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Minireview

Reverse genetics and influenza virus research

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Introduction

Members of the family *Orthomyxoviridae* contain a single-stranded, negative-sense RNA genome organized into 8 segments that encode viral polymerases (PB1, PB2, PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins 1 and 2 (M1, M2), and non-structural proteins (NS1 and NEP) (Fig. 1). RNA of individual segments is surrounded by the molecules of NP and associated with the polymerase proteins forming a ribonucleoprotein (RNP) complex. Introduction of the genomic RNA segments into cytoplasm of permissible cells does not result into a formation of the infectious virus as it is true in case of the positive-strand RNA viruses (Palese and Shaw, 2007). Since the negative-strand vRNA does not serve as a direct template for proteosynthesis, vRNA is transcribed into mRNA by the associated polymerase complex. At the same time, vRNA serves as a template for the synthesis of complementary RNA (cRNA) during the replication. New genomic vRNA is then synthesized from cRNA (Fig. 2). Thus, vRNP complexes can be considered as basic functional units in the process of viral replication (Fig. 3). These basic units have to be delivered into the cell nucleus during the production of influenza virions (Neumann and Kawaoka, 2001). In 1980-ies, the evidence about virion formation led to the first creation of recombinant influenza virus and laid the background for a new method called reverse genetics. From the times of the first experiments to the stage of a great advance, reverse genetics became a highly developed and sophisticated method, which enables the formation of a fully functional virus particles using cloned plasmid cDNA.

Strategies with a helper virus

System using a helper virus has unique role in the progress of reverse genetics as well as in the understanding of biology of many viruses including influenza virus. In order to create

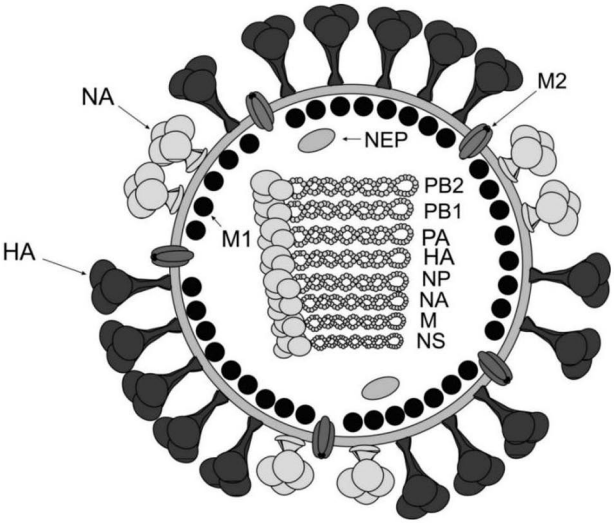

Fig. 1

Structure of the influenza virus

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Abbreviations: HA = hemagglutinin; NES = nuclear export signal; NP = nucleoprotein; RNP = ribonucleoprotein

