Reverse genetics of influenza viruses

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Reverse genetics for generation of influenza viruses is a powerful tool allowing for the direct manipulation of the genome of viruses. It has the potential to allow for the generation of safer and more immunogenic vaccines and for a better understanding of the virus replication cycle. Reverse genetics is essential tool for producing a vaccine against the highly pathogenic avian influenza virus.

The term reverse genetics is used to define the directed modification of cDNA for functional or phenotypic analysis [1]. Reverse genetics is used in molecular virology to generate infectious RNA viruses possessing genomes derived from cloned cDNA that have been modified in order to study the consequent effects on viral phenotype.

The first reverse genetics systems described were for positive-sense RNA viruses [2, 3]. The transfection of full-length genomic RNA from positive-sense RNA viruses into eukaryotic cells resulted in the RNA acting as mRNA(s) for the translation of viral proteins and, in turn, generation of infectious virus. Reverse genetics is widely used in vaccine research, viral protein interaction studies, recombinant protein expression and gene therapy.

In contrast, the genomes of negative-sense RNA viruses have no messenger function and are non-infectious. Initiation of RNA transcription from viral RNA requires the presence of the viral ribonucleoprotein (RNP) complex. RNP is essential for the transcription of the viral RNA into mRNA and the replication of the virus genome. The role of RNP was first shown for vesicular stomatitis virus, where purified polymerase protein was only active in the presence of virion RNP [4-6].

Genetic manipulation of influenza viruses

Reverse genetics for influenza viruses is far more complex than for other negative-stranded viruses since the viral genome consists of eight segments and requires four different viral proteins for RNA transcription and replication. Also, viral RNA replication and transcription occur in the nuclei of influenza virus infected cells; *in vitro* generated vRNP transfected to cells must be transported to the nucleus.

The development of methods for the rescue of influenza viruses from cloned cDNA has been refined over the past decade to the point where it is now possible to rescue influenza entirely from cloned cDNA [7, 8]. In the system reported by Fodor *et al.* [7], cDNA copies of the eight genome segments are cloned in a negative orientation between the human RNA polymerase I promoter and the hepatitis delta virus ribozyme (Figure 1).

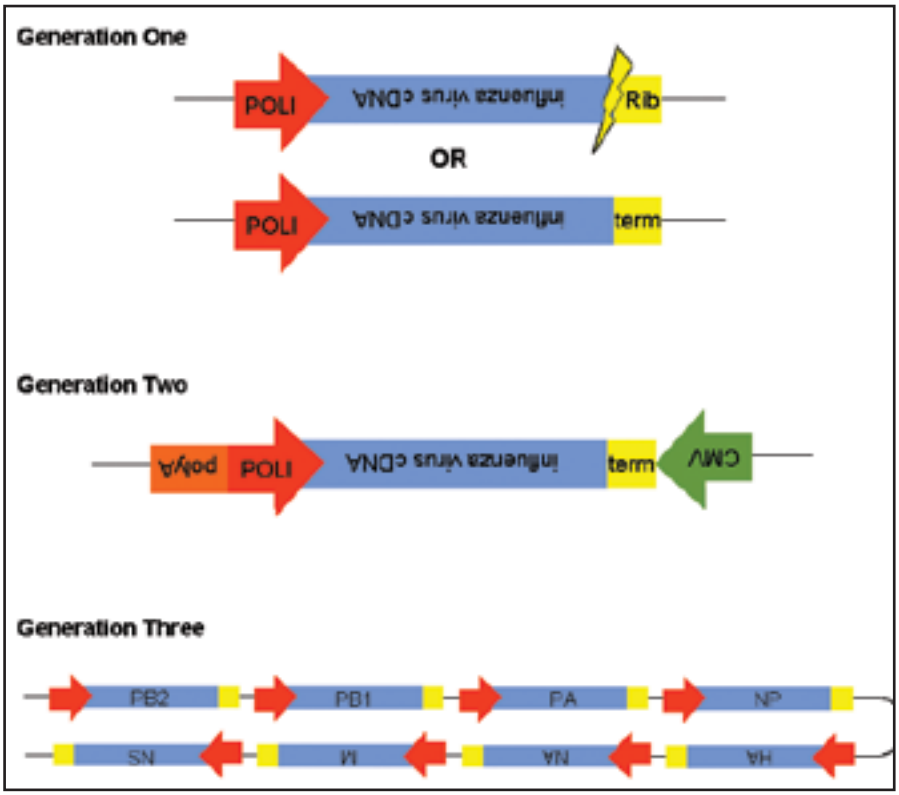

Figure 1. Schematic diagrams of RNA polymerase I promoter containing plasmids encoding influenza vRNAs. Generation one plasmids use RNA polymerase I to transcribe vRNA, with either ribozyme cleavage of the RNA or a murine RNA polymerase I terminator giving a correct 3' end. Generation two plasmids transcribe RNA in both directions, RNA polymerase I generates vRNA as in generation one vectors and a CMV immediate early promoter generates mRNA encoding the influenza proteins. The generation three plasmid is similar to generation one; however, in this plasmid, all eight of the vRNA producing plasmids are coupled end to end in one plasmid.

