mutations into the influenza A virus genome.

Generation of infectious influenza virus containing mutations in the PA gene. To produce viruses possessing mutations in the PA gene, two silent mutations were introduced creating new recognition sequences for restriction endonucleases (Bsp120I at position 846 and PvuII at position 1284 of the mRNA). Previously, it was not possible to modify this gene by reverse genetics, because of the lack of a reliable selection system. Transfectant viruses, PA-T846C and PA-A1284 were recovered. The recovered transfectant viruses were biologically cloned by two consecutive limiting dilutions. To verify that the recovered viruses were indeed transfectants with mutations in the PA gene, cDNA for the PA gene was obtained by reverse transcriptase-PCR. PA-T846C and PA-A1284C viruses had the expected mutations within the PA gene, as demonstrated by the presence of the newly introduced restriction sites. PCR of the same viral samples and primers without the reverse transcription step failed to produce any products (data not shown), indicating that the PA cDNA was indeed originated from vRNA instead of the plasmid used to generate the viruses. These results illustrate how viruses with mutated genes can be produced and recovered without the use of helper viruses.

Discussion

The reverse genetics systems described herein allows one to efficiently produce influenza A viruses entirely from cloned cDNAs. Bridgen and Elliott (1996) also used reverse genetics to generate a Bunyamwera virus (*Bunyaviridae* family), but it contains only three segments of negative-sense RNA, and the efficiency of its production was low, 10^2 pfu/10^7 cells. Although the virus yields differed among the experiments, consistently > 10^3 pfu/106 cells was observed for influenza virus, which contains eight segments. There are several explanations for the high efficiency of the reverse genetics system described hereinabove. Instead of producing RNPs *in vitro* (Luytjes et al., 1989), RNPs were generated *in vivo* through intracellular synthesis of vRNAs using RNA polymerase I and through plasmid-driven expression of the viral polymerase proteins and NP. Also, the use of 293T cells, which are readily transfected with plasmids (Goto et al., 1997), ensured that a large population of cells received all of the plasmids needed for virus production. In addition, the large number of transcripts produced by RNA polymerase I, which is among the most abundantly expressed enzymes in growing cells, likely contributed to the overall efficiency of the system. These features led to a correspondingly abundant number of vRNA transcripts and adequate amounts of viral protein for encapsidation of vRNA, formation of RNPs in the nucleus, and export of these complexes to the cell membrane, where new viruses are assembled and released.

Previously established reverse genetics systems (Enami et al., 1990; Neumann et al., 1994; Luytjes et al., 1989; Pleschka et al., 1996) require helper-virus infection and therefore selection methods that permit a small number of transfectants to be retrieved from a vast number of helper viruses. Such strategies have been employed to generate influenza viruses that possess one of the following cDNA-derived genes: PB2 (Subbarao et al., 1993), HA (Enami et al., 1991: Horimoto et al., 1994), NP (Li et al., 1995), NA (Enami et al., 1990), M (Castrucci et al., 1995; Yasuda et al., 1994), and NS (Enami et al., 1991). Most of the selection methods, except for those applicable to the HA and NA genes, rely on growth temperature, host range restriction, or drug sensitivity, thus limiting the utility of reverse genetics for functional analysis of the gene products. Even with the HA and NA genes, for which reliable antibody-driven selection systems are available, it is difficult to produce viruses with prominent growth defects. In contrast, the reverse genetics system described herein does not require helper virus and permits one to generate transfectants with mutations in any gene segment or with severe growth defects. Having the technology to introduce any viable mutation into the influenza A virus genome enables investigators to address a number of long-standing issues, such as the nature of regulatory sequences in nontranslated regions of the viral genome, structure-function relationships of viral proteins, and the molecular basis of host-range restriction and viral pathogenicity.

Although inactivated influenza vaccines are available, their efficacy is suboptimal due partly to their limited ability to elicit local IgA and cytotoxic T cell responses. Clinical trials of cold-adapted live influenza vaccines now underway suggest that such vaccines are optimally attenuated, so that they will not cause influenza symptoms, but will still induce protective immunity (reviewed in Keitel & Piedra, 1998). However, preliminary results indicate that these live virus vaccines will not be significantly more effective than the best inactivated vaccine (reviewed in Keitel & Piedra, 1998), leaving room for further improvement. One possibility would be to modify a cold-adapted vaccine with the reverse genetics system described above. Alternatively, one could start from scratch by using reverse genetics to produce a “master” influenza A strain with multiple attenuating mutations in the genes that encode internal proteins. The most intriguing application of the reverse genetics system described herein may lie in the rapid production of attenuated live-virus vaccines in cases of suspected pandemics involving new HA or NA subtypes of influenza virus.

