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 3-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 therapy 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. 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. The non-coding sequences and adjacent nucleotides serve as a viral packaging signal and are necessary for viral RNA replication and virion assembly. In the course of influenza viral infection, all RNA gene segments are synthesized in the cell nucleus where they are transported following viral entry. Influenza virus mRNA synthesis is dependent on host cellular RNA polymerase II (Pol II) activity, but vRNA and cRNA are synthesized by the ribonucleoprotein. Pol II is an enzyme found 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 RNA: however, Selection systems were still required to isolate recombinant virus from great amounts of helper virus. A breakthrough in influenza virus 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. Neumann’s influenza reverse genetics system is consisted of 17 plasmids: 8 for the influenza reverse genetics system was further improved to require only 12 plasmids. 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. 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 virus reverse genetics system and designated it as the influenza virus reverse genetics system. The core of this influenza virus rescue system is plasmid pHW2000, an “ambisense cassette” consisting of (i) RNA I polymerase and terminator sequence to transcribe negative sense vRNA and (ii) the polymerase II promoter from human CMV along with a polyadenylation sequence from the bovine growth hormone to yield viral mRNA. The

The influenza virus replication and transcription of influenza virus has made the generation of influenza viruses from DNA a reality.

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. Soon after, Palese and colleagues pioneered a new era of recombinant RNA viruses from cloned cDNA. 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. Hobom and colleagues brought light of the possibility of using influenza reverse genetics by manipulating RNA polymerase I (Pol I). 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 RNA: however, Selection systems were still required to isolate recombinant virus from great amounts of helper virus.
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. 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. 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, sialic acid receptor-unspecific vaccine strain of avian influenza virus that can tolerate insertions of up to 250 amino acids has been created as tools for tracing and deciphering the steps involved in infection and replication of influenza viruses.

In addition, the non-structural (NS) gene segment can also tolerate insertions of up to 250 amino acids. 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. NS2 mRNA is synthesized via alternative splicing of NS1 mRNA and functions in influenza virus transcription and replication, including nucleocyttoplasmic export of viral RNAs. 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. 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. 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.

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. 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. The attenuated
Table 1. Evolution of influenza virus reverse genetics

| Year | Helper Virus | Plasmids |
|------|--------------|----------|
| 1994 | Yes          | RNA poly 1 is manipulated to transcribe influenza viral RNA |
| 1999 | No           | Viruses are generated entirely from cloned cDNA encoding 8 genes |
| 1999 | No           | Reduction in plasmid number leads to higher efficiency transfections and increased viral titers |
| 2000 | No           | Introduction of mRNA for influenza virus reverse genetics |
| 2005 | No           | Three-plasmid system: 1) rRNA-transcription units, 2) polymerase proteins, and 3) NP |
| 2006 | No           | One-plasmid system produces high titers in chicken cells |

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. Intra nasal 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. Kittel et al. suggested that their recombinant virus generated a respiratory immune response against CAT in mice with intranasal inoculation. 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.

It is also possible to express full-length foreign proteins through an additional gene segment. Using reverse genetics technologies, 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. 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. 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. 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. 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 sRNA 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 vector-dose 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 and non-pathogenic products.

Different, even 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 use of these 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 R01AI072339 from the National Institutes of Health and Infectious Diseases, NIH and an internal fund from Texas Tech University Health Sciences Center Paul L. Foster School of Medicine.

