Influenza Reverse Genetics—Historical Perspective

Gabriele Neumann

Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, Wisconsin 53711, USA

Correspondence: gabriele.neumann@wisc.edu

The generation of wild-type, mutant, and reassortant influenza viruses from viral cDNAs (reverse genetics) is now a basic molecular virology technique in many influenza virus laboratories. Here, I describe the original RNA polymerase I reverse genetics system and the modifications that have been developed in past years. Together, these technologies have made possible many advances in basic and applied influenza virology that would not have been otherwise attainable, including the revival and study of extinct influenza viruses, the rapid characterization of emerging influenza viruses, the generation of conventional influenza vaccines, and the development of novel influenza vaccines.

Reverse genetics—that is, the generation of influenza viruses from cloned cDNAs—is now a basic procedure in many influenza research laboratories around the world. However, until 20 years ago, the artificial generation and modification of influenza viruses was considered an insurmountable task. First, the viral RNAs (vRNAs) of influenza viruses are of negative polarity and, as a consequence, are not sufficient to initiate viral replication and transcription. Rather, the four protein components of the viral replication machinery (i.e., the three polymerase subunits PB2, PB1, and PA, plus the nucleoprotein NP) have to be provided to initiate the replication and transcription of the vRNAs. As a further complication, in contrast to nonsegmented negative-sense RNA viruses, the genomes of influenza viruses are comprised of six to eight vRNAs, all of which are required

for influenza virus generation. For example, influenza A viruses contain eight vRNA segments; therefore, influenza A virus reverse genetics systems require 12 components be artificially supplied—eight are the required vRNA segments and four are the components of the viral replication machinery. Moreover, it was not known at the time if the amounts and ratios of these components, and the kinetics of their synthesis, are critical for influenza virus generation. As an added obstacle to establishing a reverse genetics system, influenza viruses replicate in the nucleus of infected cells (in contrast to most other RNA viruses), so it follows that the vRNAs need to be synthesized in, or delivered to, this cellular compartment. The combination of these factors presented major hurdles to the development of reverse genetics systems for influenza viruses.

THE ERA BEFORE HELPER-VIRUS-FREE REVERSE GENETICS

As a first step toward the development of reverse genetics systems for influenza viruses, studies in the 1980s showed the isolation of functional viral ribonucleoprotein (vRNP) complexes—comprised of vRNA and the viral polymerase and NP proteins—from disrupted viruses (Plotch et al. 1981; Honda et al. 1987) or infected cells (Beaton and Krug 1986), showing that these viral components are sufficient for vRNA replication. Shortly thereafter, the in vitro reconstitution of functional vRNPs was achieved from purified polymerase and NP proteins, which were able to transcribe short synthetic vRNAs or full-length vRNAs isolated from virions (Parvin et al. 1989; Honda et al. 1990). These studies identified the critical viral components of influenza virus replication and showed that these components can be reconstituted into vRNP complexes with polymerase activity.

The first system for the modification of influenza viruses was established in the late 1980s (Luytjes et al. 1989; Enami et al. 1990). A virus-like RNA (encoding a reporter protein) was transcribed in vitro by T7 RNA polymerase; the 3′ end of the virus-like RNA was generated with the help of a restriction endonuclease. The in vitro synthesized virus-like RNA was mixed with purified polymerase and NP proteins to reconstitute vRNP complexes, which were then transfected into eukaryotic cells. Subsequent infection of the vRNP-transfected cells with helper influenza virus provided the remaining seven vRNP complexes, resulting in recombinant influenza virus possessing an artificially generated vRNA. This approach allowed for the modification of an individual vRNA, but it relied on helper-virus infection and efficient selection methods to isolate the modified viruses from the large background of wild-type virus. During the following years, several selection systems were established based on neutralizing antibodies (Enami and Palese 1991; Horimoto and Kawaoka 1994; Barclay and Palese 1995), host-range restriction (Enami et al. 1990; Subbarao et al. 1993), temperature sensitivity (Enami and Palese 1991; Yasuda et al. 1994), or drug resis-

tance (Castrucci and Kawaoka 1995). However, these systems were of low efficiency, technically demanding, and limited to specific viral strains and viral proteins for which selection systems were available.