This new reverse genetics system will likely enhance the use of influenza viruses as vaccine vectors. The viruses can be engineered to express foreign proteins or immunogenic epitopes in addition to the influenza viral proteins.

One could, for example, generate viruses with foreign proteins as a ninth segment (Enami et al., 1991) and use them as live vaccines. Not only do influenza viruses stimulate strong cell-mediated and humoral immune responses, but they also afford a wide array of virion surface HA and NA proteins (e.g., 15 HA and 9 NA subtypes and their epidemic variants), allowing repeated immunization of the same target population.

Influenza VLPs possessing an artificial vRNA encoding a reporter gene have been produced by expressing viral structural proteins and vRNA with the vaccinia-T7 polymerase system (Mena et al., 1996). Using reverse genetics, one can now generate VLPs containing vRNAs that encode proteins required for vRNA transcription and replication (i.e., PA, PB1, PB2, and NP), as well as vRNAs encoding proteins of interest. Such VLPs could be useful gene delivery vehicles. Importantly, their lack of genes encoding viral structural proteins would ensure that infectious viruses will not be produced after VLP-gene therapy. Since the influenza virus genome is not integrated into host chromosome, the VLP system would be suitable for gene therapy in situations requiring only short-term transduction of cells (e.g., for cancer treatment). In contrast to adenovirus vectors (Kovesdi et al., 1997), influenza VLPs could contain both HA and NA variants, allowing repeated treatment of target populations.

The family *Orthomyxoviridae* comprises influenza A, B, and C viruses, as well as the recently classified Thogotovirus. The strategy for generating infectious influenza A viruses entirely from cloned cDNAs described herein would apply to any orthomyxovirus, and perhaps to other segmented negative-sense RNA viruses as well (e.g., *Bunyaviridae, Arenaviridae*). The ability to manipulate the viral genome without technical limitations has profound implications for the study of viral life cycles and their regulation, the function of viral proteins and the molecular mechanisms of viral pathogenicity.

Example 2

To develop a reverse genetics system for influenza A/Puerto Rico/8/34, viral RNA was extracted from the allantoic fluid of A/Puerto Rico/8/34 (H1N1), Madison high grower variant (PR8HG), using RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using MMLV-

RTase (Promega) and Uni12 primer. The cDNAs were amplified overnight by PCR using the following:

Primer sets

PB1: Ba PB1-1 and PB1-1735R (front fragment) and PB1-903 and Ba-PB1-2341R (rear fragment)

Ba-PB1-1 CACACACGGTCTCCGGGAGCGAAAGCAGGCA (SEQ ID NO:9)
173PB1-1735R GGGTTTGTATTTGTGTGTCACC (SEQ ID NO:10)

233PB1-903 CCAGGACACTGAAATTTCTTTCAC (SEQ ID NO:11)
Ba-PB1-2341R CACACAGGTCTCCTATTAGTAGAAACAAGGCATTT (SEQ ID NO:12)

PB2: Ba PB2-1 and B2 1260R (front fragment) and WSN PB2 seq-2 and Ba-PB2-2341R (rear fragment)

Ba-PB2-1 CACACAGGTCTCCGGGAGCGAAAGCAGGTC (SEQ ID NO:13)
B2 1260R CACACACGTCTCCATCATACAATCCTCTTG (SEQ ID NO:14)

WSN PB2 seq-2 CTCCTCTGATGGTGGCATAC (SEQ ID NO:15)
Ba-PB2-2341R CACACAGGTCTCCTATTAGTAGAAACAAGGTCGTTT (SEQ ID NO:16)

PA:

Bm-PA-1 CACACACGTCTCCGGGAGCGAAAGCAGGTAC (SEQ ID NO:17)
Bm-PA-2233R CACACACGTCTCCTATTAGTAGAAACAAGGTACTT (SEQ ID NO:18)

HA:

Bm-HA-1: CACACACGTCTCCGGGAGCAAAAGCAGGGG (SEQ ID NO:19)
Bm-NS-890R: CACACACGTCTCCTATTAGTAGAAACAAGGGTGTTTT (SEQ ID NO:20)

NP:

Bm-NP-1 CACACACGTCTCCGGGAGCAAAAGCAGGGTA (SEQ ID NO:21)

