Bioengineered 4:1, 9–14; January/February 2013; © 2013 Landes Bioscience

Engineering influenza viral vectors

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Keywords: influenza virus, reverse genetics, viral vector, gene delivery, pseudo-typed virus, microRNA delivery, vaccine, gene therapy

The influenza virus is a respiratory pathogen with a negative-sense, segmented RNA genome. Construction of recombinant influenza viruses in the laboratory was reported starting in the 1980s. Within a short period of time, pioneer researchers had devised methods that made it possible to construct influenza viral vectors from cDNA plasmid systems. Herein, we discuss the evolution of influenza virus reverse genetics, from helper virus-dependent systems, to helper virus-independent 17-plasmid systems, and all the way to 3- and 1-plasmid systems. Successes in the modification of different gene segments for various applications, including vaccine and gene therapies are highlighted.

Introduction

The influenza virus is a member of the family Orthomyxoviridae and contains a segmented RNA genome of negative polarity. The genome of the influenza A virus has eight gene segments which encode a minimum of 10 proteins, but some encode 11 or 12 proteins.[1-4] Each gene segment codes at least one protein and includes 5' and 3' terminal non-coding sequences. The 5' and 3' terminals contain 13 and 12 extremely conserved nucleotides, respectively.[5] The non-coding sequences and adjacent nucleotides serve as a viral packaging signal and are necessary for viral RNA (vRNA) replication and virion assembly.[6-9] In the course of influenza viral infection, all RNA gene segments are synthesized in the cell nucleus where they are transported following viral entry, fusion and uncoating along with ribonucleoproteins: PB1, PB2, PA and nucleoprotein (NP).[10] The conserved 13 nucleotides at the 5' end and 12 nucleotides at the 3' end of vRNA form hairpin loops.[11] The loop is critical for the initiation of RNA replication. The vRNA serves as a template for both mRNA and cRNA. Influenza virus mRNA synthesis is dependent on host cellular RNA polymerase II (*Pol*II) activity,[12,13] but vRNA and cRNA are synthesized by the ribonucleoprotein. *Pol*II is an enzyme found in eukaryotic cells that catalyzes the transcription of DNA to synthesize precursors of mRNA. In contrast to mRNAs, newly synthesized cRNAs and vRNAs are encapsidated.[14] Following virus replication, newly formed RNP complexes are assembled in the nucleus and exported to the cytoplasm. The elucidation of RNA replication and transcription of influenza virus has made the generation of influenza viruses from DNA a reality.

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Submitted: 07/16/12; Revised: 08/22/12; Accepted: 08/23/12
http://dx.doi.org/10.4161/bioe.21950

Influenza Virus Reverse Genetics

Techniques for influenza virus reverse genetics have developed dramatically since its conception, as illustrated in **Figure 1**. Attempts to generate influenza virus in the laboratory have been reported since 1980. Early studies show that vRNP complexes are sufficient to transcribe and replicate influenza viral RNA in vitro.[15] Soon after, Palese and colleagues pioneered a new era of recombinant RNA viruses from cloned cDNA.[16] In the early influenza reverse genetics system, a helper virus was needed to provide the remaining vRNPs, but the main disadvantages of this method were that the majority of viral progeny were helper viruses and low efficient generation of progeny. Selection of the recombinant viruses would require a lot of labor.[17,18] Hobom and colleagues brought light of the possibility of using influenza reverse genetics by manipulating RNA polymerase I (*Pol*I).[19] RNA *Pol*I is abundant in eukaryotic cells and transcribes ribosomal RNA (rRNA), which like influenza vRNA, does not contain a 5'-cap or 3' poly (A) structures. Thus, a new system was established to generate influenza viral RNAs.[19] However, selection systems were still required to isolate recombinant virus from great amounts of helper virus.