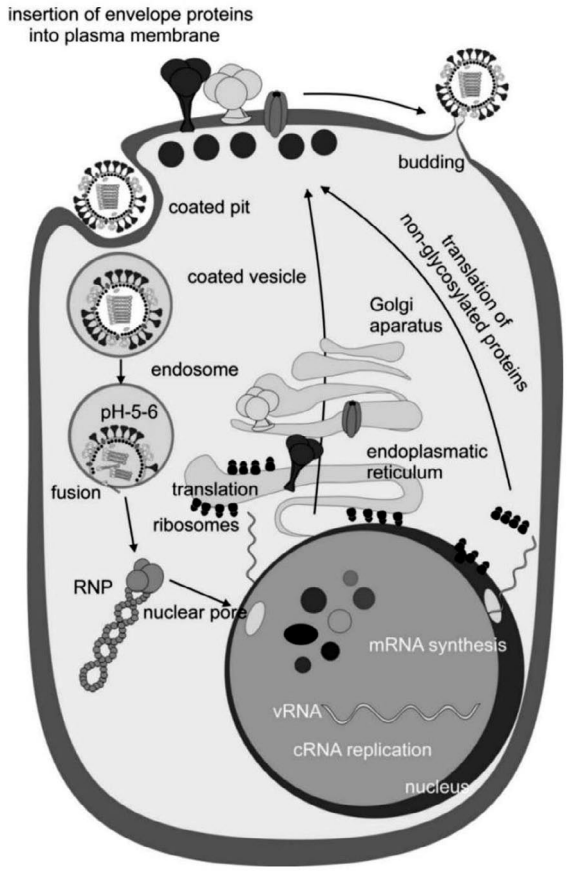

Fig. 2

Replication cycle of the influenza virus

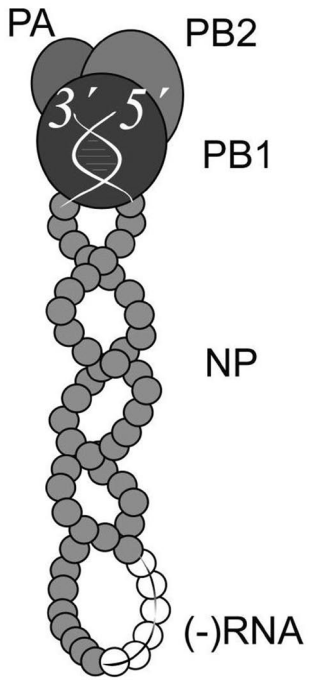

Fig. 3

Ribonucleoprotein complex of influenza virus

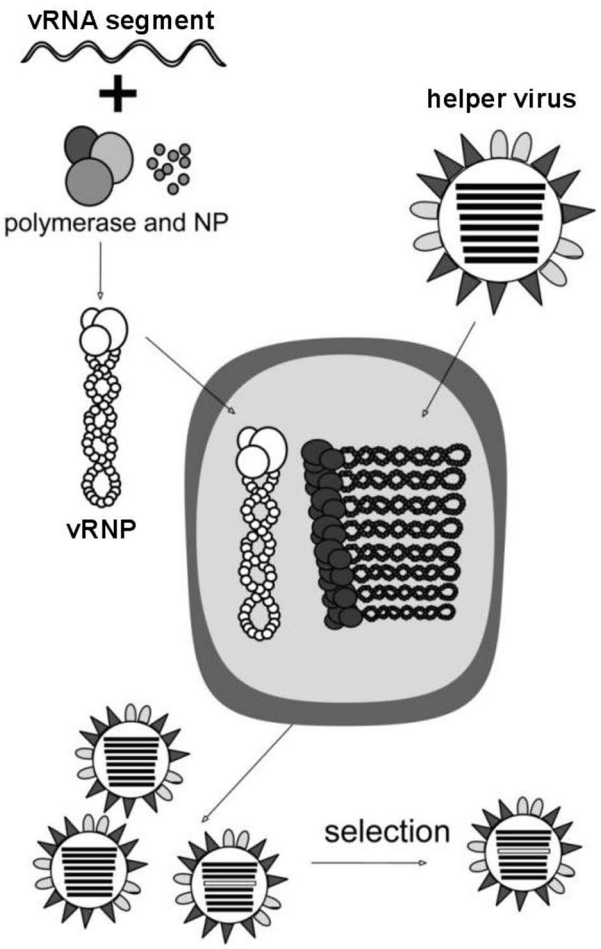

Fig. 4

Ribonucleoprotein-transfection method of influenza virus construction

an influenza virion *in vitro*, it was necessary to modify the genome (8 artificial vRNA segments) into functional vRNPs and introduce them into cells. While the first experiments were focused on the isolation of vRNP from the virions or infected cells (Plotch *et al*., 1981, Beaton and Krug, 1986), later experiments carried out independently by Parvin *et al*. (1989) and Honda *et al*. (1990) led to the *in vitro* assembly of functional vRNP.

Based on these facts, so-called ribonucleoprotein transfection method for the construction of influenza virus was developed (Luytjes *et al*., 1989) and vRNA was derived from a cloned cDNA (Fig. 4). This original method consists in the assembly of a functional vRNP complex and its transfection into eukaryotic cells. To acquire vRNP, cDNA is cloned and subsequently, the transcribed vRNA is mixed together with the viral polymerase and NP to complete the process of the vRNP assembly *in vitro*. Using one RNA segment is not