Bm-NP-1565R
CACACACGTCTCCTATTAGTAGAAACAAGGGTATTTTT (SEQ ID NO:22)

NA:

Ba-NA-1: CACACAGGTCTCCGGGAGCAAAAGCAGGAGT (SEQ ID NO:23)
Ba-NA-1413R:
CACACAGGTCTGGTATTAGTAGAAACAAGGAGTTTTTT (SEQ ID NO:24)

M:

Bm-M-1 CACACACGTCTCCGGGAGCAAAAGCAGGTAG (SEQ ID NO:25)
Bm-M-1027R
CACACACGTCTCCTATTAGTAGAAACAAGGTAGTTTTT (SEQ ID NO:26)

NS:

Bm-NS-1 CACACACGTCTCCGGGAGCAAAAGCAGGGTG (SEQ ID NO:27)
Bm-NS-890R
CACACACGTCTCCTATTAGTAGAAACAAGGGTGTTTT (SEQ ID NO:28)

DNA polymerase: *pfu* Native DNA polymerase (Stratagene)

The PCR products were separated by gel electrophoresis and extracted from the agarose gel using a gel extraction kit (Qiagen). The extracted genes were ligated into pT7Blue blunt vector (Novagen) using a Takara ligation kit ver. II (Takara). After 5 hours, the ligated genes were transformed into JM109 (PB2, M, and NS genes) or DH5alpha (PA, PB1, and NP). Six colonies for each gene were cultured in TB for 8 hours. The plasmids were extracted from the bacteria culture, and four clones per gene were sequenced.

The PA, NP, M, and NS genes in pT7Blue were excised by *Bsm* BI enzyme (New England Biolabs). The PB1 gene was excised by *Bsa* I (New England Biolabs). The excised genes were ligated overnight with pPolIR vector which contains the human RNA polymerase I promoter and the mouse RNA polymerase I terminator which had been digested with *Bsm* BI. The front fragment of the PB2 gene in pT7Blue was excised by *Bsr* GI (New England Biolabs) and *Bam* HI (Roche), and the rear fragment was excised by *Bsr* GI (New England Biolabs) and *Spe* I (Roche). The excised fragments were mixed and digested by *Bsa* I. After 6 hours, the digested genes were purified using a PCR purification kit (Qiagen) and ligated overnight between the *Bsm* BI sites of the pPolIR vector.

The ligated PB1, PA, NP, M, and NS-pPolIR genes were used to transform JM109 (M and NS genes) or DH5alpha (PB1, PA and NP genes) overnight. The colonies of transformed bacteria were cultured in LB overnight. The ligated PB2-pPolIR was used to transform JM109 overnight.

The plasmids were extracted from the bacterial cultures and gene inserts were confirmed by enzyme digestion. The colonies of bacteria transformed by PB2-PolIR were cultured in LB for 8 hours. The plasmids were then extracted and the gene insertion was confirmed by enzyme digestion. All pPolI constructs were sequenced to ensure that they did not contain unwanted mutations.

The pPolIR constructs for PR8HG were transfected into 293T human embryonic kidney cells with A/WSN/33(WSN)-HA and NA, A/Hong Kong/483/97(HK)-HAavir and NA, or A/Kawasaki/01(Kawasaki)-HA and NA PolI constructs and four protein-expression constructs for the polymerase proteins and NP of A/WSN/33. The supernatants from transfected 293T cells were serially diluted (undiluted to 10^{-7}) and infected into the allantoic cavities of 9-day-old embryonated chicken eggs. The allantoic fluids of the infected eggs were harvested and their virus titers tested by HA assay (Table 3).

Table 3

Virus possessing PR8 genes together with the following HA and NA genes	HA titer (HAU/ml) of allantoic fluid from eggs inoculated with 293T supernatants diluted at:							
	undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
WSN-HA NA	<1	<1	200	<1	<1	<1	<1	<1
HK-HAavir NA	100	<1	<1	<1	<1	<1	<1	<1
Kawasaki-HA NA	<1	<1	<1	<1	<1	<1	<1	<1

HA-positive samples (virus with WSN-HA NA at 10^{-2} and virus with HK-HAavir NA at undiluted) were diluted serially from 10^{-2} to 10^{-8} and 100ul of each dilution was infected into embryonated chicken eggs. The allantoic fluids of the infected eggs were harvested and their virus titers tested by HA assay

(Table 4). The 50% egg infectious dose (EID_{50}) of A/Puerto Rico/8/34 (H1N1) prepared from plasmids was $10^{10.33}$/ml, and the HA titer was 1:3200.