A breakthrough in influenza viral reverse genetics came in 1999 when Neumann's group and Fodor's group independently generated recombinant influenza viruses entirely from cloned cDNA encoding eight gene segments.[20,21] Neumann's influenza reverse genetics system is consisted of 17 plasmids: 8 for the synthesis of RNA segments and 9 for protein expression. The influenza reverse genetics system was further improved to require only 12 plasmids.[22] Reduction in the plasmid number resulted in a higher efficiency of transfection and dramatically increased the infectious viral titer. In contrast to Neuman's system, Fodor et al. did not use RNA polymerase I terminator, but used hepatitis delta virus ribozyme with autocatalytic activity to splice RNA transcript at the correct site. In 2000, Hoffmann et al. modified influenza viral reverse genetics system and designated it as the RNA polymerase I/II system.[23] The core of this influenza virus rescue system is plasmid pHW2000, an "ambisense cassette" consisting of (1) RNA I polymerase and terminator sequence to transcribe negative sense vRNA and (2) the polymerase II promoter from human CMV along with a polyadenylation sequence from the bovine growth hormone to yield viral mRNA. The

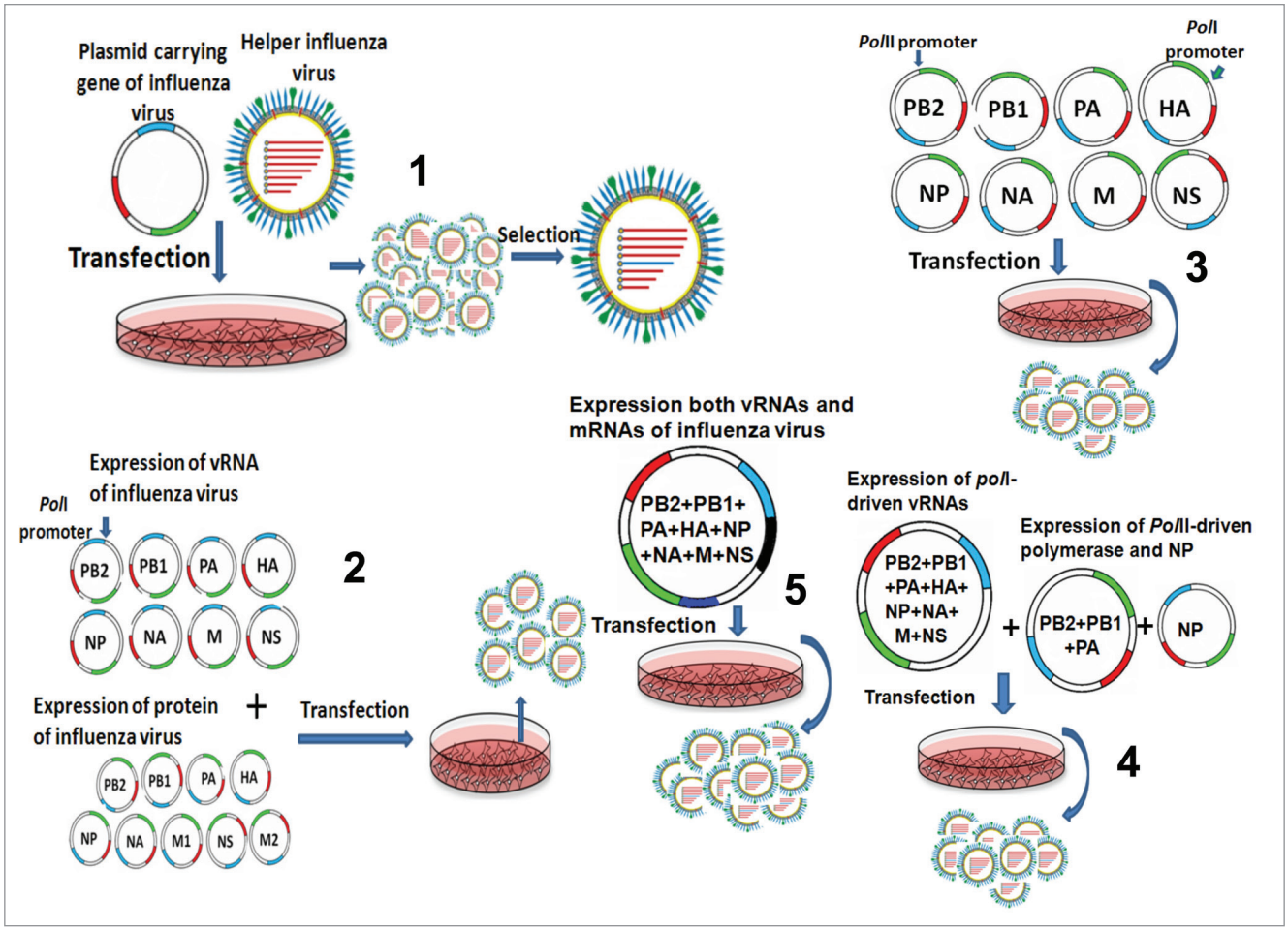

Figure 1. Technique evolution of influenza virus reverse genetics. Helper virus depended influenza reverse genetics system; (2) 17-plasmid influenza reverse genetics system. Eight plasmid for synthesis of RNA segments, and 9 plasmids for expression of structural proteins; (3) 8-plasmid influenza reverse genetics system. Each plasmid has *Pol*II and *Pol*I promoter sequence flanked by influenza gene segments; (4) 3-plasmid influenza reverse genetics system. One plasmid carries eight *Pol*I promoter-driven vRNA transcription units. The second plasmid encodes polymerases, and the third one expresses NP; (5) one-plasmid influenza reverse genetics system.

