Published in final edited form as:

Virus Res. 2011 December ; 162(1-2): 8–11. doi:10.1016/j.virusres.2011.09.035.

Taming influenza viruses

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Abstract

Plasmid-based reverse genetics systems allow the artificial generation of viruses with cloned cDNA-derived genomes. Since the establishment of such systems for influenza virus, numerous attempts have been made to tame this pathogenic agent. In particular, several types of viruses expressing foreign genes have been generated and used to further our knowledge of influenza virus replication and pathogenicity and to develop novel influenza vaccines. Here, we review these achievements and discuss future perspectives.

Keywords

Influenza virus; reverse genetics; foreign gene expression

1. Introduction

In the last century, we experienced three influenza A virus pandemics: Spanish influenza in 1918, Asian influenza in 1957, and Hong Kong influenza in 1968. The Spanish influenza virus alone killed 20–50 million people worldwide (Wright et al., 2007). These events, together with annual influenza epidemics, have compelled us to develop effective measures to control influenza virus infection.

The genome of influenza A viruses, which are members of the *Orthomyxoviridae* family, comprises eight segmented negative-strand RNAs (PB2, PB1, PA, HA, NP, NA, M, and NS gene segments) that encode at least 11 viral proteins (Palese and Shaw, 2007). Each viral RNA (vRNA) consists of a 3'-end non-coding sequence, an internal coding sequence, and a

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5'-end non-coding sequence: 12- and 13-terminal nucleotides at the 3' and 5' ends, respectively, are highly conserved among the eight vRNAs (Fig. 1). Influenza virus is unique among RNA viruses in that its vRNAs are transcribed and replicated in the nucleus not in cytoplasm of infected cells.

Advances in molecular biological technologies have enabled the development and improvement of prophylactic and therapeutic interventions against influenza. In particular, since we (Neumann *et al.*, 1999) and others (Fodor *et al.*, 1999) established plasmid-based reverse genetics systems, a molecular technique for the artificial generation of viruses with cloned cDNA-derived genomes, for influenza viruses, our knowledge about influenza have been greatly extended and countermeasures against influenza have been dramatically improved. Indeed, this DNA engineering-based technique is now used in the preparation of live attenuated vaccines against seasonal and highly pathogenic H5N1 influenza viruses (Karron *et al.*, 2009; Subbarao and Katz, 2004).

Such molecular techniques also open the door to a new field of influenza virology: creating foreign gene-expressing viruses that include not only influenza, but also some well-studied viruses such as retroviruses, lentiviruses, and adenoviruses. These 'tamed' influenza viruses have been used to gain new insights into influenza virus replication and pathogenicity and to develop new types of influenza vaccines. Moreover, because influenza viruses do not produce DNA intermediates in their replication cycle and are potent immune response stimulators, influenza viral vectors are considered to be a promising vaccine platform. Here, we summarize the efforts that have been made to generate influenza A viruses that express foreign genes and discuss the application of such studies.

2. Influenza viruses expressing a foreign gene

The first influenza virus harboring a foreign gene was reported by Luytjes *et al.* (Luytjes *et al.*, 1989). They synthesized a virus-like RNA that encoded a reporter gene flanked by the 3' and 5' non-coding sequences of the NS vRNA by means of T7 polymerase-mediated *in vitro* transcription and transfected cultured cells with the RNA construct along with purified viral polymerase subunits (PB2, PB1, and PA) and nucleoprotein NP. Helper virus infection prior to the transfection ensured that the recombinant virus-like RNA was packaged into progeny viruses and expressed in cells that were subsequently infected with these viruses. These results indicate that both ends of the non-coding sequences serve as key elements in the transcription, replication, and incorporation of a foreign gene-encoding vRNA. Further, these findings also accelerated the engineering of influenza viruses harboring genes of interest (Enami *et al.*, 1990).