These plasmids, together with four protein-expressing plasmids encoding the three polymerase subunits and the nucleoprotein, are then transfected to Vero cells, resulting in vRNA synthesis from the RNA polymerase I constructs by cellular RNA polymerase I. The expressed influenza polymerase complex then recognises these RNA polymerase I transcripts as identical to influenza virus vRNA and begin RNA replication and transcription, leading to virus assembly and release (Figure 2).

In a similar approach, Neumann *et al.* [8] cloned cDNA from each of the eight genome segments in a negative orientation between the human RNA polymerase I promoter and mouse RNA polymerase I terminator (Figure 1). If these eight RNA-producing plasmids, together with nine protein-expressing plasmids (PB2, PB1, PA, HA, NP, NA, M1, M2 and NS2) are co-transfected in human-derived cell line (293T cells), yields of over 10^7 pfu/mL of virus are produced within 48 hours [8]. Since helper virus is not required for the generation of recombinant virus, the cumbersome selection process described in earlier studies involving RNP was not necessary.

Further improvements to these systems were reported by Hoffmann *et al.* [9, 10], in which only eight plasmids were required. The plasmids contained cDNA of genome segments that had been cloned in a negative orientation between a human RNA polymerase I promoter and a mouse RNA polymerase I terminator (Figure 1). Upstream of the RNA polymerase I terminator was a CMV immediate-early promoter and downstream a polyadenylation sequence.

In this system, the eight plasmids are transfected to a mixed culture of 293T cells (required for human RNA polymerase I activity) and MDCK cells. Cellular RNA polymerase I transcribes the cDNA into vRNA and the CMV promoter drives protein expression from each clone. Expressed viral protein and vRNA are then assembled and bud from the cell membrane as progeny virus. These progeny viruses then infect other (MDCK) cells, replicate and produce yields of 10^5-10^7 pfu/mL within 72 hours.

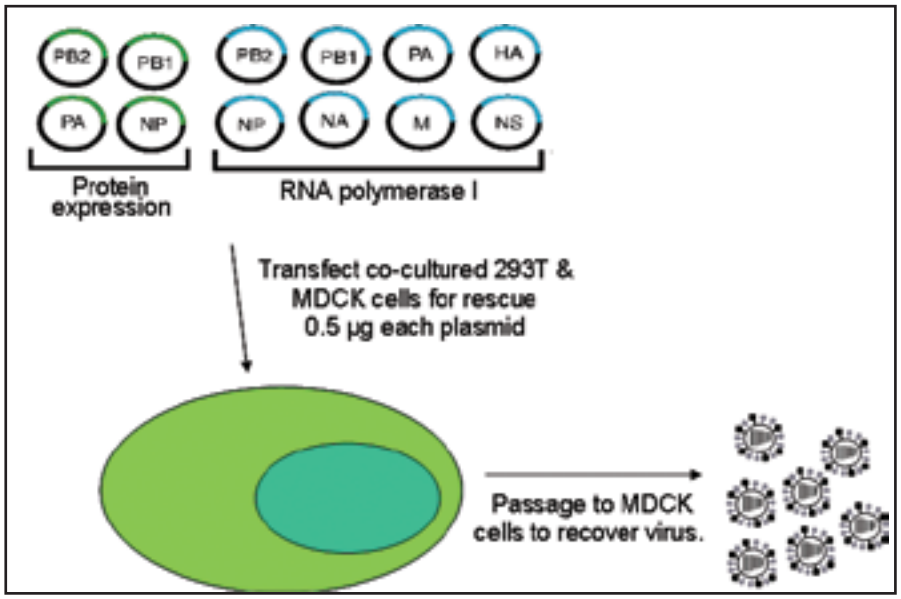

Figure 2. Schematic representation of the generation of influenza virus entirely from cDNA. Cells are co-transfected with plasmids encoding all eight segments of vRNA under the control of RNA polymerase I. Cellular RNA polymerase I synthesises vRNAs that are replicated and transcribed by the viral polymerase and NP proteins, all provided either by the 4 protein expressing plasmids (pictured) or by plasmids under the control of both RNA polymerase I and RNA polymerase II for production of both vRNA and mRNA.