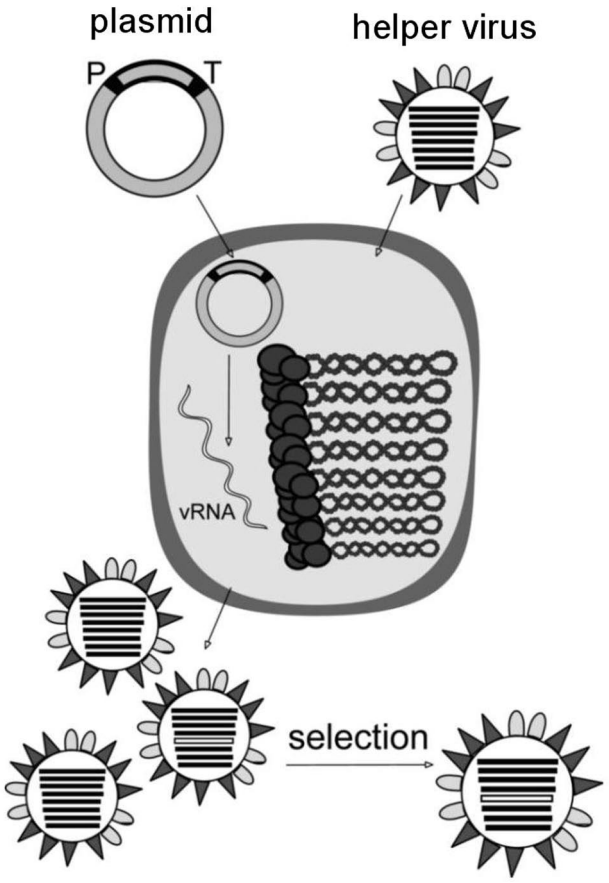

Fig. 5

Method using RNA polymerase I and helper virus

P – promoter, T – terminator.

sufficient for the formation of influenza virion and it is necessary to deliver the other 7 segments into transfected cells. Donor of the remaining 7 segments is a helper influenza virus, which is used to infect cells prior to the transfection with *in vitro*-formed vRNP. There are several limitations to this rather complicated system, particularly the use of an *in vitro* transcription, protein purification (of viral polymerase and NP), *in vitro* assembly of RNP, high degree of instability, and low efficiency of virus particle formation. A major drawback of this method is the necessity of a selection system for separation of the recombinant virus from helper virus, which dominates in the pool of rescued viruses.

Using the same system, Enami *et al*. (1990) constructed an influenza virus with mutations introduced into the haemagglutinin gene. *In vitro*-assembled vRNP comprised mutated vRNA in complex with polymerase proteins and NP. This complex was transfected into eukaryotic cells, which were superinfected with a helper influenza virus providing the remaining vRNP.

A significant progress in the reverse genetics presented the work of Neumann *et al*. (1994), which allowed to leave behind complicated methods used in transcription, purification, and assembly of vRNP complexes *in vitro*. Since cellular synthesis of vRNA is catalyzed by RNA polymerase I, this enzyme was used to obtain an influenza virus. Cells were transfected with a recombinant plasmid containing cDNA (encoding vRNA of influenza virus) under the control of RNA polymerase I promoter and terminator (Fig. 5). RNA polymerase I is found in the nucleus and is responsible for the transcription of precursor ribosomal RNA. RNA polymerase I starts and ends the transcription in non-transcribed promoter and terminator regions, respectively. As a result, transcripts produced by RNA polymerase I do not contain 5'- and 3'- added nucleotides. After transfection of the cells with recombinant plasmid, the cellular RNA polymerase I synthesizes vRNA. Similar to the RNP transfection method this method also requires infection of the cells with a helper influenza virus to provide the rest of the genetic material. Consequently, this method also requires a proper selection system to separate recombinant viruses from the progeny of helper influenza virus. Several strategies are used for this purpose such as host restriction (Subbarao *et al*., 1993; Grassauer *et al*., 1998), temperature sensitivity (Enami and Palese, 1991; Li *et al*., 1995; Egorov *et al*., 1998), antibody-mediated negative selection (Li *et al*., 1992; Barclay and Palese, 1995), and antibody-mediated virus-trapping (Horimoto and Kawaoka, 1994).

Strategies with plasmid systems

A main disadvantage of these methods was a low efficiency of the recombinant progeny formation in helper virus-dependent systems and consequently, a new strategy was explored to improve the recombinant virus production.

Schnell *et al*. (1994) were successful in construction of the rabies virus (*Rhabdoviridae*) exclusively from a cloned cDNA. Obtained construct encoding antigenomic positive-strand cRNA used T7 RNA polymerase promoter and hepatitis delta ribozyme as a terminator sequence. Transfection of plasmid DNA was preceded by the infection of cells with a recombinant vaccinia virus that provided the expression of T7 RNA polymerase. At the same time, the cells were co-transfected with protein-expressing plasmids that encoded the components of viral transcription complex.