Neumann *et al.* (Neumann *et al.*, 1994) established a plasmid transfection-based system to express influenza virus-like RNAs. They cloned the cDNA of the virus-like RNA between the promoter and terminator sequences of RNA polymerase I (PolI), which localizes in nucleoli and normally catalyzes ribosomal RNA transcription. The virus-like RNA was expressed in the nuclei by transfection with the gene cassette-encoding plasmid and then transcribed and replicated by subsequent infection with helper viruses. This alternative approach eliminated several cumbersome processes, such as viral protein purification and *in vitro* RNA transcription; however, the need for helper virus infection to rescue the foreign gene-harboring viruses from the vast background of helper viruses restricted the application of this technique to viruses that could be picked up by selective pressure.

Helper virus-free systems for influenza virus generation were subsequently developed by Neumann *et al.* (Neumann *et al.*, 1999) and Fodor *et al.* (Fodor *et al.*, 1999). In place of helper virus infection, the eight vRNAs were provided from plasmids possessing the cDNAs of the viral genes under the control of the PolI promoter and the PolI terminator (Neumann

et al., 1999) or the hepatitis delta virus-derived ribozyme (Fodor et al., 1999). In both systems, protein expression plasmids for the viral polymerase subunits and NP were transfected into cells to support initial transcription and replication of the vRNAs. These modifications extended the range of applications for this influenza virus engineering technique: theoretically, influenza viruses possessing any foreign genes could now be generated.

One of the most critical insights for the generation of foreign gene-expressing influenza viruses was obtained by Fujii *et al.* (Fujii et al., 2003) who found that in addition to non-coding sequences, partial coding sequences at both the 3' and 5' ends of the NA vRNA contribute to the efficient and selective packaging of the NA vRNA into virions. This concept was then demonstrated to be true for the remaining seven gene segments (Dos Santos Afonso et al., 2005; Fujii et al., 2005; Fujii et al., 2009; Gog et al., 2007; Liang et al., 2005; Liang et al., 2008; Marsh et al., 2007; Marsh et al., 2008; Muramoto et al., 2006; Ozawa et al., 2007; Ozawa et al., 2009; Watanabe et al., 2003) (Fig. 1). These findings allowed the design of recombinant influenza vRNAs suitable for efficient and stable incorporation into virions.

3. Replication-competent vs. -incompetent viruses

To date, several types of foreign gene-expressing influenza viruses have been created. These viruses are categorized into two groups based on their self-replication ability: replication-competent and -incompetent viruses. Both types of virus have strengths and limitations (Table 1). The replication-competent foreign gene-expressing viruses are an attractive experimental tool to investigate virus replication, especially *in vivo*, although their risk of revertant generation and potential pathogenicity can cause biosafety concerns. By contrast, their biologically contained replication-incompetent counterparts have fewer biosafety issues and the potential to accelerate *in vitro* studies, although they usually require special growth conditions to compensate for their replication-incompetency and allow their efficient replication.

3.1. Replication-competent viruses

Kittel *et al.* (Kittel et al., 2004) and Shinya *et al.* (Shinya et al., 2004) generated replication-competent influenza viruses possessing the green fluorescent protein (GFP) gene in the NS and NA vRNAs, respectively. Both viruses replicated efficiently under modified cell culture conditions; however, their replication was severely hampered under normal culture condition because a portion of the authentic coding sequences of these viruses was replaced with the GFP gene. Therefore, their replication kinetics and *in vivo* tropism did not necessarily reflect those of authentic viruses, although the NA-deficient GFP-expressing viruses were used to detect neutralizing antibodies against H5-subtype viruses (Rimmelzwaan et al., 2011).