References
1. Aoki SC, Maserejian SN, Yu SN, Schachter O, Nabhani N, Green K. Replication-competent influenza A virus that encodes a single-fragment protein-tagged PKI peptide plasmid allows live-cell imaging of the viral life cycle. PLoS Pathog 2012; 8:e1002468; PMID:23214355; http://dx.doi.org/10.1371/journal.ppat.1002468. 2. Chen W, Cai H, Makita D, Gehrke P, Schenk S, Beck S, et al. Novel influenza A virus microchondrial protein that infects chick cells. Nat Med 2001; 7:1306-12; PMID:11372070; http://dx.doi.org/10.1038/nm0301-1306.
3. Gilks D, Makita D, Heimann E, Benard JR, Sowdell JW. The influenza A virus F12 protein targets the inner mitochondrial membrane via a predicted basic amphipathic fold that disrupts mitochondrial func-
tion. J Virol 2003; 77:7224-34; PMID:12835982; http://dx.doi.org/10.1128/JVI.77.15.7224-7234.2003.
4. Westaway D, Lai H, Timmis KCM, Howard W, et al. A comprehensive map of modification of a novel PKI-related protein translated from influenza A virus 2' ADNAS. J Virol 2003; 77:8021-31; PMID:12809068; http://dx.doi.org/10.1128/JVI.77.16.8021-8031.2003.
5. Shokat K, Mayer M. Shuffled receptors at the Y terminus of influenza virus RNA's and their transcripts. Nucleic Acids Res 1978; 5:2071-19; PMID:652519; http://dx.doi.org/10.1093/nar/5.9.1407.
6. Togari G, Grace H, Watanabe S, Nakada Y, Tanaka Y. Selective incorporation of influenza RNA segments into segments. Proc Natl Acad Sci U S A 2005; 102:2002-7; PMID:12574509; http://dx.doi.org/10.1073/pnas.1250377102.
7. Watanabe T, Watanabe S, Noda T, Ishida T. Isolation and characterisation of influenza A virus RNAs terminal structure. RNA 1996; 2:1046-57; PMID:8734016; http://dx.doi.org/10.1017/S1355838296883361.
8. Muramoto Y, Muranaka T, Shima O, Endo K, et al. A green fluorescent protein-tagged PB2 protein of influenza virus in vitro. Arch Virol 2009; 154:821-32; PMID:19242243; http://dx.doi.org/10.1007/s00705-009-0587-z.
9. Palm R, Neumann G, Hoffmann E, N⇔ane M, Hoben H. Prominent elements in the influenza A virus terminal structure. RNA 1996; 2:1046-57; PMID:8449780.
10. Sugasawa W, Brown G, Hofschneider PH. Direct evidence for membrane activity of influenza virus RNA. Proc Natl Acad Sci U S A 1973; 70:28-33; PMID:435376; http://dx.doi.org/10.1073/pnas.70.1.28.
11. Glenn HE, McGush D, Barry RD. Characterization of the mRNA of influenza A virus. J Virol 1975; 16:1659-63; PMID:12022433.
12. Brinker M, Morgan MG, Shababi AJ, King RM. Cap-
ter proximal nucleotides of native mRNA primes are incorporated into influenza viral complementary RNA during transcription in vitro. J Virol 1979; 32:855-64; PMID:531285.
13. Zavitz JD, Palos H, Honda A, Ishihama A, Koyd M. Prominent analysis of influenza virus RNA polymerase. J Virol 1989; 63:1542-51; PMID:29059461.
14. Inayama W, Koyd M, Taira M, Tair JD, Palos P. Amplification, expansion, and packaging of foreign genes by influenza virus. J Virol 1991; 65:1037-43; PMID:19958261; http://dx.doi.org/10.1128/JVI.65.4.1037-1043.1991.
15. Barley WJ, Palos P. Influenza A virus with unique replication introduced into the HA gene. J Virol 1999; 63:1275-6; PMID:10395005.
16. Heinzen T, Tanaka Y. Reverse genetics provides direct evidence for a correlation of homologation cleavability and virulence of an avian influenza A virus. J Virol 1999; 73:1017-25; PMID:10313051.
17. Neumann G, Haber H, Greene RA. Somatic mutations introduced into the HA gene of influenza virus. J Virol 1994; 68:3210-9; PMID:76157177.
18. Neumann G, Watanabe T, Ishida T, Watanabe S, Goto H, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 1995; 92:15694-9; PMID:8849780; http://dx.doi.org/10.1073/pnas.92.32.15694.
19. Sugasawa W, Brown G, Hofschneider PH. Direct evidence for membrane activity of influenza virus RNA. Proc Natl Acad Sci U S A 2000; 97:6108-13; PMID:10801978; http://dx.doi.org/10.1073/pnas.109701797.
20. Zavitz JD, Palos H, Honda A, Ishihama A, Koyd M. Prominent analysis of influenza virus RNA polymerase. J Virol 1989; 63:1542-51; PMID:29059461.
21. Yamanaka H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 1999; 96:8804-9; PMID:10430844; http://dx.doi.org/10.1073/pnas.96.16.8804.
22. Yamanaka H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 1999; 96:8804-9; PMID:10430844; http://dx.doi.org/10.1073/pnas.96.16.8804.
23. Yamanaka H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 1999; 96:8804-9; PMID:10430844; http://dx.doi.org/10.1073/pnas.96.16.8804.
Garulli B, Kawaoka Y. Hai R, García-S astré A, Swayne DE, Palese P. A reassortment-incompetent live attenuated influenza virus vaccine for protection against pandemic virus strains. J Virol 2011; 85:6832-43; PMID:21543486; http://dx.doi.org/10.1128/JVI.01018-10.