vRNA and mRNA are produced from the same viral cDNA template and the negative-strand vRNA is synthesized in the forward direction, while the positive-strand mRNA is produced in the reverse direction. In this influenza rescue system, the additional protein expression plasmids are not required. Thus, producing recombinant influenza virus became faster and easier. This eight-plasmid influenza rescue system is still used broadly.

Several studies aimed at decreasing the plasmid number and enhancing the transfection efficiency were later presented. In 2005, a three-plasmid influenza rescue system was developed:[24] one plasmid carries eight *Pol*I promoter-driven vRNA transcribing units, the second plasmid encodes three polymerase proteins under the *Pol*II promoter, and the third plasmid encodes NP protein. By 2009, Zhang et al. had introduced a one-plasmid influenza rescue system that was more efficient than the eight-plasmid system.[25] This novel one-plasmid system can produce high titers of influenza virus in chicken cells. In addition, it is a much easier method for generating influenza viral seeds. These low plasmid number systems have the potential to revolutionize and facilitate how influenza vaccines are produced and formulated, especially since it is currently very important to change the hemagglutinin (HA) and neuraminidase (NA) antigen formulations of influenza virus vaccines annually because of antigen drift.

Replication-Competent Influenza Virus-Vectored Vaccines

Because there is no DNA phase during the replication of negative-sense RNA viruses, there is no danger that the viral genome will integrate into the host genome when influenza virus is used as a viral vector.[26] This would provide for a dramatic increase in safety as compared with other viral vectors with the potential to integrate their viral DNA into the host genome. Moreover, the RNA genome of the influenza virus is a potent trigger of innate immunity and can stimulate RIG-I and induce IFN-β production.[27] It may also be possible that influenza virus, when used as a viral vector, could help to induce strong humoral and cellular immune response after immunization as will be discussed in later