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 PR8HG was prepared. The titer of the recombinant virus was $10^{10.67}$ EID_{50}/ml, and the HA titer was 1:1600

Table 4

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

Sequences of PR8 genes:

PA

AGCGAAAGCA GGTACTGATC CAAAATGGAA GATTTTGTGC GACAATGCTT
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
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
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
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
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
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
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
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
TGCCTGATTA ATGATCCCTG GGTTTTGCTT AATGCTTCTT GGTTCAACTC
CTTCCTTACA CATGCATTGA GTTAGTTGTG GCAGTGCTAC TATTTGCTAT
CCATACTGTC CAAAAAAGTA CCTTGTTTCT ACT
(SEQ ID NO:1)

PB1

AGCGAAAGCA GGCAAACCAT TTGAATGGAT GTCAATCCGA
CCTTACTTTT CTTAAAAGTG CCAGCACAAA ATGCTATAAG CACAACTTTC
CCTTATACTG GAGACCCTCC TTACAGCCAT GGGACAGGAA CAGGATACAC
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
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ACCCTGAACA CAATGACCAA AGATGCTGAG AGAGGGAAGC TAAAACGGAG
AGCAATTGCA ACCCCAGGGA TGCAAATAAG GGGGTTTGTA TACTTTGTTG

AGACACTGGC AAGGAGTATA TGTGAGAAAC TTGAACAATC AGGGTTGCCA
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GATGACCAAT TCTCAGGACA CCGAACTTTC TTTCACCATC ACTGGAGATA
ACACCAAATG GAACGAAAAT CAGAATCCTC GGATGTTTTT GGCCATGATC
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ATCCTGAATC TTGGACAAAA GAGATACACC AAGACTACTT ACTGGTGGGA
TGGTCTTCAA TCCTCTGACG ATTTTGCTCT GATTGTGAAT GCACCCAATC
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
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
CGTTTATGCA ACCCACTGAA CCCATTTGTC AGCCATAAAG AAATTGAATC
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T
(SEQ ID NO:2)

PB2

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GAGCAAGGAC
AAACTTTATG GAGTAAAATG AATGATGCCG GATCAGACCG
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GGGAATGATT GGGATATTGC CCGACATGAC TCCAAGCATC GAGATGTCAA
TGAGAGGAGT GAGAATCAGC AAAATGGGTG TAGATGAGTA CTCCAGCACG
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CACCAAAGCA AAGTAGAATG CAGTTCTCCT CATTTACTGT GAATGTGAGG
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TAACTGAAGA CCCAGATGAA GGCACAGCTG GAGTGGAGTC CGCTGTTCTG
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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
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TCAGTGATTA TGAGGGACGG TTGATCCAAA ACAGCTTAAC AATAGAGAGA
ATGGTGCTCT CTGCTTTTGA CGAAAGGAGA AATAAATACC TTGAAGAACA
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GTGGGATCAA TGATCGGAAC TTCTGGAGGG GTGAGAATGG ACGAAAAACA
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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
ATGCCATTCT GCCGCATTTG AAGATCTAAG AGTATTAAGC TTCATCAAAG
GGACGAAGGT GCTCCCAAGA GGGAAGCTTT CCACTAGAGG AGTTCAAATT
GCTTCCAATG AAAATATGGA GACTATGGAA TCAAGTACAC TTGAACTGAG
AAGCAGGTAC TGGGCCATAA GGACCAGAAG TGGAGGAAAC ACCAATCAAC
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AGAAATCTCC CTTTTGACAG AACAACCATT ATGGCAGCAT TCAATGGGAA
TACAGAGGGG AGAACATCTG ACATGAGGAC CGAAATCATA AGGATGATGG
AAAGTGCAAG ACCAGAAGAT GTGTCTTTCC AGGGGCGGGG AGTCTTCGAG
CTCTCGGACG AAAAGGCAGC GAGCCCGATC GTGCCTTCCT TTGACATGAG
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AAAGAAAAAT ACCCTTGTTT CTACT
(SEQ ID NO:4)

M

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CACTATTGCC
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(SEQ ID NO:5)

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CTACT (SEQ ID NO:8)

