Many ways to make an influenza virus – review of influenza virus reverse genetics methods

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Methods to introduce targeted mutations into a genome or, in the context of virology, into a virus are subsumed under the term reverse genetics (RG). Influenza viruses are important human pathogens that continue to surprise us. The development of RG for influenza viruses has greatly expanded our knowledge about influenza virus and enabled researchers to generate influenza viruses with rationally designed genotypes. Currently, a wide array of influenza virus RG methods is available. These can all be traced to fundamental principles essential in any RG system for negative-strand RNA viruses. This review gives an overview of these principles and of the multitude of RG methods, categorising them by technical characteristics.

Keywords Candidate vaccine viruses, influenza virus, methods, reverse genetics.

Introduction

Influenza viruses are the most prominent members of the family *Orthomyxoviridae*. Their genome consists of eight (influenza A and B) or seven (influenza C) negative-sense RNA segments, each encoding one or more viral proteins. Owing to the segmented nature of the influenza virus genome, reassortment of RNA segments can occur upon co-infection of one cell with two or more viruses, resulting in viruses with new genetic constellations. Reassortment has been used to investigate functions of viral proteins and to generate candidate vaccine viruses for the production of influenza vaccines for decades. However, reassortment does not allow the targeted engineering of viral genes, limiting the scope of investigations. In contrast, reverse genetics (RG) in the context of viruses describes 'the ability to engineer deliberate genetic change into a viral genome'.[1]

Isolated influenza virus RNA, when introduced into cells, is non-infectious. The minimal transcriptionally active unit of the influenza virus is the viral ribonucleoprotein complex (RNP), containing in addition to viral genomic RNA a set of at least four viral proteins, namely nucleoprotein (NP) and the trimeric viral RNA-dependent RNA polymerase (RdRp) consisting of one subunit each of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). Influenza virus RG methods, that is, the generation of influenza viruses from cloned DNA, therefore have to provide both viral genomic RNA and the set of essential proteins to a susceptible cell. RG has been crucial in elucidating various aspects of the basic biology of influenza viruses and has also aided the development of candidate vaccine viruses (CVVs), in particular those derived from highly pathogenic avian influenza viruses such as H5N1 and H7N7 identified in poultry outbreaks and sporadically in humans.[2–5]

Influenza A virus was the first negative-strand RNA virus that became amenable to genetic manipulation through the development of a reverse genetics method.[6,7] This first method allowed the genetic manipulation of one influenza genomic RNA segment at a time and depended on the use of a helper virus and a selection system to select for the newly generated recombinant (also known as 'transfectant') influenza virus, or against the respective RNA segment of the helper virus. A major breakthrough in influenza virus reverse genetics was achieved in 1999 when two groups independently published methods to generate influenza viruses entirely from cDNA, without the use of helper viruses and thus obviating the need for a selection system.[8,9]

This literature review collates information on RG methods for influenza virus that are in the public domain and attempts to categorise them based on various technical aspects of these methods.

Methods

PubMed was searched for relevant articles using the keywords 'influenza' 'reverse' 'genetics'. Relevant articles were reviewed in detail. Additional relevant publications were extracted from the bibliographies of reviewed papers and from the author's files. Information from patents and patent applications was retrieved using freely available online databases maintained by the US Patent and Trademark Office (USPTO), World Intellectual Property Organisation (WIPO) and European Patent Office (EPO).

Results

Based on the literature reviewed during this study, two main groups of influenza virus RG methods can be discerned: (i) helper virus-dependent and (ii) helper virus-independent (Figure 1).

Helper virus-dependent methods

These were the first influenza virus reverse genetics methods developed.[6,7] While they proved successful and of extremely high value to the field of influenza research, their weakness is their dependence on a helper influenza virus