Manicassamy *et al.* generated replication-competent GFP-expressing viruses of greater potency by using the porcine teschovirus-1-derived 2A protease cleavage site to express the NS1-GFP fusion protein and 'almost' authentic NS2 protein from a single recombinant NS vRNA (Manicassamy et al., 2010). This cloning strategy allowed the recombinant virus to express a full-set of functional viral proteins, and the recombinant virus grew reasonably in normal cell culture. Moreover, this GFP-expressing virus was lethal to mice, although its pathogenicity was 100-fold lower than that of authentic virus. These results highlight the potential applications of replication-competent foreign gene-expressing influenza viruses for the *in vivo* study of influenza virus pathogenicity. Li *et al.* (Li et al., 2010) also generated replication-competent viruses that expressed the GFP gene from the recombinant NA vRNA. Although they also preserved the authentic NA coding sequence and inserted the

GFP gene as an 'extra gene element' along with a 2A peptide sequence (origin unknown), *in vivo* growth kinetics or pathogenicity of their viruses was not reported.

Other virus-derived glycoproteins have also been accommodated in replication-competent recombinant influenza viruses. Watanabe *et al.* (Watanabe et al., 2003) cloned the vesicular stomatitis virus glycoprotein (VSVG) gene into the HA vRNA. Since VSVG protein could functionally substitute for influenza viral envelope proteins, (i.e., HA and NA), the NA gene segment was open for insertion of an additional foreign gene. These authors therefore chose to generate recombinant influenza viruses possessing the VSVG and GFP genes in the HA and NA vRNAs, respectively, and demonstrated the stability of the inserted genes during serial virus passages. To compensate for the lack of NA function, Maeda *et al.* (Maeda et al., 2005) replaced the ectodomain of NA with that of Sendai virus-derived hemagglutinin-neuraminidase (HN) protein. The resultant NA-HN chimeric protein not only supported NA-deficient virus replication but also generated a recombinant virus that simultaneously displayed influenza viral (HA) and Sendai viral (HN) major protective antigens on virions and infected cells. The bivalent vaccine potency of this replication-competent virus was validated in a mouse model, although its potential pathogenicity was observed (Maeda et al., 2005).

3.2. Replication-incompetent viruses

Since most of influenza viral proteins are indispensable for virus replication, single gene-knockouts render influenza viruses replication-incompetent. Marsh *et al.* (Marsh et al., 2007) established a cell line stably expressing HA protein and demonstrated that this cell line supported efficient replication of viruses that possessed either the GFP or the red fluorescent protein (RFP) gene in their HA vRNA. These authors did not, however, report on the replicative ability of their HA-knockout (HA-KO) reporter gene-possessing viruses in normal cell culture. Martínez-Sobrido *et al.* (Martínez-Sobrido et al., 2010) followed up this study by establishing cell lines expressing HAs derived from various virus strains, including highly pathogenic H5-subtype and 'Spanish influenza' pandemic viruses. They showed that their HA-KO GFP-expressing virus was replication-incompetent without *in trans* HA expression. Although the stability of the reporter HA gene during virus replication remains unclear, this HA-KO system was demonstrated to be a safe and powerful tool to screen influenza viral HA-specific neutralizing antibodies.

While studying the mechanism underlying the spread of oseltamivir-resistant mutants, Bloom *et al.* developed a PB1-KO system (Bloom et al., 2010) and demonstrated that a virus possessing either the GFP or the mCherry gene in the PB1 vRNA replicated efficiently in cells expressing the PB1 protein. However, these replication-incompetent viruses were not characterized in detail in their study and several questions, such as the stability of the reporter PB1 gene during virus passages, remain unanswered.

Recently, we developed a PB2-KO system by replacing the viral PB2 gene with reporter genes and by establishing a PB2-expressing cell line (Ozawa et al., 2011). The growth kinetics of the PB2-KO viruses in the PB2-expressing cells was comparable to those of authentic virus, and no progeny viruses were recovered from normal cell culture. HA and/or NA genes could be replaced with other virus strain counterparts without preparing additional cell lines that expressed the viral proteins. More importantly, unlike the HA-KO system (Marsh et al., 2007), a seven-segmented virus lacking the PB2 vRNA did not undergo multiple replication cycles even in cells expressing the PB2 protein *in trans*, and thus the reporter gene-encoding PB2 vRNA was stably inherited in progeny viruses. We also demonstrated the potential of the PB2-KO system as a novel vaccine platform and obtained promising preliminary data in a mouse model (unpublished data). These results demonstrate

the superior reliability and greater potential of the PB2-KO reporter gene-expressing viruses over other replication-incompetent influenza viruses.