RNA POLYMERASE I—OPENING THE DOOR FOR A REVERSE GENETICS SYSTEM FOR INFLUENZA VIRUSES

The breakthrough in influenza virus reverse genetics was achieved by using a cellular enzyme, RNA polymerase I, for the intracellular synthesis of the influenza vRNAs. RNA polymerase I possesses several features that make it an ideal candidate for the intracellular synthesis of influenza vRNAs: (1) It localizes to the nucleus of eukaryotic cells and the site of influenza virus replication and transcription; (2) RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences, allowing the synthesis of transcripts with the desired 5′ and 3′ ends; (3) RNA polymerase I transcripts do not possess 5′-cap or 3′-poly(A) structures and resemble the "cap-less" and "tail-less" influenza vRNAs in this regard; (4) RNA polymerase I transcribes ribosomal RNA (rRNA) and is thus highly expressed in growing cells; and (5) RNA polymerase I synthesizes rRNA transcripts of >20 kb in length; hence, the length of the influenza vRNAs (ranging from 0.9 to 2.3 kb in length) should not be a limiting factor.

Despite the appealing properties of RNA polymerase I for the intracellular synthesis of influenza vRNAs, several critical questions and issues remained at the onset of the project: (1) Does RNA polymerase I transcribe "foreign" (non-rRNA) sequences cloned between the RNA polymerase I promoter and terminator sequences? Or are "foreign" sequences not transcribed because they lack regulatory sequences located within the rRNA template sequences? (2) The RNA polymerase I promoter is composed of core and enhancer elements. Which of these elements are necessary and sufficient for efficient transcription? (3) The detailed mechanism of 3′-end formation of RNA polymerase I transcripts was still under investigation

at the time this system was established; thus, it was not clear whether the 3′ ends of vRNAs generated by RNA polymerase I would be identical to those generated by the viral replication machinery.

Initial in vitro studies focused on the open questions described above. Influenza virus–like RNAs cloned between the RNA polymerase I promoter and terminator sequences showed that "foreign" sequences were transcribed with defined 5′ and 3′ ends, resulting in transcripts indistinguishable from authentic influenza vRNAs (Zobel et al. 1993; Neumann et al. 1994). In the next step, eukaryotic cells were transfected with a plasmid possessing RNA polymerase I promoter and terminator sequences separated by cDNA encoding an influenza-like RNA expressing a reporter protein (Neumann et al. 1994). Following influenza helper infection (which provides the remaining seven vRNP complexes and viral proteins required for transcription and replication), the reporter protein was successfully expressed and the virus-like RNA was incorporated into progeny virus (Neumann et al. 1994). These findings established that RNA polymerase I can be used to generate functional influenza vRNAs in eukaryotic cells. Pleschka et al. (1996) used this approach for the intracellular generation of an influenza vRNP encoding NA, followed by helper-virus infection.

GENERATION OF INFLUENZA VIRUSES FROM CLONED cDNA

The RNA Polymerase I System

In the 1990s, it was generally assumed that the transfection of eukaryotic cells with several plasmids resulted in low percentages of cells transfected with all plasmids. The transfection of cells with 12 different plasmids (as would be needed for influenza A virus reverse genetics) thus seemed impossible. However, the high transfection efficiency of human embryonic kidney (293T) cells enabled the development of experimental systems that require the transfection of cells with multiple plasmids (Neumann et al. 1999).

The first de novo synthesis of an influenza A virus from cloned cDNA was achieved in 1999 (Neumann et al. 1999). Cloned cDNAs of all eight vRNAs of an influenza A virus were individually flanked by human RNA polymerase I promoter and mouse RNA polymerase I terminator sequences. These eight plasmids were transfected into 293T cells. The cells were also transfected with four protein expression plasmids encoding the viral polymerase and NP proteins; we expressed all four proteins under the control of the strong chicken β-actin promoter (Niwa et al. 1991) to ensure robust levels of the viral proteins critical for viral transcription and replication.

In cells simultaneously transfected with all 12 plasmids, the viral polymerase and NP proteins will replicate and transcribe the eight vRNAs synthesized by cellular RNA polymerase I, resulting in authentic live influenza virus. To detect the presence of replicating influenza viruses, the supernatant from transfected 293T cell was collected at different times after transfection and propagated in Madin–Darby canine kidney (MDCK) cells, a cell line commonly used for this task. Indeed, replicating virus was detected, marking the first generation of influenza virus from cloned cDNA. In this study, we also generated two novel mutant viruses, hence showing the ability to modify the genome of influenza viruses (Neumann et al. 1999). (For a detailed protocol of influenza virus reverse genetics, see Neumann et al. 2012.)

It is important to note that the protein expression plasmids do not need to match the polymerase and NP proteins of the virus to be rescued. The proteins expressed from the protein expression plasmids "kick-start" viral replication and transcription, but their genetic sequences will not be incorporated into the newly generated viruses. We routinely use the polymerase and NP proteins of the laboratory-adapted A/WSN/1/33 (WSN; H1N1) or A/Puerto Rico/8/34 (PR8; H1N1) viruses for reverse genetics, independent of the subtype and host origin of the virus to be rescued. The polymerase and NP proteins of the WSN and PR8 viruses typically facilitate efficient replication and transcription. Moreover, by using sets

of core protein expression plasmids, there is no need to generate new protein expression plasmids for every virus to be rescued.