A further improvement on influenza rescue systems was reported by Neumann *et al.* [11], in which the eight RNA polymerase I plasmids were combined into one plasmid. Surprisingly, transfection of this single plasmid resulted in recovery of recombinant virus in the absence of co-expressed influenza polymerase proteins. It was concluded that the transfection of the plasmid resulted in "leaky" protein expression, allowing for the virus generation.

Reverse genetics and improved influenza vaccines

Reverse genetics has the potential to improve and shorten the process required for inactivated vaccine preparation [12, 13]. Current influenza vaccines are produced by reassortment of epidemic viruses with a high-yielding donor strain. Progeny virus from the reassortment is screened for the presence of virus that contain the haemagglutinin (HA) and neuraminidase (NA) of the epidemic strain and the internal genes of the high-yielding parent that specify high growth [14].

These procedures are time consuming and unpredictable; co-infection of eggs with two different viruses theoretically can generate 2^8 or 256 different reassortants. Comprehensive screening and selection procedures must be undertaken to obtain the desired reassortant, a process that takes approximately 6-8 weeks [15]. Timelines for vaccine preparation are generally tight and are primarily dependent on the number of embryonated eggs required for growth of the vaccine virus. Egg supplies must be anticipated many months in advance. Potential problems that interrupt their supply or problems with vaccine manufacture can have a major effect on the timeline for vaccine delivery [16].

A reverse genetics approach to vaccine production is been considered essential in the event of a pandemic caused by a new human influenza virus. Human infections with highly pathogenic H5N1 avian influenza strains have been observed in many countries over the past few years. These highly pathogenic strains are lethal for chickens and chicken embryos [17-19]. Lethality has been associated with the presence of a multiple basic amino acid motif adjacent to the cleavage site of the HA glycoprotein. The presence of this motif increases the range of target organs that can support the growth of these viruses [17]. Unless this cleavage site is removed from the HA, it is not possible to grow vaccine viruses in embryonated eggs and alternative methods of cultivation, including cell culture, would be necessary.

In addition, the large-scale growth of unmodified viruses constitutes a potential public health problem. Examples of approaches to produce suitable vaccine candidates against highly pathogenic influenza strains have been reported by Webby *et al.* [20] and Govorkova *et al.* [21]. Reverse genetics was used in these studies to remove the multiple basic amino acid motif from the 2003 Asian virus. These viruses were prepared with the modified HA and NA derived from the H5N1 avian strain and the internal genes from A/PR/8/34 and replicated to high titre in embryonated eggs. Viruses containing altered HAs were attenuated compared with the *wt* avian parent [22].

Recently, the Food and Drug Administration in the USA has granted approval to MedImmune to use reverse genetics to prepare their seasonal influenza vaccines, including FluMist (an intranasal administered live attenuated influenza virus vaccine) and the next-generation, refrigerator-stable formulation.

As exclusive licensee of the reverse genetics technology for human influenza vaccines, MedImmune has offered other influenza vaccine manufacturers non-exclusive licenses for use in manufacturing seasonal or pandemic vaccines. Using this technology, a live attenuated H5N1 vaccine candidate has been prepared based on FluMist. Recently the National Institutes of Health in the USA have began enrolling participants in a phase 1 study of this vaccine. It is hoped that this vaccine candidate will be as effective against potential pandemic A strains as it has been shown against seasonal matched and mismatched A strains of influenza.

Reverse genetics, although still a relatively new tool for the manipulation of negative-strand RNA viruses, is widely regarded as having great potential for the preparation of vaccines against influenza. Reverse genetics systems allow the direct modification of the genome, providing a powerful means for studying virus replication and assembly by allowing the function(s) of viral proteins to be studied after modification or deletion of single or multiple genes.

Future applications should provide much more information than is currently available on the role of individual genes in virulence and on mechanisms of viral pathogenicity. This will allow a more rational basis for the selection of live vaccine strains than has been used in the past, where selection has been often based on purely empirical criteria. For inactivated vaccines against influenza, reverse genetics could be used to more precisely identify specific sequences concerned with enhanced growth of a vaccine reassortant in eggs or cell culture, which could contribute significantly to the cost and availability of pandemic vaccines.

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MICRO-FACT

The neuraminidase inhibitors, Relenza and Tamiflu, are the two best drugs ever developed against influenza; they arose from Australian research.