Example 3

Influenza virus A/Hong Kong/213/2003 (H5N1, HK213) replicates systemically in chickens, causing lethal infection. Furthermore, this virus is lethal to chicken embryos. Thus, although its surface proteins are highly related to the currently circulating pathogenic avian influenza viruses, HK213 cannot be used as a vaccine strain as attempts to grow it in embryonated chicken eggs result in the production of poor-quality allantoic fluid. Additionally, the use of this highly virulent virus in the production of vaccines is unsafe for vaccine workers. To test the feasibility of using A/PR/8/34 as a master vaccine strain, the cleavage site of the hemagglutinin (HA) gene of HK213 (containing multiple basic amino acids) was mutated from a virulent to an avirulent phenotype (from RERRRKKR (SEQ ID NO:9) to ----TETR). A virus containing the mutated HA gene produced non-lethal, localized infection in chickens. Additionally, the mutated virus was non-lethal to chicken embryos. Thus, growth of the mutated virus in embronated eggs yielded high-quality allantoic fluid, and in this attenuated form, the virus is safe for vaccine producers.

A recombinant virus containing the neuraminidase (NA) and mutated HA genes from HK213, and all the remaining genes from high-titer A/PR/8/34 (H1N1, HG-PR8) virus (Example 2), which grows 10 times better than other A/PR/8/34 PR8 strains in eggs (10^{10} EID_{50}/ml; HA titer: 1:8,000), was generated in embryonated chicken eggs. This recombinant virus, which expresses surface proteins related to the currently circulating pathogenic avian influenza virus, grew to high titers in embryonated chicken eggs (Figure 4). Thus, replacement of the HA and NA genes of HG-PR8 with those of a currently circulating strain of influenza virus resulted in a vaccine strain that can be safely produced, and demonstrates the use of PR8-HG as a master vaccine strain.

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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 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.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition capable of producing influenza virus comprising a plurality of influenza virus vectors, comprising:

a) vectors capable of producing vRNA comprising a promoter operably linked to an influenza virus PA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB1 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus PB2 cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus HA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NP cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus NA cDNA linked to a transcription termination sequence, a vector comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, or a vector comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, wherein the cDNA for PB1, PB2, PA, NP, M and NS includes sequences that correspond to sequences that encode a polypeptide having at least 90% amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID NOs: 1-6, wherein the cDNAs for HA and NA do not have sequences corresponding to SEQ ID NOs: 7 and 8, and wherein the vectors of a) comprise a RNA polymerase I promoter and a RNA polymerase I terminator; and

b) vectors capable of producing mRNA comprising a vector comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, or a vector comprising a promoter operably linked to a DNA segment encoding influenza virus NP.

2. The composition of claim 1 wherein the vectors of b) further include a vector comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector comprising a promoter operably linked to a DNA

segment encoding influenza virus NA, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus M2, or a vector comprising a promoter operably linked to a DNA segment encoding influenza virus NS2.

3. The composition of claim 1 wherein the HA is a type A HA.

4. The composition of claim 1 wherein the HA is a type B HA.

5. The composition of claim 1 wherein the RNA polymerase I promoter is a human RNA polymerase I promoter.

6. The composition of claim 1 wherein all of the vectors of a) comprise a RNA polymerase II promoter.

7. The composition of claim 1 wherein each vector of a) is on a separate plasmid.

8. The composition of claim 1 wherein each vector of b) is on a separate plasmid.

9. The composition of claim 1 wherein the each of the vectors of b) further comprise a RNA transcription termination sequence.

10. The composition of claim 1 further comprising a vector comprising a promoter linked to 5' influenza virus sequences comprising 5' influenza virus noncoding sequences linked to a cDNA of interest linked to 3' influenza virus sequences comprising 3' influenza virus noncoding sequences linked to a transcription termination sequence.

11. The composition of claim 10 wherein the cDNA of interest is in the sense orientation.

12. The composition of claim 10 wherein the cDNA of interest is in the antisense orientation.

13. The composition of claim 10 wherein the cDNA of interest comprises an open reading frame encoding an immunogenic polypeptide or peptide of a pathogen or a therapeutic polypeptide or peptide.

14. A method to prepare influenza virus, comprising: contacting a cell with the composition of claim 1 in an amount effective to yield infectious influenza virus.

15. The method of claim 14 further comprising isolating the virus.

16. A method to prepare a gene delivery vehicle, comprising: contacting cells with the composition of claim 10 in an amount effective to yield influenza virus, and isolating the virus.