sections. Thus, it was a great milestone when the reverse genetics of the negative-stranded virus was successfully developed. Many important achievements were produced by using this novel virus rescue system, including studies with influenza viruses. Scientists can now mutate specific nucleotides in the viral genome to explore the functions of the proteins or amino acid domains, or elucidate the nature of regulatory sequences. Furthermore, the influenza virus reverse genetics system may also be a very important tool in generating influenza vaccines. Of significance, a cold-adapted, seasonal influenza vaccine which stimulates potent humoral and T cell immune responses was created for human use.[28] Influenza virus reverse genetics may also be used to produce effective vaccines to prevent influenza pandemics.[29,30] In addition, influenza viral vectors have several potential advantages against various disease pathogens. These advantages include the ability for large-scale production of influenza virus in embryonated chicken eggs. Since influenza HA and NA surface antigens continuously evolve,[31] another advantage of influenza virus-vectored vaccines is that they may be used frequently for immunization.[32] Moreover, as a major respiratory system disease pathogen, influenza virus stimulates potent mucosal and systemic immune responses.[33] Finally, as we have discussed in earlier sections, highly efficient, reverse genetic systems of influenza virus have been established.

The majority of studies on the use of influenza virus as a vaccine vector are centered on modifying an influenza virus to incorporate a heterologous gene. Several influenza gene segments can be modified in the process of producing recombinant influenza viruses by inserting a foreign gene into a gene segment of the influenza viral genome. Such a rescued influenza virus was reported in 1994, and expressed a conserved epitope ELDKWA from gp41 ectodomain of HIV-1. The epitope was inserted into the loop of antigenic site B of influenza HA. This was the first study to generate a chimeric influenza virus carrying a heterologous amino acid epitope.[34] Intranasal immunization with this chimeric virus induces a significant mucosal antibody response and induces strong immune responses in the respiratory, vaginal, and intestinal tracts. Sustained levels of secretory immunoglobulin A (sIgA) were detectable for more than 1 y after immunization.[35] In another study, *Bacillus anthracis* protective antigen (PA) domain 1 and domain 4 were inserted into the C-terminal flank of the HA signal peptide or into the HA1 subunit of HA. Both chimeric proteins show similar HA function to that of wild-type HA. Furthermore, immunization with the plasmids encoding the chimeric proteins and the recombinant virus induce immune responses against both HA and PA. Genes encoding exogenous protein domains can also be inserted into the HA gene segment of recombinant influenza virus without decreasing the function of HA.[36,37] Intranasal immunization with live influenza expressing the receptor domain of *Bacillus anthracis* protective antigen (PA) or the lethal and edema factor binding domain (domain 1) induce strong and specific immune responses. To reduce the possibility of recombination of the rescued influenza virus with natural influenza virus, Hai et al. generated a recombinant influenza B virus to express the ectodomain of influenza A virus HA protein. Immunization of mice with these viruses protected mice against lethal influenza virus infection.[38]

Besides the influenza virus HA gene segment, the NA gene segment may also be modified to express heterologous amino acids. In fact, Castrucci and colleagues inserted a cytotoxic T lymphocyte epitope specific to the lethal lymphocytic choriomeningitis virus (LCMV) nucleoprotein into the NA stalk region and found that as many as 58 amino acids could be inserted into the NA stalk region without obvious effects on the function of NA.[39] In contrast, Mishin et al. generated a NA-deficient mutant influenza A virus by replacing the NA stalk region with an enhanced derivative of green fluorescent protein (GFP), also known as eGFP. The results from this study in ferrets suggested that NA stalk region-lacking mutants can be used as either a live attenuated influenza virus vaccines or as viral vectors.[40] Moreover, Efferson et al. generated a recombinant influenza virus expressing the immunodominant HER-2 CTL epitope KIF (E75) in the stalk region of NA protein. It was the first report that demonstrated a live attenuated recombinant influenza vector carrying the immunodominant HER-2 CTL E75 could induce human epitope-specific CTLs against a tumor-associated antigen.[41] These results suggest that influenza virus may be used as anti-tumor viral vector. In another study, a recombinant influenza virus containing an HIV-1 epitope in the NA stalk region induced strong, durable immune responses in female mice when administered to the mucosa of the vaginal tract.[42] Finally, recombinant viruses with eGFP insertions that do not cause loss of NA function have been created as tools for tracing and deciphering the steps involved in infection and replication of influenza viruses.[43,44]