A milestone in the *in vitro* assembly of influenza virus represents independent studies conducted by Neumann *et al*. (1999) and Fodor *et al*. (1999). Both systems enable to rescue a virus exclusively from the cloned cDNA.

Neumann *et al*. (1999) developed a system that did not involve the superinfection of transfected cells with a helper virus and consequently, did not require the use of selection

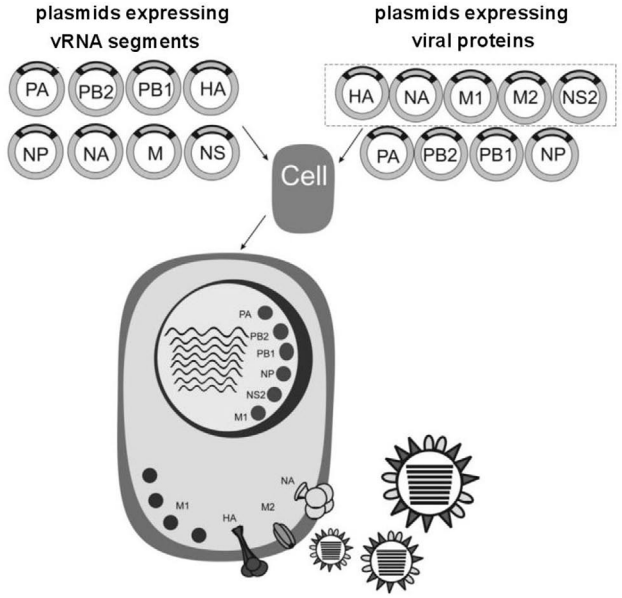

Fig. 6

Plasmid system RNA polymerase I

The scheme describes two alternative experiments (see text). In the initial experiments all plasmids were utilized, in the advanced system five plasmids drawn in dashed rectangle were not included.

procedure to rescue a recombinant virus. This approach based on utilizing RNA polymerase I places cloned cDNA encoding all 8 segments between human RNA polymerase I promoter and murine RNA polymerase I terminator. Plasmid is then transfected into human embryonic kidney cells 293T, where RNA polymerase I synthesizes vRNA. Because the presence of polymerase complex and NP is required for the assembly of influenza virions, the cells have to be co-transfected with expression plasmids. In first experiments, cells were transfected with 9 expression plasmids encoding structural proteins PB2, PB1, PA, HA, NP, NA, M1, M2, and NEP/NS2, where M1 and M2 proteins were products of the alternative splicing of one common segment and with 8 transcription plasmids containing complementary sequences of all vRNA segments. Altogether 17 plasmids were used for transfection. Further experiments showed that a transfection with 4 structural protein-encoding plasmids was sufficient for the rescue of infectious virus from infected cells. These structural proteins included the polymerase complex and NP (PB1, PB2, PA, NP) (Fig. 6). Reduction in the number of plasmids from 17 to 12 resulted into a higher efficiency of transfection that in turn led to an increase of the infectious virus titer, e.g. from 1 x 10^7 to 1 x 10^8 PFU/ml (Neumann and Kawaoka, 2001).

Similar system was presented by Fodor *et al*. (1999), where human RNA polymerase I promoter was used to initiate the transcription and to control the expression of cloned cDNA encoding each individual vRNA segment. Unlike the preceding system, the system by Fodor *et al*. (1999) does not utilize RNA polymerase I terminator, but a hepatitis delta virus ribozyme with an autocatalytic activity that ensures a splicing of RNA transcript at the correct site. Transcription plasmids together with the expression plasmids for the polymerase proteins and NP were transfected into Vero cells.

Further improvement of the reverse genetics was made by Hoffmann *et al*. (2000). Transfection of various cell lines showed that the limits of RNA polymerase I system were in the efficiency of some cell lines. To enhance the transfection efficiency of cells Hoffmann *et al*. (2000) developed a system employing RNA polymerase I/II (Fig. 7), where cDNA encoding vRNA is cloned between RNA polymerase