The above-described reverse genetics system is highly efficient for the great majority of influenza viruses to be generated. As described above, plasmids encoding the polymerase and NP proteins are sufficient for influenza viruses generation; however, in cases of low virus rescue efficiency, additional protein expression plasmids (such as plasmids encoding the HA and NA proteins) can increase the efficiency of virus generation (Neumann et al. 1999). Increases in reverse genetics efficiencies have also been achieved with co-cultures of 293T and MDCK cells, in which the 293T ensured high transfection efficiency, and the MDCK cells facilitated efficient influenza virus replication (Hoffmann et al. 2000).

Use of a Ribozyme to Generate the 3′ Ends of Viral RNAs

Fodor et al. (1999) also used the human RNA polymerase I promoter but combined it with the hepatitis delta virus ribozyme sequence to generate the 3′ end of the vRNAs. The resulting plasmids were transfected into African green monkey kidney (Vero) cells, together with protein expression plasmids for the polymerase and NP proteins. The efficiency of influenza virus generation described in the original report was low, perhaps due of the limited transfection efficiency of Vero cells, and/or the limited effectiveness with which the ribozyme generated the 3′ ends of vRNAs. In fact, one study reported that influenza virus generation is substantially more efficient with the mouse RNA polymerase I promoter as compared with the hepatitis delta ribozyme sequence (Feng et al. 2009). Another study did not detect appreciable differences in transient in vitro assays between vRNA 3′-end generation by the mouse RNA polymerase I terminator and the hepatitis delta virus ribozyme (de Wit et al. 2004).

Bidirectional Reverse Genetics Systems

Hoffmann et al. (2000) designed plasmids in which the viral cDNAs were inserted in nega-

tive-sense orientation between human RNA polymerase I promoter and mouse RNA polymerase I terminator sequences. This transcription cassette was then flanked in positive-sense orientation by an RNA polymerase II promoter (i.e., the truncated immediate-early promoter of the human cytomegalovirus and the polyadenylation signal of the gene encoding bovine growth hormone). In this system, RNA polymerase I transcription yields negative-sense vRNA, whereas RNA polymerase II transcription yields positive-sense mRNA from the same plasmid. Consequently, the four protein expression plasmids are no longer needed, so that influenza virus generation can be achieved from only eight RNA polymerase I/II plasmids. The lower number of plasmids required for influenza virus generation may result in a higher percentage of cells transfected with all plasmids, potentially leading to higher rescue efficiencies as compared with the 12-plasmid reverse genetics system published by Neumann et al. (1999). On the other hand, the bidirectional eight-plasmid system provides less flexibility if reverse genetics efficiencies are low. For example, the polymerase complex of avian influenza viruses is restricted in its replicative ability in mammalian cells. The generation of avian influenza viruses could thus be limited with the bidirectional eight-plasmid system, in which the mRNAs encoding the polymerase and NP proteins are of avian influenza virus origin. In contrast, the 12-plasmid system provides the opportunity to perform the reverse genetics experiments with the polymerase and NP proteins of WSN, PR8, or any other human influenza virus to "kick-start" the viral life cycle. Overall, both the 12- and eight-plasmid systems have proven to be very robust and have been used widely.

Alternative Approaches

In addition to the above-described, widely used reverse genetics systems, several alternative approaches have been described. To reduce the number of plasmids required for the generation of influenza viruses, several groups combined multiple cassettes for influenza vRNA synthesis, or for vRNA/mRNA synthesis on one plas-

mid (Neumann et al. 2005; Zhang et al. 2009; Zhang and Curtiss 2015) or bacmid (Chen et al. 2014), so that as little as one plasmid or bacmid resulted in influenza virus generation. In another approach, an adenovirus-based RNA polymerase I reverse genetics system was developed (Ozawa et al. 2007) that allowed the highly efficient generation of influenza viruses after transduction of Vero cells with the recombinant adenovirus vectors.

Influenza viruses can also be generated with the help of a T7 RNA polymerase–based system in which influenza viral cDNAs are flanked by T7 RNA polymerase promoter sequences and hepatitis delta virus ribozyme and T7 RNA polymerase terminator sequences (de Wit et al. 2007). The efficiency of influenza virus generation was lower than with the RNA polymerase I–based reverse genetics systems, but in contrast to the species-specific RNA polymerase I promoter (discussed in more detail below), T7 RNA polymerase is not restricted to particular cell lines, allowing influenza virus generation in human, canine, and avian cells (de Wit et al. 2007).