which in turn necessitates the use of a selection system that allows the isolation of the desired recombinant/transfectant influenza virus. The stringency of the employed selection system determines, to a large extent, the ease of generating, or finding, the desired transfectant virus. During the period when helper virus-dependent systems were the only available RG systems for influenza virus, selection systems for only six of the eight influenza A virus genomic RNA segments were described (reviewed for example in[10,11]). This meant that two RNA segments, PB1 and PA, could not be genetically manipulated. Furthermore, different selection strategies varied in their stringency and thus in their usefulness. The dependence on a selection system may also mean that certain virus genes, or certain mutations in virus genes, cannot be rescued into viable virus owing to incompatibility with the selection strategy. For instance, a selection system based on a temperature-sensitive (ts) helper virus, as described for the NS genome segment, cannot be used to generate viruses with mutations in the relevant gene that confer a ts phenotype.[12] Therefore, the number of mutant or reassortant viruses generated using this system was considerably lower than the number generated using helper virus-independent systems, once these were established.
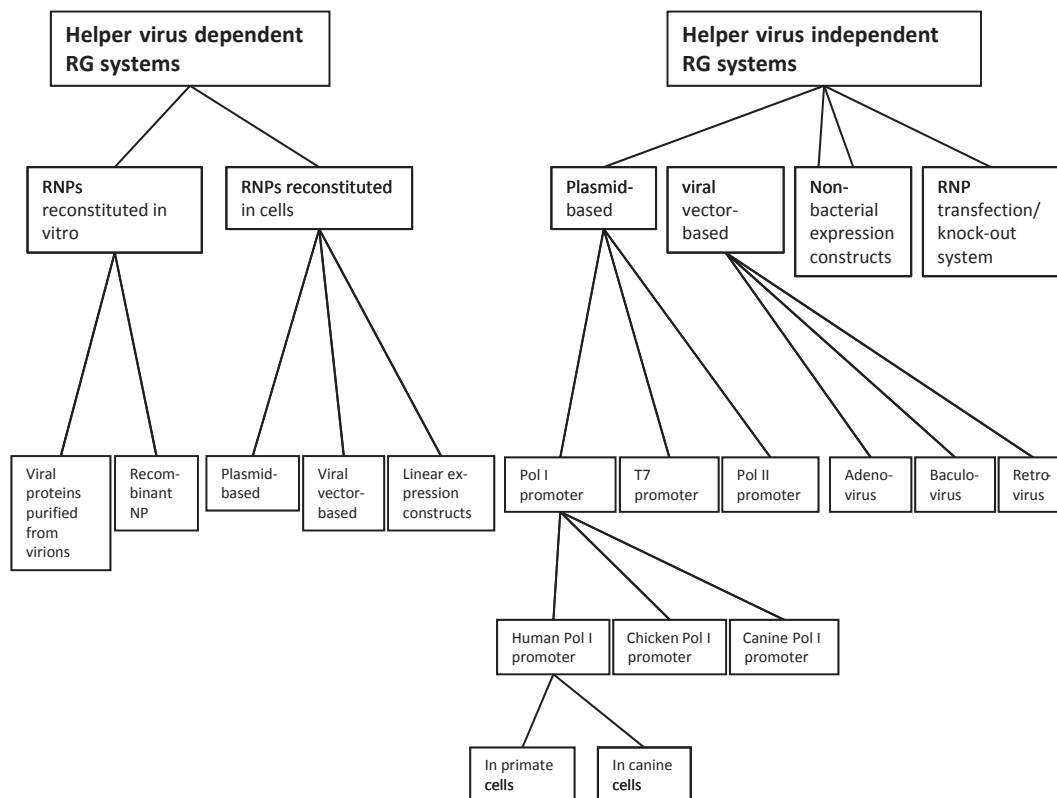

Figure 1. Schematic overview of influenza reverse genetics methods by main characteristics of methods.

Helper virus-dependent methods can be further subdivided based on the way the cDNA-derived gene is provided.

RNP transfection

In the original method, an *in vitro* reconstituted RNP was transfected into helper virus-infected cells; these RNPs were generated by combining RNA produced by *in vitro* run-off transcription (using a phage promoter) and purified viral proteins [NP and polymerase proteins (Ps)] necessary for the formation of viral RNPs. The purification of the viral proteins was technically demanding and not highly reproducible.[13,14] A variation of this method used recombinant NP, expressed in insect cells using a baculovirus vector encoding the viral NP, instead of purified viral proteins from virus preparations.[15]