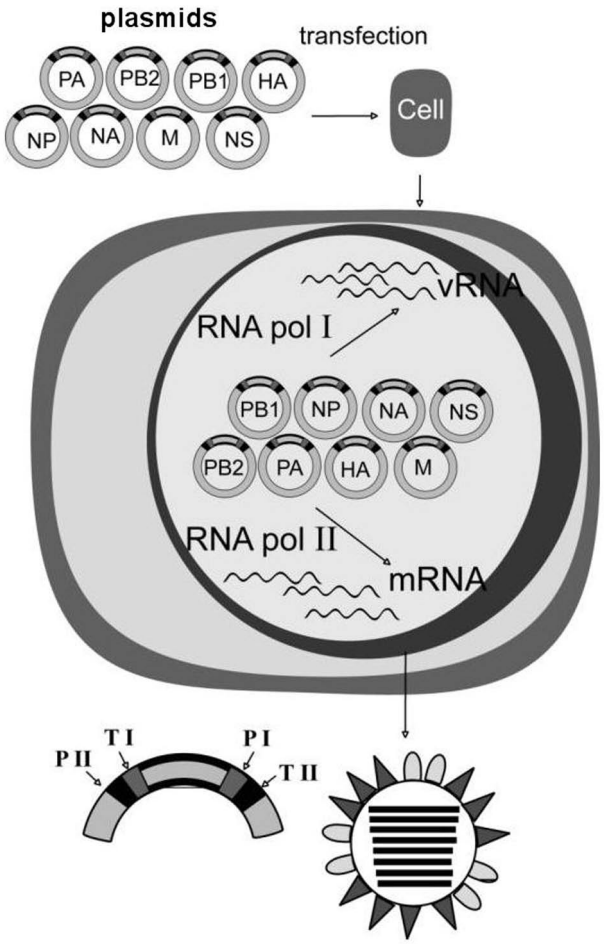

Fig. 7

Plasmid system RNA polymerase I/II

PI, TI – promoter and terminator of polymerase I; PII, TII – promoter of polymerase II and polyadenylation signal.

I promoter and terminator. This transcription unit is then cloned between human cytomegalovirus RNA polymerase II promoter and polyadenylation signal from the bovine growth hormone gene. As a result, one cDNA template contains two transcription units and a negative-strand vRNA is synthesized in forward direction and a positive-strand mRNA in reverse direction (Hoffmann *et al*., 2000). The synthesis of vRNA and mRNA from the same matrix leads to the reduction of the plasmid number from 12 to 8. A system that does not require the transfection of cells with expression plasmids enables a fast and easy rescue of reassortant influenza viruses, which can be subsequently used for preparation of vaccines or for pathogenicity studies.

The aim of further studies was to enhance the transfection efficiency while decreasing the number of plasmids necessary for the influenza virus assembly in MDCK and Vero cells. Outstanding results in this problem solving achieved Neumann *et al*. (2005), who were able to obtain a recombinant virus after transfection of cells with only one plasmid. Such a plasmid consisted of 8 RNA polymerase I transcription cassettes and each cassette contained the respective cDNA in the negative orientation. Each cDNA was flanked by human RNA polymerase I promoter and murine RNA polymerase I terminator. Virus formation was detected after transfection of 293T cells. The reconstruction of viruses proceeded not only after the transfection of cells with one plasmid, but also after the transfection with a combination of 3 plasmids, where one of them contained two transcription cassettes encoding HA and NA and the second one six cassettes encoding the rest of proteins. The third plasmid contained three RNA polymerase II transcription cassettes necessary for the expression of polymerase complex subunits. Combination of the cassettes resulted in a reduction of the plasmid number necessary for virus formation and consequently, in a different transfection efficiency in eukaryotic cells.

Application of reverse genetics

Many of the results obtained in recent years from the studies of influenza virus arose from the use of reverse genetics. This method allows incorporation of the specific mutations into viral genome through a cloned cDNA. The use of recombinant viruses enable to elucidate a relationship between structure and function of proteins, to explain a nature of viral regulatory sequences, pathogenicity of the influenza virus, and a tropism of host cell. The reverse genetics has a huge potential in the development of live attenuated vaccines. Another exciting potential of this system is the use of influenza virus as a vector containing foreign genes. Because reverse genetics is currently applied into many areas of research, we do not intend to bring up all data obtained using this system in the field of influenza virus or other viruses. In this paper we describe only several illustrative applications that show some possibilities of the reverse genetics.

Methods of the reverse genetics strongly affected research of the influenza virus proteins. One example represents the study of the essential function of M2 protein as an ion channel during virus replication (Watanabe *et al*., 2001). Using the 17-plasmid system, the recombinant viruses with a mutation in the ion-forming transmembrane domain of M2 were constructed. The recombinant virus was able to replicate in the cell culture, but was highly attenuated in mice. This outcome confirmed the important role of M2 activity as an ion channel in the process of virus replication.