17. A method to prepare influenza virus, comprising contacting a cell with a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus PA cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus PB1 cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus PB2 cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus HA cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus NP cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus NA cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus M cDNA linked to a transcription termination sequence, a vector capable of producing vRNA comprising a promoter operably linked to an influenza virus NS cDNA linked to a transcription termination sequence, a

vector capable of producing mRNA comprising a promoter operably linked to a DNA segment encoding influenza virus PA, a vector capable of producing mRNA comprising a promoter operably linked to a DNA segment encoding influenza virus PB1, a vector capable of producing mRNA comprising a promoter operably linked to a DNA segment encoding influenza virus PB2, and a vector capable of producing mRNA comprising a promoter operably linked to a DNA segment encoding influenza virus NP, so as to yield infectious virus, wherein the cDNA encoding PB1, PB2, PA, NP, M, and NS includes sequences that correspond to sequences that encode a polypeptide having at least 90% amino acid sequence identity to a corresponding polypeptide encoded by one of SEQ ID NOs:1-6, wherein the cDNAs for HA and NA do not have sequences corresponding to SEQ ID NO:7 and 8, and wherein the vectors for vRNA production comprise a RNA polymerase I promoter and a RNA polymerase I terminator.

18. The method of claim 17 further comprising a vector comprising a promoter operably linked to a DNA segment encoding influenza virus HA, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus NA, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus M1, a vector comprising a promoter operably linked to a DNA segment encoding influenza virus M2, and a vector comprising a promoter operably linked to a DNA segment encoding influenza virus NS2.

19. The method of claim 17 or claim 18 further comprising a vector comprising a promoter linked to 5' influenza virus sequences comprising 5' influenza virus noncoding sequences linked to a cDNA of interest or a fragment thereof linked to 3' influenza virus sequences comprising 3' influenza virus noncoding sequences linked to a transcription termination sequence.

20. The method of claim 19 wherein the cDNA of interest comprises an open reading frame encoding an immunogenic polypeptide or peptide of a pathogen or a therapeutic polypeptide or peptide.

21. The method of claim 19 wherein the cDNA of interest is in the sense orientation.

22. The method of claim 19 wherein the cDNA of interest is in the antisense orientation.

23. The method of claim 17 further comprising isolating the virus.

24. The composition of claim 1 wherein the cDNA for HA is a H5 subtype.

25. The composition of claim 1 wherein the cDNA for PB1, PB2, PA, NP, M, and NS has at least 90% nucleotide sequence identity to SEQ ID NOs:1-6 or the complement thereof.

26. The method of claim 17 wherein the cDNA for HA is a H5 subtype.

27. The method of claim 17 wherein the cDNA for PB1, PB2, PA, NP, M, and NS has at least 90% nucleotide sequence identity to SEQ ID NOs:1-6 or the complement thereof.

28. The method of claim 17 wherein the cDNA for PB1, PB2, PA, NP, M, and NS encodes a polypeptide with one or more conservative substitutions relative to a corresponding polypeptide encoded by one of SEQ ID NOs: 1-6.

29. The composition of claim 24 wherein the H5 HA is a mutant H5 with an avirulent cleavage site.

30. The composition of claim 1 wherein the cDNA for PB1, PB2, PA, NP, M, and NS encodes a polypeptide with one or more conservative substitutions relative to a corresponding polypeptide encoded by one of SEQ ID NOs: 1-6.

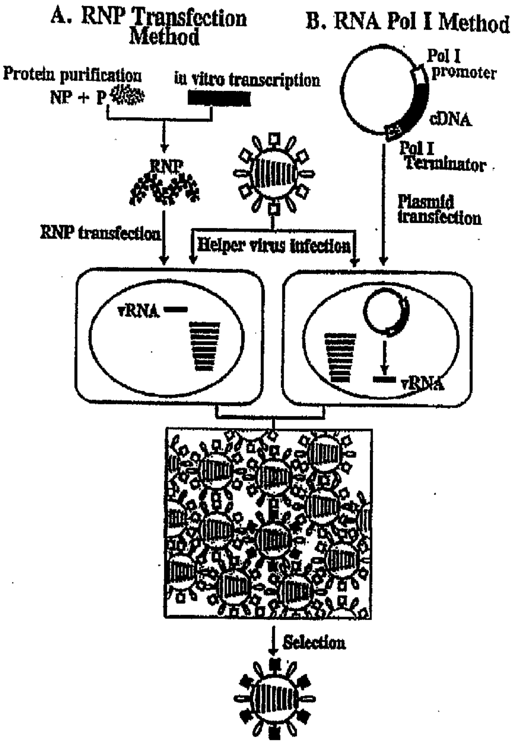
31. The composition of claim 1 wherein the cDNAs for PB1, PB2, PA, NP, M, and NS have a coding region for a polypeptide encoded by one of SEQ ID NOs:1-6.