Nucleic acid-based RNP reconstitution in cells

Ribonucleoprotein complex can also be reconstituted within cells, by transfecting cells with a plasmid expressing a viral-like RNA. Expression of vRNA within cells was achieved using the promoter recognised by the cellular DNA-dependent RNA polymerase I (Pol I).[16–18] Generation of the correct 3' end of the expressed vRNA occurred through the activity of a ribozyme, encoded immediately downstream of the cDNA copy of the viral RNA, or through the use of a Pol I terminator sequence.[16–18] These viral-like, intracellularly expressed RNAs are recognised by viral polymerase proteins and NP and subsequently packaged into RNPs, replicated and transcribed. Provision of the four essential viral proteins, PB1, PB2, PA and NP, can occur through infection with a helper influenza virus or through transfection of plasmids expressing these proteins, usually under the control of a cellular DNA-dependent RNA polymerase II (Pol II)-dependent promoter (for brevity referred to as Pol II promoter in this review). For the generation of infectious recombinant viruses, a helper virus is required, regardless of whether or not the viral polymerase proteins and NP are expressed from plasmids. A recent patent application[19] describes transfection of cells with linear expression constructs instead of plasmids for the expression of a viral RNA inside cells, again utilising a Pol I promoter for driving expression of the viral RNA. Furthermore, the described linear construct also contains a Pol II promoter and polyadenylation site flanking the Pol I transcription unit (for details of this type of dual promoter construct see below). This method circumvents the need to generate plasmid DNA and allows the direct use of linear DNA generated, for example, by PCR, potentially saving time.

Viral vector-based RNP reconstitution in cells

Another method of providing cDNA for the expression of a vRNA within mammalian cells used a baculovirus vector containing a Pol I transcription unit (Pol I promoter and hepatitis delta virus ribozyme sequence).[20] Cells were first infected with the recombinant baculovirus expressing a full-length vRNA and later superinfected with an influenza virus. A selection system was required to select for the recombinant virus containing the transfected gene.

Owing to the disadvantage of having to use selection strategies, helper virus-dependent RG methods have been largely replaced by helper virus-independent methods (see following section).

Helper virus-independent methods

In the majority of these methods, all viral genomic RNA segments are expressed inside cells, using various plasmids or other vector systems, together with the necessary viral 'helper' proteins. Selection strategies are not required because no helper viruses are used and therefore do not need to be removed. A different approach has been described by Enami and Enami[21]; this method, the application of which has not been reported in the literature following its initial description, uses purified RNPs from influenza virus preparations, but not infection with helper virus.

Plasmid-only reverse genetics systems

Expression of viral RNA segments and the required viral proteins is most often achieved by the transfection of appropriate plasmids into cells (plasmid-based RG systems). Plasmid-only reverse genetics systems can be divided into subgroups depending on various technical features, such as type of promoter used to express vRNAs in cells, numbers and types of plasmids, transcription control elements other than promoters, and cell types (species), with overlaps between these subgroups. Plasmid-based RG systems have been developed for influenza A, B and C viruses (see references below) as well as for the tick-transmitted orthomyxovirus, Thogoto virus.[22]

Promoters used to express viral RNA

The first helper virus-free plasmid-only reverse genetics systems employed plasmids containing a human Pol I promoter to express viral RNAs.[8,9] The 3' end of the expressed viral RNAs was generated through the activity of a ribozyme or a (murine) Pol I terminator sequence. Pol I promoters are generally believed to be species-specific, that is, to work only in the species of origin of the promoter sequence, or in related species.[23,24] Thus, the human Pol I promoter has been used for reverse genetics purposes in human 293T[9] or PER.C6[25] and monkey Vero[8] or COS-1[26] cells, with or without coculture of cells more susceptible to influenza virus infection such as MDCK[26–28] or chicken embryo cells.[29] However, recently, it was reported that reverse genetics plasmids containing a human Pol I

promoter were successfully used to generate influenza viruses in canine MDCK cells.[30] This report is in contrast to previous work by others reporting failure to generate influenza viruses in MDCK cells transfected with reverse genetics plasmids containing human Pol I promoters.[31,32]

Canine Pol I promoters and chicken Pol I promoters have been described for use in MDCK cells[31,32] and avian cells [chicken embryo fibroblasts (CEF)],[33,34] respectively. While the murine Pol I promoter was the first one to be used for the expression of vRNAs or vRNA-like RNAs in cells,[16,18] its use for a plasmid-only reverse genetics system has not been described, possibly owing to the lack of easily transfectable murine cell lines.

To address the problem of species specificity of Pol I promoters, a universally applicable plasmid-based reverse genetics system was designed using the bacteriophage T7 promoter to express viral RNA segments in cells.[35] This system depends on the expression, within the transfected cells, of the T7 RNA polymerase protein; this was achieved by transfection of plasmids expressing either a cytoplasmic or a nuclear version of T7 RNA polymerase, with the latter showing higher efficiency of influenza virus generation. The T7 reverse genetics system was used to generate influenza viruses in human 293T, canine MDCK and quail QT6 cells. A recent patent[36] proposes the use of Pol II promoters to express viral RNAs for reverse genetics; in this system, transcription of the viral RNAs is driven by a Pol II promoter and both the 5′ and 3′ end of the viral RNAs are generated by self-cleaving ribozymes, a hammerhead ribozyme at the 5′ end and a hepatitis delta ribozyme at the 3′ end.