In addition, the non-structural (NS) gene segment can also tolerate insertions of up to 250 amino acids.[45] The NS gene segment is the smallest segment, encoding two proteins, NS1 and NS2. NS1 is an important factor for effective influenza virus replication and serves as an IFN α/β antagonist.[45] NS2 mRNA is synthesized via alternative splicing of NS1 mRNA and functions in influenza virus transcription and replication, including nucleocytoplasmic export of viral RNPs.[46] Different approaches may be utilized for genetic manipulation of the NS gene segment. Several studies report the expression of full-length exogenous proteins in influenza virus via the insertion of an internal ribosomal entry site (IRES) into the recombinant RNA gene segment to generate a bicistronic vRNA.[47] Another method used to generate multicistronic vectors is to introduce a short 2A cleavage site. These 2A cleavage sites have been identified in several RNA viruses, including foot and mouth disease virus, equine rhinitis A virus and porcine teschovirus.[48] The 2A peptide facilitates co-translational cleavage of large viral polyproteins at a single site, but can also mediate co-translational cleavage in synthetic polyproteins.[49]

Furthermore, as a determinant of influenza virus pathogenesis, the NS gene segment is the most popular gene for modification. Attenuated influenza A and B viruses can be produced via alteration of the NS gene segment as Talon and colleagues first reported.[50] In a later study, Ferko and colleagues generated a hyper-attenuated recombinant influenza virus containing 137 C-terminal amino acid residues of human immunodeficiency virus type-1 Nef protein within the NS gene. Animal experiments show that this hybrid influenza virus can induce strong Nef and influenza-specific $CD8^+$-T cell responses.[51] The attenuated

Table 1. Evolution of influenza virus reverse genetics

Year	Milestone	Helper Virus	Plasmids
1994[19]	RNA pol I is manipulated to transcribe influenza viral RNA	Yes	
1999[20]	Viruses are generated entirely from cloned cDNA encoding 8 genes	No	17
1999[22]	Reduction in plasmid number leads to higher efficiency transfections and increased viral titers	No	12
2000[23]	Introduction of RNA pol I/II system for influenza virus reverse genetics	No	8
2005[24]	Three-plasmid system: 1) vRNA-transcription units, 2)polymerase proteins, and 3) NP	No	3
2009[25]	One-plasmid system produces high titers in chicken cells	No	1

influenza viruses with altered NS genes markedly increase the safety of a live influenza vaccine.

Specific modifications of the NS gene segment can also be used to improve the immunogenicity of live influenza vaccines. In one study, the NS gene segment was modified to express human interleukin-2 (IL-2) in an attenuated, cold-adapted influenza virus.[52] Intranasal immunization of mice with this modified influenza virus dramatically enhanced mucosal and cellular immune responses compared with its wild-type counterpart. In another study, Wolschek et al. replaced the interferon antagonist NS1 of influenza A viral vector with human IL-2, human interleukin-24 (IL-24), or human CCL20.[53] Kittel et al. also generated a recombinant influenza virus expressing biologically active human interleukin-2 from within the NS1 open reading frame. An insertion of an overlapping UAAUG stop-start codon cassette into NS gene gives this bicistronic influenza vector the ability to replicate in mouse lungs and secrete biologically functional interleukin-2.[26] These influenza virus vectors expressing cytokines are potential viral vectors for cancer treatments.