32. The composition of claim 1 wherein the cDNAs for PB1, PB2, PA, NP, M, and NS correspond to SEQ ID NOs:1-6.

33. An isolated reassortant influenza virus comprising viral genomic RNAs corresponding to the same nucleotide sequences as SEQ ID NOs:1-6, and viral genomic RNAs for HA and NA that do not have sequences corresponding to sequences in SEQ ID NOs:7-8, wherein the viral genomic RNA for HA is for a H5 subtype, and wherein the reassortant virus grows in eggs to a titer of at least 10^{10} EID_{50}/ml.

34. The isolated reassortant influenza virus of claim 33 wherein the H5 HA is a mutant H5.

35. The composition of claim 1, the method of claim 14, 16 or 17, or isolated reassortant influenza virus of claim 33, substantially as hereinbefore described with reference to the accompanying examples and/or figures.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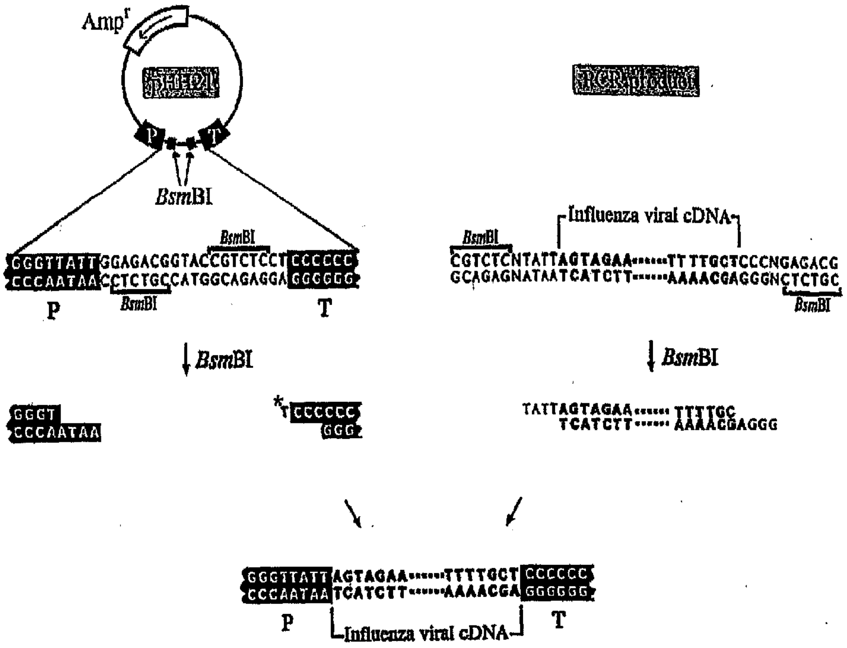
vRNA
Selection

Fig. 1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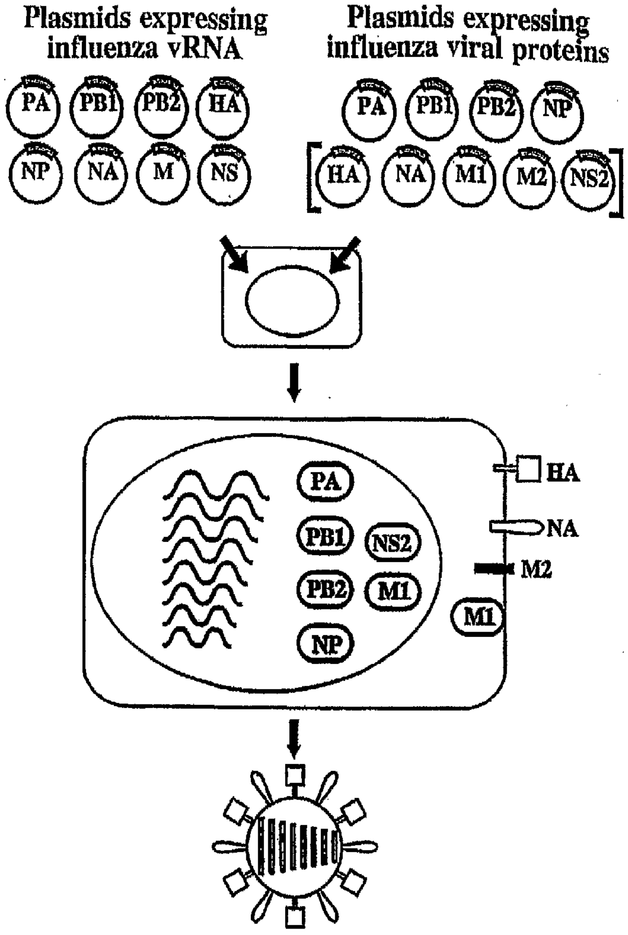
Influenza viral cDNA
T