Generation of correct 3′ end of expressed vRNA

Two basic approaches have been developed to generate the correct 3′ end of vRNA expressed inside transfected cells: (i) use of a ribozyme that cleaves the transcribed RNA such that the ribozyme is released from the transcript and (ii) use of a Pol I terminator sequence that directs the transcribing RNA polymerase I to stop transcription. The hepatitis delta virus genomic[8,17] and antigenomic ribozyme[37] have been used in reverse genetics plasmids. In all described RG systems employing a Pol I terminator element, a murine Pol I terminator sequence has been used, which has proved functional in primate[9,27], canine[30–32] and chicken cells.[33,34]

Numbers and types of plasmids

The minimal transcriptionally active unit of influenza virus is the RNP, consisting of viral RNA complexed with viral nucleoprotein (NP) and the viral RdRp, which is made up of one subunit each of the three proteins PB1, PB2 and PA. Because the viral genome is of negative sense and is usually expressed in negative orientation in reverse genetics systems, these four influenza proteins have to be co-expressed together with the complement of eight viral RNA segments to 'kick-start' an infectious cycle in transfected cells. The strategies for provision of these four proteins, and occasionally other viral proteins, differ between various published plasmid-based RG systems.

In the pioneering studies establishing plasmid-based RG systems for influenza virus, four[8,9] or more[9] plasmids expressing viral proteins under the control of a Pol II promoter were co-transfected into cells together with eight plasmids expressing the eight viral RNA segments. Expression of more than the essential four RNP-associated viral proteins from additional plasmids increased efficiency of virus generation, resulting in a total of 17 plasmids that were transfected into cells.[9] A number of subsequent studies employed transfection of 12 plasmids (eight vRNA expressing plasmids and four protein-expression plasmids) for the generation of influenza A and B viruses de novo.[8,28,29,31,37–40] Koudstaal et al.[25] expressed the four essential viral helper proteins from only two plasmids, using a Pol II promoter and an internal ribosomal entry site (IRES) to express two proteins from one plasmid; this RG system thus requires transfection of 10 plasmids into cells. The T7 promoter-based RG system described earlier requires transfection of 13 plasmids, because of the need to express T7 RNA polymerase in addition to the viral helper proteins.[35] Because influenza C virus has only seven genomic RNA segments, plasmid-based RG systems for influenza C virus use transfection of 11 or 16 plasmids, depending on whether or not more proteins than the essential set of polymerase proteins and nucleoprotein are expressed from plasmids.[41,42]

Hoffmann et al. developed approaches that combined Pol I and Pol II transcription cassettes onto a single plasmid.[26,27,43,44] The approach that proved more efficient and has been used more in subsequent studies, used Pol I and Pol II transcription units in opposing directions; full-length vRNA (negative sense) was expressed under the control of a human Pol I promoter, with a murine terminator defining the 3′ end of the transcript; this transcriptional unit was flanked by a Pol II promoter and a polyadenylation signal, placed at opposite ends of the Pol I transcription unit, such that positive-sense transcripts (mRNA) were produced by cellular Pol II.[27,43] This system is known as 'bidirectional'. A 'unidirectional' strategy, with positive-sense cRNA as well as (positive sense) mRNA being transcribed in the same direction because of the positioning of a Pol I promoter and a Pol II promoter upstream of the inserted cDNA, was also successful in generation of influenza virus de novo, but the efficiency of this system was lower than the bidirectional system.[26] Both systems require transfection of eight plasmids into cells. The bidirectional approach has been adopted by others to specific requirements, for instance for use with other Pol I promoters

(chicken, canine),[32,33] with human Pol I promoter in canine cells,[30] for generation of influenza B and C viruses[32,44–46] and even for viral vector-based systems.[47,48] Interestingly, it has been reported that the bidirectional approach did not work when combined with the T7 promoter system.[35]

Attempts have been made to further reduce the number of plasmids that have to be transfected into cells. Neumann et al.[49] described several variations of a system that combines more than one Pol I transcription unit on one plasmid. Generation of influenza viruses was achieved using plasmids containing up to eight Pol I units per plasmid. A variation that may be more useful for the generation of reassortant viruses for vaccine production used two vRNA expressing plasmids, one expressing HA and NA vRNAs, the other expressing the remaining six vRNAs. These plasmids did not contain Pol II promoters for the expression of viral proteins, and thus, separate plasmids expressing one or more viral proteins were cotransfected. Surprisingly, in control transfections, transfection of one plasmid containing all eight Pol I transcription units, or of two plasmids containing two and six Pol I transcription units, resulted in generation of influenza virus *de novo* even without the supply of Pol II-dependent protein expression plasmids. A one plasmid RG system for chicken cells was described recently.[34] In this system, four transcription units under the control of a chicken Pol I promoter were combined with four bidirectional Pol I/Pol II units (for NP and three Ps) in one plasmid.