Iwatsuki-Horimoto *et al*. (2004) employed Neumann's system of "RNA polymerase I" in the study of virus proteins. They succeed in elucidation of the role of highly conserved nuclear export signal (NES) motif of the NEP (NS2) protein in the export of viral RNP complexes from the nucleus mediated by cellular export factor CRM1. To study the importance of the NES for efficient export of vRNP during virus replication, random mutations were introduced into the cDNA in the NES region. It was found that the intact NES sequence is not necessary for its own function, but some regions of NES play a critical role in the export of the protein from cell nucleus. NES motif can be an attractive aim for introduction of attenuating mutations in production of viruses serving as a live vaccine.

Using the method of reverse genetics employing 12 plasmids, Conenello *et al*. (2007) constructed chimeric recombinant viruses and proved that protein PB1-F2 carrying the amino acid substitution N66S was the factor that increased virulence of the avian influenza virus A/Hong Kong/156/97 (H5N1) as well as a recombinant virus containing PB1-F2 from the pandemic strain of 'spanish flu' A/Brevig Mission/18. Introduction of reverse mutation S66N in PB1-F2 resulted in a markedly decreased virulence of both viruses in the mouse model (LD_{50} was lower by three orders of magnitude).

An interesting result was obtained by using the system "RNA polymerase I/II" in a study that tested the relationship between NS1 gene and virulence of the avian H5N1 influenza virus (Li *et al*., 2006). To carry out this study, 4 recombinant viruses were constructed, which contained 1 gene of a non-conserved sequence from a non-pathogenic virus (GS/GD/2/96) and the remaining 7 gene segments originated from a highly pathogenic virus (GS/GD/1/96). It was found that NS1 gene from a non-pathogenic virus inhibited the replication of a recombinant pathogenic virus, while changes in the genes for PA, NP, and M did not result in a change of the high pathogenicity of the virus GS/GD/1/96. Interestingly, a recombinant of non-pathogenic virus containing NS1 gene from the pathogenic virus was able to replicate and to cause a disease or death of experi-

mental animals. These results demonstrated that the gene encoding NS1 significantly affects pathogenicity of the recombinant virus.

Currently, the research direction is focused on the preparation of a universal live attenuated vaccine that would not require a selection of currently circulating virus strains and at the same time would have a reliable protective effect. Today, seasonal vaccines (mostly inactivated) are prepared by a reassortment technique that is based on the preparation of a reassortant virus using vaccination strain of the influenza virus (e.g. A/PR/8/34 (H1N1)) and a currently circulating strain. The obtained reassortant virus contains HA and NA gene segments from the wild-type seasonal virus and the remaining segments are derived from the vaccination strain A/PR/8/34. However, the reassortment techniques used for the preparation of a vaccine are painstaking and time-consuming. In contrast, the method of reverse genetics offers an elegant and fast solution for construction of the viruses using "pre-made" cloned plasmids containing gene segments of the parental virus and plasmids encoding HA and NA derived from currently circulating influenza viruses.

A similar approach to improve the live attenuated vaccines was employed by Maassab and Bryant (1999), who created a live attenuated cold-adapted influenza vaccine. The vaccine was based on a construction of two stabile mutants of influenza virus type A and B that were used for the preparation of reassortant viruses using currently circulating influenza viruses. Next, the resulting reassortant virus was assembled from 6 genome segments of "pre-made" mutant and 2 segments – HA and NA – of current virus strain.

Suguitan *et al*. (2006) constructed a virus suitable as live attenuated vaccine using the reverse genetics. This virus included a gene encoding for a modified H5 HA and N1 NA of wild type virus A/H5N1 isolated in Hong Kong and Vietnam in 1997, 2003, and 2004. The remaining gene segments were derived from the cold-adapted influenza A vaccine A/Ann Arbor/6/60 (H2N2). Eight plasmids containing cloned cDNA in the system RNA polymerase I/II were transfected into Vero cells by electroporation.