Reporter influenza viruses have also been produced by inserting a tetracysteine[54] or GFP[45] tag into the NS gene segment. Use of the tetracysteine tag allowed for the real-time visualization of NS1 protein transport in infected cells by Li et al.,[54] while Kittel et al. suggested that their GFP-expressing virus construct could be used in the future to track viral infection in animals and for rapid screening of drugs against influenza virus.[45] In addition, Takasuka et al. inserted the chloramphenicol acetyltransferase (CAT) reporter gene into the NS gene segment and demonstrated that their recombinant virus generated a respiratory immune response against CAT in mice with intranasal inoculation.[55]

As a respiratory disease pathogen, live attenuated influenza virus may be considered as a potential vector against other respiratory pathogens, such as *Mycobacterium tuberculosis* and respiratory syncytial virus. Indeed, an attenuated influenza virus has been generated to express the *M. tuberculosis* secretory antigenic target protein within the influenza NS segment. This construct provides potent protection against challenge with tuberculosis to mice and guinea pigs when administered via the intranasal route. Moreover, protection by this influenza virus is similar to that induced by the *Mycobacterium bovis* Bacillus Calmette-Guérin strain (BCG), and has a synergistic effect when used with chemotherapy.[56,57]

It is also possible to express full-length foreign proteins through an additional gene segment. Using reverse genetics techniques, Gao et al. generated a recombinant A/PR/8/34/ with a ninth gene segment encoding GFP or the HA gene of A/Hong Kong/1/68.[58] This ninth gene segment can be used further to generate bivalent attenuated influenza vaccines against other pathogens.

Pseudo-Typed Influenza A Virus Vector Vaccine

Modulation of a highly pathogenic influenza virus requires a high safety level facility. To decrease the risk caused by highly pathogenic influenza viruses, Martinez-Sobrido and colleagues established a HA-expressing stable MDCK cell line along with a stable, infectious influenza virus encoding GFP in place of HA. The GFP gene in this construct is flanked by the replication, transcription, and packing cis-acting sequences of the HA gene.[59] Thus, this HA-deficient recombinant influenza virus can replicate and be passaged in the MDCK cell line constitutively expressing HA protein. By using this strategy, gene-deficient influenza viruses can be generated, and related experiments can be performed under BSL-2 condition. A pseudotyped influenza virus expressing a foreign protein can also be constructed as a vaccine against multiple pathogens.

MicroRNA Delivery Using Influenza Viral Vectors

Influenza viruses can be attenuated by incorporating non-avian microRNA response elements into the open-reading frame of the influenza viral nucleoprotein.[60] Influenza viruses can also be designated as a tool for microRNA delivery. Varble et al. reported the modification of an influenza virus using reverse genetics to express cellular microRNA-124 that did not affect viral replication.[61] These results demonstrate that influenza virus can be engineered to produce functional microRNAs and thus, microRNA delivery using viral vectors is not limited to DNA viruses. Furthermore, this study shows replication or transcription from vRNA does not affect the formation of functional microRNA. It may also be possible to use influenza virus as a shRNA vector to deliver siRNA into the respiratory system.

Conclusions

Emerging reverse genetics methodologies and technologies have made it possible to construct recombinant influenza viruses from cDNA plasmid systems without the requirement of helper viruses (**Table 1**). It is now possible to modify different gene segments

to encode foreign peptide sequences, proteins and even microRNAs. Because many of the influenza gene segments are now encoded into a number of cDNA plasmids that lend themselves to modifications, it is possible to produce a multivalent vaccine using a single recombinant influenza viral vector for delivery. It is also possible to encode immunological adjuvants into the influenza virus vectored-vaccines as has been shown with IL-2. Besides IL-2, it would be fascinating to study the effects of other adjuvants, including other cytokines and chemokines, Toll-like receptor agonists, bacterial toxins and plant-derived products. Furthermore, different gene modifications result in influenza viruses with attenuated pathogenicity, thus increasing the safety profile of the influenza viral vector as used for vaccines, cancer gene therapy, or as tool for discovery in a laboratory setting. The ability to use various permutations of viral strains and vectors coupled with genetic manipulations offer limitless possibilities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the research grant R01AI072139 from the National Institute of Allergy and Infectious Diseases, NIH and an internal fund from Texas Tech University Health Sciences Center Paul L. Foster School of Medicine.

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