Cell lines used
Owing to the species specificity of the Pol I promoter (but see[30] for an exception), the choice of Pol I promoter limits the choice of cell lines that can be used. Cell lines successfully used in plasmid-based reverse genetics systems include human cells (293T, PER.C6),[9,25–27,35,49] monkey cells (Vero, COS-1),[8,26,48,49] avian cells (CEF, QT6)[33–35] and canine cells (MDCK).[30–32,35] Some systems employ coculture of two different cell lines, or addition of a second cell line following transfection into a first cell line.[26–29,37,38,44] Coculture/addition systems are often used to increase yields of influenza virus generated by reverse genetics. It should be noted that the choice of cell lines for the generation of candidate vaccine viruses by reverse genetics is very limited because, according to WHO guidance, cells 'approved for human vaccine production should be used' for this purpose.[50] Such qualification has only been achieved for a small number of cell lines.

Other distinguishing elements of plasmid-based RG systems
Apart from the features described earlier that can be used to categorise plasmid-based RG systems into (overlapping) subgroups, there are minor technical differences between plasmid-based RG systems that could be used for further subdivision. Such features include (i) the way viral cDNAs are inserted into RG plasmids, for example, by the use of restriction endonucleases and ligases (in most plasmid-based RG systems), by homologous recombination *in vitro* or in bacterial cells,[51–53] or by target-primed plasmid amplification;[54] (ii) the way of introducing plasmids into cells, for example, by transfection (the most commonly used method) or electroporation.[32]

Use of non-bacterial expression constructs
Expression constructs other than plasmids have been described for use in reverse genetics in recent patent applications. Eight linear expression constructs containing two transcription cassettes, a Pol I cassette for the expression of viral RNA and a Pol II cassette, flanking the Pol I unit, for the expression of viral mRNA/protein were transfected into cells, from which influenza virus could be recovered.[55] Non-bacterial expression constructs, in particular linear constructs, containing coding sequences for more than one viral genome segment for reverse genetics have been claimed in another patent application.[56] These methods that do not require the generation of plasmids may save time, for example, when a candidate vaccine virus needs to be produced rapidly in the case of a newly emerging virus of pandemic concern.

Viral vector-based RG systems
Expression of viral RNA segments and the required viral proteins can also be achieved by transduction of cells with viral vectors containing the relevant expression cassettes.

Adenovirus based RG system
Ozawa et al.[48] described the development of two related RG systems using adenoviral vectors to deliver expression units/cassettes into host cells such that viral RNA segments and the four minimally required viral proteins (PA, PB1, PB2 and NP) are expressed in these cells. In the first system, 12 replication-incompetent adenovirus type 5-based vectors were used; eight contained human Pol I promoter – Pol I terminator units expressing all eight viral RNA segments and four were Pol II promoter-dependent protein expression vectors expressing the four viral RNP components. In their second system, bidirectional constructs (Pol I transcription unit inserted between Pol II promoter and polyadenylation site) were used, resulting in the need to use only eight adenovirus vectors for the generation of influenza virus de novo. The 8-vector system was found to be more efficient than the 12-vector system.

Baculovirus-based RG system
A reverse genetics system for influenza B virus reported by Nakowitsch et al.[47] used eight baculovirus vectors containing bidirectional expression cassettes. The baculovirus

vectors were used to infect mammalian cells (FreeStyle 293 cells) in which all viral RNA segments as well as viral proteins were expressed and influenza B virus was generated de novo.

Other viral vector systems

The use of retroviral vectors for reverse genetics has been suggested in a recent patent application,[57] but this system has not yet been published in the scientific literature.