Fig. 2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PA
PB1
PB2
HA
NP
NA
M
NS
Plasmids expressing influenza viral proteins
PA
PB1
PB2
NP
HA
NA
M1
M2
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HA
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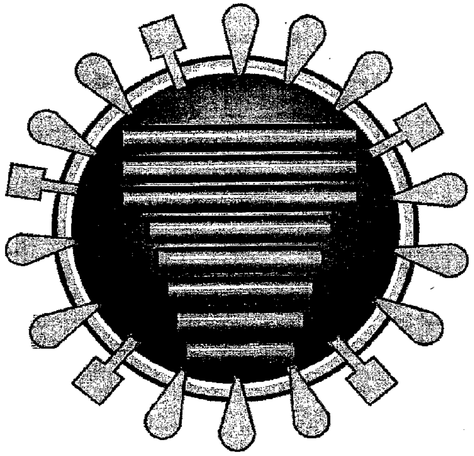
M2

Fig. 3

A/PR/8/34 (H1N1)



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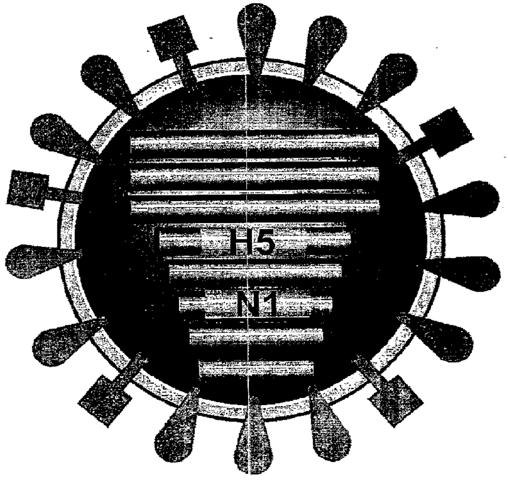
HA titer: 1:8,000

H5N1-PR8



10^{10} EID_{50}/ml
HA titer: 1:3,200

SEQUENCE LISTING

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Therapy

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<210> 2
<211> 2341
<212> DNA
<213> Influenza virus

<400> 2
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<210> 3
<211> 2341
<212> DNA
<213> Influenza virus

<400> 3
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aagaagtaca catcaggaag acaggagaag aacccagcac ttaggatgaa atggatgatg     180
gcaatgaaat atccaattac agcagacaag aggataacgg aaatgattcc tgagagaaat     240
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cctgtccatt ttagaaacca agtcaaaata cgtcggagag ttgacataaa tcctggtcat     480
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t                                                                    2341
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<210> 4

<211> 1565

<212> DNA

<213> Influenza virus

<400> 4

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agagcatccg tcggaaaaat gattggtgga attggacgat tctacatcca aatgtgcacc     180
gaactcaaac tcagtgatta tgagggacgg ttgatccaaa acagcttaac aatagagaga     240
atggtgctct ctgcttttga cgaaaggaga aataaatacc ttgaagaaca tcccagtgcg     300
gggaaagatc ctaagaaaac tggaggacct atatacagga gagtaaacgg aaagtggatg     360
agagaactca tcctttatga caaagaagaa ataaggcgaa tctggcgcca agctaataat     420
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ctact                                                                1565

<210> 5
<211> 1027
<212> DNA
<213> Influenza virus

<400> 5
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ttctact 1027

<210> 6
<211> 890
<212> DNA
<213> Influenza virus

<400> 6
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<210> 7
<211> 1775
<212> DNA
<213> Influenza virus

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<210> 8

<211> 1413

<212> DNA

<213> Influenza virus

<400> 8

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<210> 9
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> A synthetic primer

<400> 9
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<210> 10
<211> 22
<212> DNA
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<220>
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