Alternatives to Classical Cloning Strategies

To expedite the generation of influenza viruses, several approaches have been established that no longer necessitate the traditional cloning strategies. In RecA-positive *Escherichia coli* strains, homologous sequences undergo recombination, resulting in the fusion of the two DNA fragments. Based on this principle, reverse transcription polymerase chain reaction (RT-PCR)-derived viral cDNAs and RNA polymerase I vectors were generated that possess short identical sequences comprised of the conserved termini of influenza vRNAs and/or the RNA polymerase promoter and terminator regions (Wang et al. 2008; Zhou et al. 2009). The overlapping vector and insert fragments can then be joined in a recombination-based, ligation-independent process in vitro or in bacteria (Wang et al. 2008; Zhou et al. 2009; Shao et al. 2015). In a slightly different approach, viral cDNA inserts and RNA polymerase I vectors were generated with sequence overlaps, an-

nealed, and elongated by using PCR (Stech et al. 2008).

To date, two "plasmid-free" reverse genetics systems for influenza viruses have been described (Chen et al. 2012a; Dormitzer et al. 2013). In these approaches, the viral cDNAs are generated by overlapping RT-PCRs (Chen et al. 2012a) or from overlapping oligonucleotides (Dormitzer et al. 2013), followed by the PCR-mediated fusion of RNA polymerase I promoter and terminator sequences to the linear viral cDNA fragments. The resulting linear DNA fragments are then transfected into eukaryotic cells, thus eliminating the need for the generation, sequencing, and amplification of plasmids (Chen et al. 2012a; Dormitzer et al. 2013). However, mutations introduced by PCR errors will result in mutant viruses.

Species Specificity of the RNA Polymerase I Promoter

The RNA polymerase I promoter is species-specific, so that the human RNA polymerase I promoter was expected to function only in human cells. However, it has now been shown that the human RNA polymerase I promoter allows influenza virus generation in canine MDCK cells (Suphaphiphat et al. 2010), in African green monkey Vero (Neumann et al. 2005) and COS-1 (Hoffmann and Webster 2000) cells, and in swine kidney cells (Chen et al. 2014). Moreover, additional influenza virus reverse genetics systems have now been established based on the RNA polymerase I promoter from (1) mice (Zhang and Curtiss 2015), with the mouse RNA polymerase I promoter also being functional in baby hamster kidney BHK21 and in Chinese hamster ovary CHO cells (Zhang and Curtiss 2015); (2) chickens (Massin et al. 2005; Zhang et al. 2009); (3) dogs (Wang and Duke 2007; Murakami et al. 2008); (4) nonhuman primates (Song et al. 2013); (5) horses (Lu et al. 2016); (6) swine (Wang et al. 2017); and (7) salmon (Toro-Ascuy and Martín 2017). In addition, the earlier-described T7 RNA polymerase–based reverse genetics system (de Wit et al. 2007) is not species-specific and was used for influenza virus generation in hu-

man, canine, and quail cells. Collectively, these systems allow for influenza virus generation in a wide range of cell lines.

Reverse Genetics Systems for Other Members of the Orthomyxoviridae Family

In addition to reverse genetics systems for influenza A viruses, similar systems have now been established for influenza B (Hoffmann et al. 2002; Jackson et al. 2002), influenza C (Crescenzo-Chaigne and van der Werf 2007; Muraki et al. 2007), influenza D (Yu et al. 2019), Thogoto virus (Wagner et al. 2001), and infectious salmon anemia virus (Toro-Ascuy and Martín 2017), allowing a wide range of members of the Orthomyxoviridae family to be studied by reverse genetics–based approaches. All of these systems are based on the RNA polymerase I–mediated synthesis of vRNAs.

CONCLUDING REMARKS

The ability to generate and modify influenza viruses has profoundly changed influenza virus research. In the era before reverse genetics, researchers were limited to studying natural isolates and reassortants generated in the laboratory. Reverse genetics has resulted in thousands of publications that span the entire range of influenza virus research from basic virology to the development of novel influenza vaccines. Selected examples include the generation of the extinct pandemic 1918 (H1N1) influenza virus (Taubenberger et al. 1997; Reid et al. 1999), the generation of bat influenza viruses that have not been isolated in nature (Moreira et al. 2016), the identification of major influenza virus pathogenicity markers (Hatta et al. 2001) and highly pathogenic H5 influenza viruses that acquired the ability to transmit among ferrets (Chen et al. 2012b; Herfst et al. 2012; Imai et al. 2012; Zhang et al. 2013), the development of vaccines to highly pathogenic influenza viruses, and the development of novel influenza viruses.

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