Due to repeated cases of an avian influenza infection and ongoing threat of pandemic outbreak, a strategy to prepare a vaccine with fast and effective protective effect is permanently explored. Song *et al*. (2007) focused on a development of the new generation of modified live attenuated avian influenza viruses, which could be potential candidates for a poultry vaccine. Using methods of the reverse genetics, the mutation responsible for temperature sensitivity was introduced into the genes for PB1 and PB2 of the avian influenza virus A/Guinea Fowl/Hong Kong/WF10/99 (H9N2). The resulting recombinant designated as WF10 was a background for other genetic modifications and for a substitution in the genes NA and HA derived from a H7N2 virus.

A fundamentally different and promising possibility of the reverse genetics application is the introduction of foreign genes into an influenza virus to create a vaccination vector.

One of the first studies in this field (Muster *et al*., 1994) presented a chimeric influenza virus, where a conservative epitope from the gp41 ectodomain of HIV-1 was inserted into antigenic site B in the HA1 region of HA. Later, Ferko *et al*. (2001) constructed a hyperattenuated recombinant influenza virus with the 137-aa C-terminal region of HIV-1 protein Nef inserted into ORF of NS1 of the influenza virus. Also, Sereinig *et al*. (2006) utilized the insertion of *Mycobacterium tuberculosis* ESAT-6 protein sequence into the influenza NS1 segment with the aim to induce a protective immune response against *M. tuberculosis* in mice and guinea pigs.

Influenza virus served as the vector for a foreign gene in another important work (Li *et al.,* 2005). Here, a gene for the long domain of the protective antigen (PA) of *Bacillus anthracis* was inserted into HA region of the influenza virus A and resulting chimeric peptide was expressed in the infectious influenza virus. To create the chimeric infectious virus, an RNA expression plasmid was constructed, where PA/HA chimeric gene was introduced. The recombinant plasmid was transfected into 293T cells together with other plasmids using the system described by Neumann *et al*. (1999). The obtained chimeric virus was used as a vector for the induction of an immune response against *B. anthracis* PA protein.

The presented examples of reverse genetics application are only a part of the large number of studies that were able to answer several basic questions and to demonstrate an enormous potential of the reverse genetics methods. Similar, if not more important, is the role of reverse genetics in applied research especially in the vaccine development.

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References

Barclay WS, Palese P (1995): Influenza B viruses with site-specific mutations introduced into the HA gene. J. Virol. 69, 1275–1279.

Beaton AR, Krug RM (1986): Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 59 capped end. Proc. Natl. Acad. Sci. USA 83, 6282–8286. doi:10.1073/pnas.83.17.6282

Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P (2007): A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to

increased virulence. PLOS Pathogens 3, 1414–1421. doi:10.1371/journal.ppat.0030141

Egorov A, Brandt S, Sereinig S, Romanova J, Ferko B, Katinger D, Grassauer A, Alexandrova G, Katinger H, Muster T. (1998): Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. J. Virol. 72, 6437–6441.

Enami M, Luytjes W, Krystal M, Palese P (1990): Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA 87, 3802–3805. doi:10.1073/pnas.87.10.3802

Enami M, Sharma G, Benham C, Palese P (1991): An influenza virus containing nine different RNA segments. Virology 185, 291–298. doi:10.1016/0042-6822(91)90776-8

Ferko B, Stašáková J, Sereinig S, Romanova J, Katinger D, Niebler B, Katinger H, Egorov A (2001): Hyperattenuated recombinant influenza A virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 Nef-specific systemic and mucosal immune responses in mice. J. Virol. 75, 8899–8908. doi:10.1128/JVI.75.19.8899-8908.2001

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A (1999): Rescue of influenza A virus from recombinant DNA. J. Virol. 73, 9679–9682.

Grassauer A, Egorov AY, Ferko B, Romanova I, Katinger H, Muster T (1998): A host restriction-based selection system for influenza haemagglutinin transfectant viruses. J. Gen. Virol. 79, 1405–1409.

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000): A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA 97, 6108–6113. doi:10.1073/pnas.100133697

Honda A, Mukaigawa J, Yokoiyama A, Kato A, Ueda S, Nagata K, Krystal M, Nayak DP, Ishihama A (1990): Purification and molecular structure of RNA polymerase from influenza virus A/PR8. J. Biochem. 107, 624–628.

Horimoto T, Kawaoka Y (1994): Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68, 3120–3128.

Iwatsuki-Horimoto K, Horimoto T, Fujii Y, Kawaoka Y (2004): Generation of influenza A virus NS2 (NEP) mutants with an altered nuclear export signal sequence. J. Virol. 78, 10149–10155. doi:10.1128/JVI.78.18.10149-10155.2004

Li SQ, Schulman JL, Moran T, Bona C, Palese P (1992): Influenza A virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. J. Virol. 66, 399–404.

Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H (2006): The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J. Virol. 80, 11115–11123. doi:10.1128/JVI.00993-06 Li ZN, Mueller SN, Ye L, Bu Z, Yang Ch, Ahmed R, Steinhauer DA (2005): Chimeric influenza virus hemagglutinin proteins containing large domains of the Bacillus anthracis protective antigen: protein characterization, incorporation into infectious influenza viruses, and antigenicity. J. Virol. 79, 10003–10012. doi:10.1128/JVI.79.15.10003-10012.2005

Li S, Xu M, Coelingh K. (1995): Electroporation of influenza virus ribonucleoprotein complexes for rescue of the nucleoprotein and matrix genes. Virus Res. 37, 153–161. doi:10.1016/0168-1702(95)00031-K

Luytjes W, Krystal M, Enami M, Pavin JD, Palese P (1989): Amplification, expression and packaging of a foreign gene by influenza virus. Cell 58, 1107–1113. doi:10.1016/0092-8674(89)90766-6

Maassab HF, Bryant ML (1999): The development of live attenuated cold-adapted influenza virus vaccine for humans. Rev. Med. Virol. 9, 237–244. doi:10.1002/(SICI)1099-1654(199910/12)9:4<237::AID-RMV252>3.0.CO;2-G

Muster T, Guinea R, Trkola A, Purtscher M, Klima A, Steindl F, Palese P, Katinger H (1994): Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. J. Virol. 68, 4031–4034.

Neumann G, Fujii K, Kino Y, Kawaoka Y (2005): An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc. Natl. Acad. Sci. USA 102, 16825–16829. doi:10.1073/pnas.0505587102

Neumann G, Kawaoka Y (2001): Reverse genetics of influenza virus. Virology 287, 243-250. doi:10.1006/viro.2001.1008

Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez D, Donis R, Hoffmann E, Hobom G, Kawaoka Y (1999): Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. USA 96, 9345–9350. doi:10.1073/pnas.96.16.9345

Neumann G, Zobel A, Hobom G (1994): RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202, 477–479. doi:10.1006/viro.1994.1365

Palese P, Shaw LM (2007): Orthomyxoviridae: The Viruses and Their Replication. Chapter 47. In: Knipe DM, Howley PM. (Eds): Fields Virology. Vol. II, 5 ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1647–1690.

Parvin JD, Palese P, Honda A, Ishihama A, Krystal M (1989): Promoter analysis of influenza virus RNA polymerase. J. Virol. 68, 5142–5152.

Plotch SJ, Bouloy M, Ulmanen I, Krug RM (1981): A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23, 847–858 doi:10.1016/0092-8674(81)90449-9

Sereinig S, Stukova M, Zabolotnyh N, Ferko B, Kittel C, Romanova J, Vinogradova T, Katinger H, Kiselev O, Egorov A (2006): Influenza virus NS vectors expressing the Mycobacterium tuberculosis ESAT-6 protein induce CD4+ Th1 immune response and protect animals against tuberculosis challenge. Clin. Vaccine Immunol. 13, 898–904. doi:10.1128/CVI.00056-06

Schnell MJ, Mebatsion T, Conzelmann KK (1994): Infectious rabies viruses from cloned cDNA. EMBO J. 13, 4195-4203.

Song H, Nieto GR, Perez DR (2007): A new generation of modified live-attenuated avian influenza viruses using a two-strategy combination as potential vaccine candidates. J. Virol. 81, 9238–9248. doi:10.1128/JVI.00893-07

Subbarao EK, Kawaoka Y, Murphy BR (1993): Rescue of an influenza A virus wild-type PB2 gene and a mutant de-

rivative bearing a site-specific temperature-sensitive and attenuating mutation. J. Virol. 67, 7223–7228.

Suguitan AL, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murény B, Swayne DE, Kemble G, Subbarao K (2006): Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in Mice and Ferrets. PLoS Medicine 3, 1541–1555. doi:10.1371/journal.pmed.0030360

Watanabe T, Watanabe S, Ito H, Kida H, Kawaoka Y (2001): Influenza A virus can undergo multiple cycles of replication without m2 ion channel activity. J. Virol. 75, 5656–5662. doi:10.1128/JVI.75.12.5656-5662.2001