RNP transfection-based helper virus-free reverse genetics system

Enami and Enami[21] described a helper virus-free reverse genetics system that is independent of introduction of DNA-based expression cassettes into cultured cells. This system uses viral RNPs purified from virus preparations; these RNPs contain all eight genomic vRNA segments of the virus, packaged as functional RNPs. To eliminate one particular viral RNA segment, the authors used the enzyme RNase H, which cleaves RNA when annealed to single-stranded complementary DNA, in conjunction with cDNA fragments corresponding to the targeted genomic segment. The targeted vRNA segment was replaced by an *in vitro* reconstituted RNP containing RNA transcribed from a linearised plasmid, as described for helper virus-dependent RNP transfection-based reverse genetics. The use of this system, which appears not to be very efficient, has not been reported in the literature since its original description.[21]

Discussion

The genetic manipulation of influenza viruses has been possible for more than two decades through the use of RG methods and has resulted in numerous new insights into the biology of influenza viruses. With the emergence of highly pathogenic avian H5N1 viruses in 1997 and again in 2003, and the threat of a pandemic, RG has become the method of choice for developing candidate vaccine viruses from highly pathogenic viruses in global efforts directed towards pandemic preparedness. The classical way of generating candidate vaccine viruses, which is still used for seasonal influenza vaccines, that is, reassortment of a wild-type virus and a high-growth laboratory-adapted strain, such as A/Puerto Rico/8/34 (PR8), cannot be used for developing candidate vaccine viruses from highly pathogenic parental viruses owing to biosafety concerns, as the polybasic cleavage site of the haemagglutinin, which is the main determinant of high pathogenicity in avian H5N1 viruses, would still be present in the resulting reassortant viruses. Only RG allows the attenuation of highly pathogenic H5 and H7 viruses by deletion of the stretch of basic amino acids at the cleavage site of the haemagglutinin.[3,4,39]

Thus, there is considerable interest in the influenza research and vaccine communities in optimised RG methods.

Influenza virus RG methods rely on a few principles that can be implemented in different ways. RG methods for all negative-strand RNA viruses have to take account of the fact that isolated viral RNA is not in itself infectious, a situation different from positive-sense RNA viruses, for which, consequently, RG methods were easier to establish and were described earlier. For negative-strand RNA viruses, supply of both functional viral RNA and a set of essential viral proteins is required to generate infectious virus *de novo*. The supplied viral RNA can be of genomic orientation (i.e. negative sense) or antigenomic orientation (positive sense); the latter has been used widely in RG systems for non-segmented negative-strand RNA viruses (for reviews see for example),[58,59] which are not subject of this review. It should be noted that important cis-acting signals are present in the terminal regions of influenza viral RNAs, and thus care must be taken to ensure the completeness and correct length of the non-coding regions at the 3′ and 5′ ends of viral genomic RNAs. The essential viral proteins, in the case of influenza virus the NP and three polymerase subunits, can be supplied in various ways; in the earliest described RG methods for influenza virus, RNPs were reconstituted *in vitro* or in cultured cells, followed by infection with a helper virus. In helper virus-free RG methods, the full genome of influenza virus and the essential viral proteins are provided to/in susceptible cells to 'kick-start' a viral life cycle and generate virus de novo. Following on from the first description of helper virus-free RG systems in 1999,[8,9] researchers have modified these in order to improve efficiency or to make them easier to work with. Different requirements of laboratories may direct them towards certain RG methods in preference over others. For instance, when generating CVVs for use in the production of human vaccines, qualified cell lines have to be used, of which there are few; this entails the use of appropriate Pol I promoters active in the chosen cell line or cell type. While some studies in the literature report comparative efficiency data, it is generally difficult to compare efficiencies of RG systems between laboratories and reports, because of the non-standardised way of assessing efficiency and the potential confounding effects of laboratory-specific variables that are not intrinsic to the employed RG system as such. Therefore, it is at this point impossible to select one or more RG methods based on highest possible efficiency of generation of influenza viruses. Other factors, such as number of plasmids to handle, cell line availability, efficiency of transfection in a particular cell line of interest, differences in viral growth between virus strains and experience with various formats of RG systems, may determine which RG method is being used in a laboratory.

The use of influenza virus RG systems has enhanced our knowledge of influenza virus and enabled a rapid vaccine response to newly emerging influenza viruses, including those of high pathogenicity. A wide array of RG methods is now available to choose from. Further technical modifications of influenza virus RG methods will likely be developed in the future; for instance, the use of synthetically made DNA rather than cDNA copies of viral gene segments, in RG systems as described earlier, has been described and is likely to be of more importance in the future.[60–63] However, all influenza virus RG systems build on the same principles which apply for RG of all negative-sense RNA viruses.

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