Li, X. L., X., L. J., Brindil N., Gurt KL., Liu Q., et al. Generally, engineered, hemagglutinidentified influenza virus allows evaluation of viral NS1 proteins in living cells. J Virol 2010; 84:7204-13; PMID:20666386; http://dx.doi.org/10.1128/JVI.00615-10.

Takahashi, N., Ems, M., Imm, A., Takemiya T. Intranasal inoculation of a reassortment-incompetent influenza virus containing envelope mutations in the NS segment induced mucosal immune responses against the swine gene product in mice. Vaccine 2002; 19:1579-87; PMID:12085665; http://dx.doi.org/10.1016/S0264-410X(01)00491-1.

Stokka M, Soini, S., Zabotinets N., Ferko B., Brink C., Romantov N., et al. Vaccine potential of influenza vector expressing Microbacterium tuberculosi ESAT-6 virus. Tuberculosis (Edinb) 2008; 88:336-40; PMID:18456124; http://dx.doi.org/10.1016/j.tube.2008.10.010.

Soini S, Stokka M, Zabotinets N, Ferko B, Brink C, Romantov N, et al. Influenza virus non-structural-protein-encoding vector allows visualization of viral NS1 protein in living animals against tuberculosis challenge. Clin Vaccine Immunol 2006; 13:808-14; PMID:16713930; http://dx.doi.org/10.1128/CVI.00056-06.

Gao Q, Lassen AC, Wang CY, Fidler P A reassortant influenza virus carrying a self-cleaving 2A peptide for bicistronic expression in transgenic mice and HIV. Proc Natl Acad Sci U S A 2004; 101:15497-502; PMID:15576665; http://dx.doi.org/10.1073/pnas.0405925101.

Martinez-Salio, L., Cadagas, R., Sout, J., Baker CE, Fidler P. Influenza virus A virus preparation is a potential vaccine against smallpox. J Virol 2002; 76:6282-9; PMID:12044726; http://dx.doi.org/10.1128/JVI.01433-09.

Basler CF, Garcia-Sastré A, Swayne DE, Palese P. A nine-segment influenza virus expressing GFP from the NS1 reading frame. Virology 2004; 324:190-5; PMID:15064769; http://dx.doi.org/10.1016/j.virol.2004.03.035.

Brennde JT, Finn AM, Lorini MH, Chua MA, Steel J, Manicassamy B, García-Sastré A, Swayne DE. Engineered RNA virus expression of microRNAs. Proc Natl Acad Sci U S A 2010; 107:13151-6; PMID:20826380; http://dx.doi.org/10.1073/pnas.1007881107.