4. Future perspectives

Despite abundant efforts to understand influenza virus pathogenicity, the mechanisms by which this pathogenic agent cause disease and kill animals and humans remain largely unclear. Replication-competent foreign gene-expressing influenza viruses are promising experimental tools to track virus-infected cell populations and to gain insights into pathogenicity. However, as exemplified by the replication-competent virus expressing the NS1-GFP fusion protein (Manicassamy *et al.*, 2010), foreign gene insertion typically causes severe virus attenuation that obfuscates data interpretation. In addition, the stability of these foreign genes needs to be improved; Manicassamy *et al.* found that GFP-negative virus populations increased over time in cell culture, reaching 45% at 84 h post-infection. Accordingly, next-generation replication-competent influenza viruses will need to retain pathogenicity comparable to that of authentic viruses and to stably express foreign genes during multiple replication cycles.

Currently, cell-based high-throughput screening plays a critical role in identifying anti-influenza viral compounds (Maddry *et al.*, 2011) and cellular factors involved in influenza virus replication (Brass *et al.*, 2009; Hao *et al.*, 2008; Karlas *et al.*, 2010; König *et al.*, 2010). Since the equipment required for high-throughput screening is often built in biologically “uncontained” facilities, the availability of replication-incompetent foreign gene-expressing influenza viruses should be of benefit to these research areas. In fact, Hao *et al.* (Hao *et al.*, 2008) and König *et al.* (König *et al.*, 2010) applied replication-incompetent foreign gene-expressing influenza viruses to their genome-wide siRNA screenings.

Replication-incompetent viruses could also serve as vaccine candidates. Although these viruses do not produce progeny viruses under normal conditions, their vRNAs are transcribed and replicated in infected cells to some extent; for example, we detected significant levels of reporter gene expression even in PB2-KO virus-infected normal cell culture (Ozawa *et al.*, 2011). These results indicate that replication-incompetent viruses have advantages over inactivated and live attenuated vaccines in terms of providing antigen and biosafety, respectively. Moreover, a foreign gene of interest can be accommodated as a secondary vaccine antigen derived from another pathogen, thereby generating bivalent vaccines. It may also be possible to combine multiple recombinant gene segments to prepare trivalent (or multi-valent) vaccines.

In conclusion, since the development of plasmid-based reverse genetics technologies, influenza viruses are no longer just pathogens, but also useful biologically engineered tools. Replication-competent and -incompetent influenza viruses that express foreign genes are likely to further our understanding of influenza virus pathogenicity and aid in the development of countermeasures against influenza viruses.

Acknowledgments

We thank Susan Watson for editing the manuscript. This work was supported, in part, by Grants-in-Aid for Specially Promoted Research and for Scientific Research, by a Contract Research Fund for the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, by ERATO (Japan Science and Technology Agency), by the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by National Institute of Allergy and Infectious Diseases Public Health Service research grants, USA.

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Fig. 1. Schematic diagram of the coding regions required for efficient genome packaging
 Noncoding and coding regions are represented by grey and yellow bars, respectively. Gene-specific coding regions required for efficient genome packaging are represented by red bars. Numbers in the red bars indicate the number of nucleotides.

Table 1

Key features of replication-competent and -incompetent viruses.

	Specific feature of:	
	Replication-competent viruses	Replication-incompetent viruses
Strength	Mirror wild-type virus behavior	Limited biosafety concerns
Limitation	Risk of reversion to a wild-type sequence	Require special growth condition
	Potential pathogenicity	Limited applications for <i>in vivo</i> study